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IMMUNOMODULATION OF HOUSE FLIES EXPOSED TO AZADIRACHTIN

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In this study the lethal and sub-lethal effects of azadirachtin on adult house flies, and the main variations in their immune-related gene expression levels were detected. Flies acquiring azadirachtin by ingestion showed different degrees of susceptibility depending on concentration and time of exposure to this compound, with LC_{50} value corresponding to 101.14 $\mu\text{g/ml}$, after five days of exposure to a dose of 7.5 $\mu\text{l/fly/day}$. Flies surviving ingestion of sub-lethal concentrations showed significant decrease in their lifespan and reproductive performance, including fecundity and percentage of egg hatching.

A significant immune-stimulation effect of lower azadirachtin concentrations (25 $\mu\text{g/ml}$), and a general immunosuppression of most AMPs (i.e. *attacin*, *cecropin*, *defensin*, *diptericin* and *muscin*) at higher concentration levels (100 $\mu\text{g/ml}$) were observed.

This study highlights the immunodeficiency potential of azadirachtin, providing new insights into understanding the physiological response of *Musca domestica* to this botanical compound at the molecular level.

KEY WORDS: *Musca domestica*, immune system, gene expression, botanical insecticide, immunosuppression.

INTRODUCTION

The triterpenoid compound azadirachtin represents the most important active substance present in seed kernels of the Indian neem tree, *Azadirachta indica* (A. Juss) (Meliaceae) (HUMMEL *et al.*, 2012). Due to its well documented insecticidal properties, different azadirachtin-based biochemical formulations are available for pest management (ISMAN, 2015). Lethal and sub-lethal effects were observed on diverse insect species exposed to variable dosages of this compound and its derivatives (MORDUE and BLACKWELL, 1993). Whilst there are numerous studies reporting the interaction of azadirachtin with insect's physiological mechanisms (i.e. growth regulation, fecundity, and fitness) and feeding behavior, little is known on its potential as immune-modulator.

The innate immune defense system of insects typically relies on physical barriers, cellular mechanisms such as phagocytosis, encapsulation, or nodulation, and on a humoral response based on the production of antimicrobial peptides (AMPs) and other substances involved in stress tolerance like the copper-containing enzyme, Phenol oxidase (PO) and the heat-shock proteins (HSPs) (LEMAITRE and HOFFMAN, 2007; ROLFF and REYNOLDS, 2011; VALANNE *et al.*, 2011; TANG *et al.*, 2012). These immune functions are typically activated in response against invading microbial agents (MANACHINI *et al.*, 2011; MASTORE *et al.*, 2015), however immunity can be modulated as a result of insect exposure to sub-lethal doses of chemicals (SHAURUB, 2012). Accordingly, immunomodulation was observed after azadirachtin treatments, thus highlighting significant suppressive effects of this compound on the cellular mechanisms of defence. For instance, a significant reduction in total haemocyte count (THC) and changes in their morphology have been observed in some insect species (AZAMBUJA *et al.*, 1991; PETER and ANANTHAKRISHNAN, 1995; AYAAD *et al.*, 2001; SHARMA *et al.*, 2003; PANDEY *et al.*, 2008). On the other hand, azadirachtin effects were reported to be immune-stimulatory

on the freshwater teleost *Oreochromis mossambicus* (Peters) and on the goldfish *Carassius auratus* Linn. (LOGAMBAL and MICHAEL, 2000; KUMAR *et al.*, 2013). Further studies are needed, especially to investigate the humoral immune response in azadirachtin-challenged insects. Such information would be very important to have a deeper understanding of the interaction of this biopesticide with the insect physiology. A better comprehension of the immune system implications deriving from the action of either botanical or synthetic insecticides would be very important for their optimal integration in pest management programs.

