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A 3D-Fluorescence Fingerprinting Approach to Detect Physiological Modifications Induced by Pesticide Poisoning in *Apis mellifera*: A Preliminary Study

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Abstract

The combined use of 3D-fluorescence spectroscopy and independent component analysis using a differential fingerprinting approach has been applied with success to detect physiological effects of dimethoate in honeybees. Biochemical determinations combined with the identification of fluorescence zones that may correspond to proteins, NADH or neurotransmitters/ neurohormones (octopamine, dopamine and serotonin) related to the physiological stress caused by the pesticide enabled phenomenological modeling of the physiological response in the honeybee using a simple and rapid method. The signals associated with the fluorophores involved in the response to stress were extracted from the fluorescence spectra using an unsupervised algorithm such as independent component analysis. The signals of different neurotransmitters were isolated on separated factorial components, thus facilitating their biochemical interpretation.

Introduction

The development of chemistry in agriculture after the Second World War improved the control of harmful insects in crops through the