Several insect species show susceptibility to azadirachtin and, more in general, to neem based products. The house fly *Musca domestica* L. (Diptera: Muscidae), an ubiquitous and very common pest of medical and veterinary importance, can be controlled with azadirachtin (MILLER and CHAMBERLAIN, 1989; KHAN and AHMED, 2000; LARRAMENDY *et al.*, 2004; RUII *et al.*, 2008, 2011). The effects of azadirachtin on the house fly immune system have not been investigated so far. Following the recent sequencing and annotation of *M. domestica* genome, several immune-related genes have been identified (SCOTT *et al.*, 2014), which provides additional knowledge for investigations targeting the immune system of this species.

The main objectives of this study were 1) to determine the lethal and sub-lethal effects of azadirachtin on adult house flies and 2) to investigate its immunomodulatory properties at the transcriptional level.

MATERIALS AND METHODS

BIOASSAYS

Newly emerged *M. domestica* adults employed in bioassays were provided by the laboratory rearing facilities of the Dipartimento di Agraria of the University of Sassari (Italy) using the methods of RUII *et al.* (2006). All experi-

ments were carried out in a bioassay room at 25 °C and a photoperiod of L14:D10.

Azadirachtin [AZ] technical mixture (CAS N. 11141-17-6, Sigma Aldrich) was used for insect diet treatments in bioassays. Initial experiments were conducted with different azadirachtin concentrations in dose-response bioassays. Before starting experiments, flies were starved for 24 h, in order to force them to a rapid food intake. The experimental design involved groups of 10 flies maintained in plastic cages (10×15×5 cm) and fed daily 75 µl saccharose solution (30%) containing azadirachtin at variable concentration, administered through three capillary tubes (25 µl each). The control was just fed the saccharose solution (30%). Mortality was assessed consecutively for 5 days. The following range of concentrations was assayed to estimate the median lethal concentration (LC₅₀) value: 20, 40, 60, 80, 100, 150, 200 µg/ml. Each treated and control group involved four replications. The whole experiment was repeated three times with different batches of flies.

Supplementary observations were conducted to determine the sub-lethal effects caused by azadirachtin, in terms of fecundity, egg viability, and longevity of treated house fly adults in comparison with untreated controls. For this purpose, five replicated groups of 10 adults (5 males and 5 females), were maintained in transparent plastic boxes (10×15×5 cm) with a gauze covered window at the bottom (2×3 cm), which allowed females to lay eggs on a milk-soaked flock of cotton underneath. Flies were fed by capillary tubes (75 µl/cage/day) containing a saccharose solution (30%) with azadirachtin at two sub-lethal concentrations (40 and 80 µg/ml) for the first 5 days, and just the saccharose solution afterwards. A pinch of milk powder (1 g) was additionally provided to each cage to ensure an adequate source of protein and fat (RUIU *et al.*, 2006). From the 6th day on, the numbers of laid eggs were recorded for a 3 weeks period. Fly mortality was assessed daily.

Egg viability was determined in three different days (10, 15 and 20) from adult emergence, by maintaining five groups of 20 eggs from each treatment in a growth chamber at 25 °C and 80% R.H. on wet filter papers inside Petri dishes (5 cm diameter). Egg hatching was verified under a stereomicroscope after 24 and 48 h.

REVERSE TRANSCRIPTION QUANTITATIVE PCR (RT-qPCR) ANALYSIS

In total two different experiments were conducted, in order to determine the immune-related gene expression levels in flies exposed to diverse concentrations of azadirachtin for different time intervals. The general experimental design involved the analyses of house fly pools (10 flies per pool) fed by capillary tubes containing a 30% saccharose solution mixed with azadirachtin. Flies were administered a daily dose of 7.5 µl/fly. Pools of flies feeding only the saccharose solution were included as controls. Three independent biological replicates for each experiment were involved in analyses.

In the first experiment, analyses focused on the following genes: *attacin*, *cecropin*, *defensin*, *MdHSP-70*, and *lysozyme*. In this case flies were exposed to two different concentrations of azadirachtin (25 and 100 µg/ml) and processed after 24 h. A second experiment was conducted including a larger number of target genes (Table 1) to analyze fly pools exposed for 6, 12, and 24 h, to a unique azadirachtin concentration (100 µg / ml).

In all cases, total RNA was extracted from homogenized fly pools using TRIzol® Reagent (Life Technologies) according to manufacturer's protocol (CHOMCZYNSKI and SACCHI, 1987). Retrotranscription and qPCR reactions were performed using the same procedures and conditions described earlier (MURA and RUIU, 2017). The PCR efficiency of each primer set shown in Table 1 was preliminary tested by standard curve and dissociation curve analyses

Table 1 – Primer pair sequences used to amplify house fly immune-related genes.

Gene	Primer pair sequence	
	Forward	Reverse
<i>Attacin</i>	5'ATCGTTTTTTTAGCCACACTGGC3'	5'CCTAAAGTGTCCAGCTGC3'
<i>Cecropin</i>	5'CTGGATGGTTGAAAAAATCGG3'	5'ACCCTTTAATGTGGCGGCAA3'
<i>Defensin</i>	5'GCTACTTGCGATTTGTTGAGC5'	5'TCAGTTACGGCAAACACAAAC3'
<i>Diptericin</i>	5'AAGTCACAGCCACCTCCACC3'	5'GACGACTATTGCCATAGGGTCCA3'
<i>Domesticin</i>	5'TTTTTCACACTCCTGGCTGCACT3'	5'CCTCCTGGCCTTCTAATTGGTGC3'
<i>MdHSP-70</i>	5'TACCCCCTGTCTTTGGGTATTGAAACC3'	5'TCTGGGTTTGCTTAGTGGGGATGGTG3'
<i>Lysozyme</i>	5'TCCAACGGTTCCAACGATTACGGT3'	5'TCCAGCCTTGTTGGGACTTGATCT3'
<i>Muscin</i>	5'ATACTCGTGGTGCTGCTAAT3'	5'CTTCGTGTTAGCGTAATGTGG3'
<i>Prophenoloxidase</i>	5'CGGTCTTCGATGTTCCCGAC3'	5'GACGATGGCGTGGCACGAA3'
<i>Tubulin</i>	5'GCCTGCTGCATGTTGTATCGTGGT3'	5'CGAATTGAATGGTGGCCTTGGTCT3'

(PFAFFL, 2001). Tubulin was used after validation as a reference gene for immune-related genes expression normalization (NAYDUCH and JOYNER, 2013).

STATISTICAL ANALYSIS

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS, 2004). Linear regression analyses were used for analyzing the relationship between the azadirachtin concentration and fly mortality. Mortality data of dose-response bioassays were also analyzed by Probit model to determine the median lethal concentration (LC_{50}) (Finney, 1971). Data for longevity, fecundity, and egg viability were compared across treatments using one-way ANOVA, followed by LSD test to separate means in each sampling interval.

The relative expression of the target genes was analyzed using the comparative $2^{-\Delta\Delta C_t}$ method (LIVAK and SCHMITTGEN, 2001). Fold changes in gene expression of azadirachtin-challenged house flies in different experiments were subjected to one-way ANOVA, followed by LSD test for post-hoc comparison of means.

RESULTS

LETHAL AND SUB-LETHAL EFFECTS

A concentration-dependent acute toxicity of azadirachtin on house fly adults ingesting treated diets was observed in preliminary experiments (Fig. I). Mortality increased over time during bioassays achieving after five days an LC_{50} (95% FL) of 101.14 (86.17-114.53) $\mu\text{g/ml}$ (Slope $\pm$ SE = 4.92 ± 0.79 ; $\chi^2 = 39.05$; $df = 1$; $P < 0.0001$).

The effects of sub-lethal concentrations of azadirachtin administered by ingestion to adult flies are shown in Table 2. Longevity was significantly affected by treatments (Males: $F_{2,12} = 14.89$, $P = 0.0006$; Females: $F_{2,12} = 10.18$, $P = 0.0026$) that determined a significant reduction for the highest concentration assayed in both males (42.9 %) and females (42.1 %), and only in males (36.5 %) for the lowest concentration assayed in comparison with control. Fecundity was significantly influenced by treatments with different azadirachtin concentrations ($F_{2,12} = 27.74$, $P < 0.0001$), with a decrease reaching around 50 % in the case of the highest concentra-

Table 2 – Means ($\pm$ SE) of longevity, fecundity, and egg viability of house flies surviving exposure to sub-lethal concentrations of azadirachtin.

Azadirachtin concentration ($\mu\text{g/ml}$) ¹	Longevity (days) ²		Fecundity (No. eggs/female)	Egg hatching ³ %
	Male	Female		
80	13.6 $\pm$ 1.3 a ⁴	16.0 $\pm$ 1.2 a	118.6 $\pm$ 7.2 a	80.0 $\pm$ 1.4 a
40	18.8 $\pm$ 1.3 b	21.4 $\pm$ 1.5 b	198.6 $\pm$ 9.7 b	90.8 $\pm$ 1.2 b
Control	23.8 $\pm$ 1.4 c	25.2 $\pm$ 1.6 b	233.4 $\pm$ 15.1 c	92.9 $\pm$ 1.1 b

¹ Azadirachtin was administered to *M. domestica* adults only during the first 5 days of the experiment. From the 6th day on, flies were fed with just 30% sucrose solution, as the control.

² Days from adult emergence to adult death.

³ Egg hatching was evaluated after 10, 15 and 20 days from adult emergence. Mean values are presented.

⁴ Means in each column followed by different letters, are significantly different (ANOVA followed by LSD test, $P < 0.05$)

tion assayed compared with control. Besides, a significant reduction in the percentage of egg hatching was determined by treatments with a diet containing an azadirachtin concentration of 80 $\mu\text{g/ml}$ ($F_{2,33} = 30.99$, $P < 0.0001$).

RELATIVE EXPRESSION OF IMMUNE-RELATED GENES

Relative expressions of *attacin*, *cecropin defensin*, *MdHSP-70*, and *lysozyme* genes in house flies exposed for 24 h to two different concentrations of azadirachtin (25 and 100 $\mu\text{g/ml}$) are shown in Fig. II. A significant increase in expression of *attacin* ($F_{1,16} = 15.17$, $P = 0.0013$), *cecropin* ($F_{1,16} = 5.22$, $P < 0.0363$), and *defensin* ($F_{1,16} = 5.89$, $P = 0.0274$) genes was observed in flies treated with the lowest azadirachtin concentration, while a decrease was recorded for the highest concentration assayed. Besides, non significant were the differences between azadirachtin treatments for *MdHSP-70* ($F_{1,16} = 4.29$, $P = 0.0548$) and *lysozyme* ($F_{1,16} = 1.83$, $P = 0.1947$) gene expression levels that didn't substantially differ from untreated controls.

Fig. I – Linear regression plots with 95% confidence intervals (shaded areas) showing the predicted relationship between azadirachtin concentration and mortality of house flies exposed to a daily dose of 7.5 $\mu\text{l/fly/day}$.

Fig. II – Comparison of relative expression fold change (mean $\pm$ SE) for selected genes of house flies exposed for 24 h to different concentrations (25 and 100 $\mu\text{g/ml}$) of azadirachtin. Statistical significance (1-way ANOVA followed by LSD test) of the relative expression ratio is indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = non significant).

The relative expression (fold change) of *attacin*, *cecropin defensin*, *dipteracin*, *domesticin*, *muscin*, *MdHSP-70*, *lysozyme*, and *prophenoloxidase* in house flies exposed to azadirachtin (100 µg / ml) for progressive time intervals (6, 12, and 24 h) are shown in Fig. III. A considerable over-expression after 6 h, followed by a significant under-expression after 12 and 24 h of exposure, compared with untreated controls, were observed for *attacin* ($F_{2,24} = 150.49$, $P < 0.0001$), *cecropin* ($F_{2,24} = 83.84$, $P < 0.0001$), *defensin* ($F_{2,24} = 54.12$, $P < 0.0001$), *muscin* ($F_{2,24} = 721.54$, $P < 0.0001$), and *prophenoloxidase* ($F_{2,24} = 34.19$, $P < 0.0001$). A similar initial over-expression, followed by a gradual decrease in expression was associated with *domesticin* ($F_{2,24} = 6.11$, $P = 0.0072$) and *lysozyme* ($F_{2,24} = 5.88$, $P = 0.0084$) genes, while more variable were variations in the expression of *dipteracin* gene after successive time intervals ($F_{2,24} = 4.01$, $P = 0.0314$). Besides, non significant over time changes were associated with *MdHSP-70* gene ($F_{2,24} = 1.94$, $P = 0.1660$).

DISCUSSION

The action of azadirachtin on insects involves feeding deterrence, reduced fecundity, growth and moulting aberrations, and mortality (ASCHER, 1993). The potency of these lethal and sub-lethal effects is dose-dependent and varies with species. As expected, house fly adults assuming azadirachtin by ingestion showed different degrees of susceptibility depending on concentration and time of exposure to this compound, with an LC_{50} value corresponding to 101.14 µg/ml, after five days of exposure to a dose of 7.5 µl/fly/day. Flies surviving ingestion of sub-lethal concentrations showed significant decrease in their lifespan and reproductive performance, including fecundity and percentage of egg hatching.

These results are in line with previous studies reporting acute toxicity of azadirachtin on different house fly stages, with a considerably higher susceptibility of larvae developing on treated breeding media, as documented by larval LC_{50} values ranging between 10.45 µg/g and 24.53 µg/g (MILLER and CHAMBERLAIN, 1989; RUIU *et al.*, 2008) depending on the azadirachtin formulation used and on the specific experimental conditions. Biocidal effects were

observed as a result of direct treatments of *M. domestica* pupae with diluted neem oil solutions, which would support the penetration of the active substances through insect cuticle (DELEITO and MOYA BORJA, 2008). Beyond immature development inhibition, teratomorphic effects on house flies were occasionally observed (NAQVI *et al.*, 2007). Detrimental consequences on fly fecundity have also been reported (SIRIWATTANARUNGSEE *et al.*, 2008).

Similar effects have been observed on a variety of insect species in different orders (SCHMUTTERER, 1990; ABEDI *et al.*, 2014a,b), even though the biochemical mechanisms, especially at cellular level, have not been clarified so far. It is known that azadirachtin may interact with insect corpus cardiacum (REMBOLD *et al.*, 1989), interfering with the activity of the molting hormone that is known to play an important role in the regulation of house fly ovarian functions (ISHAAYA *et al.*, 2007), which may partly explain the observed decrease in fecundity and egg viability (ADAMS *et al.*, 1988). Some of these effects, might more broadly be related to the properties of azadirachtin to cause significant protein and lipid metabolism changes (HUANG *et al.*, 2004; HUANG *et al.*, 2012). At cellular level, oxidative stress induced by azadirachtin is related to mitochondrial dysfunctions and apoptosis in insect cell lines (HUANG *et al.*, 2013).

Significant changes in the arthropod defense system are also reported as a consequence of azadirachtin challenges. This compound may affect insect immune reactivity as demonstrated by studies on *Rhodnius prolixus* Stal (Heteroptera: Reduviidae), wherein a significant decrease in the numbers of haemocytes and nodule formation following challenges with azadirachtin and the bacterium *Enterobacter cloacae* was observed (FEDER *et al.*, 1997). Inhibition of nodule formation might be related to interference with the activities of endogenous mediators like eicosanoids, as reported for *Poeciloceris pictus* Fab. (Acrididae: Orthoptera) (SINGH *et al.*, 2014). Cellular immune response impairment due to azadirachtin treatments has also been observed on the wolf spider, *Schizocosa episina* Gertsch & Wallace (Araneae: Lycosidae) (PUNZO, 1997) and the Greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae) (ER *et al.*, 2017). Moreover, azadirachtin antagonism with ecdysone has been reported to be a main cause of the reduction in haemocyte phagocytosis activity (FIGUEIREDO *et al.*, 2006).

Fig. III – Comparison of relative expression fold change (mean $\pm$ SE) for different immune-related genes of house flies exposed for different time intervals (6, 12 and 24 h) to azadirachtin (100 µg/ml). Statistical significance (1-way ANOVA followed by LSD test) of the relative expression ratio is indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. = non significant).

All these findings are consistent with the results of studies revealing the immunomodulatory properties of various botanical and synthetic insecticides against diverse insect species. While the humoral response of insects to pathogens has recently been investigated (KURATA, 2010; MASTORE *et al.*, 2015; HE *et al.*, 2017; TASZLOW *et al.*, 2017), the knowledge on the humoral immunity implications due to botanical extracts is still limited. Modulation of specific immune functions involving the production of heat shock proteins and the phenoloxidase cascade regulating the synthesis of oxidative species (ROS) have been associated with diverse insect growth regulators (IGRs), including buprofezin, flufenoxuron, and pyriproxifen (SALOKHE *et al.* 2006; NASR *et al.*, 2010).

Overexpression of immune related genes *attacin*, *cecropin*, and *defensin* was detected after ingestion of lower concentrations of azadirachtin (25 µg/ml) by house fly adults. On the other hand, immunosuppression of the same genes after the same time interval (24 h) was noticed in flies feeding on a diet incorporated with a higher concentration of the terpenoid (100 µg/ml). In the same experimental conditions no significant changes were associated with *MdHSP-70* and *lysozyme* genes relative expression levels, in comparison with untreated controls. In a different experiment, overexpression of *attacin*, *cecropin*, *defensin*, *muscin*, *domesticin*, *lysozyme*, and *prophenoloxidase* genes was recorded after 6 h of exposure, followed by a rapid drop in the next hours, which resulted in under expression of *attacin*, *cecropin*, *defensin*, *muscin*, and *prophenoloxidase* genes, compared to controls. Even in this case, *MdHSP-70* gene relative expression in treated flies didn't change in respect to untreated control.

Our results highlight a significant immune-stimulation effect of low azadirachtin dosages, and a general immunosuppression of most AMPs at higher intake levels. The latter condition should be achieved either in the case of longer exposure to a lower concentration (first experiment) or when flies are exposed for a short time to a higher concentration (second experiment). The ability of azadirachtin to impair house fly immune system is confirmed by the results of our previous experiments showing that when flies were exposed to an adequate concentration of azadirachtin became more susceptible to the action of the entomopathogenic bacterium *Brevibacillus laterosporus* (MURA and RUIU, 2017). This finding corroborates the additive acute toxicity previously detected in experiments involving combinations of azadirachtin and the biological control agent *Bacillus thuringiensis* Berliner against insect larvae (ABEDI *et al.*, 2014b; TRISYONO and WHALON, 1999).

On the other hand, the immune-stimulatory effects of low azadirachtin dosages on a significant number of house fly AMP genes is not controversial, as the potential of appropriate dosages of this botanical substance to induce immune system improvements is well documented in the case of other organisms like diverse fish species (LOGAMBAL and MICHAEL, 2000; KUMAR *et al.*, 2013). In the case of insects, changes in the expression level of genes related to development, stress, and immunity were noticed in larvae of the Indian meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) feeding different azadirachtin doses (KUMAR *et al.*, 2013). These included up-regulation of *prophenoloxidase* gene that we observed only after a limited exposure of flies to this compound. In line with our results, these authors didn't find significant changes associated with the heat shock protein HSP 70, whose homologous represents an important component of house fly mechanisms of protection from damage under

stress conditions (TANG *et al.*, 2012). The up-regulation of *lysozyme* gene observed soon after fly exposure to higher azadirachtin concentrations, followed by a significant decrease in its expression level may represent another indicator of azadirachtin immunomodulatory properties. However, in the case of *lysozyme*, its availability is mostly related to a post-transcriptional mechanism of control (NAYDUCH and JOYNER, 2013).

In conclusion, our study highlighted the immunodeficiency potential of azadirachtin when administered at appropriate dosage, and proved the involvement of house fly humoral defense mechanisms, thus providing new insights in understanding the physiological response of insects to this compound at the molecular level.

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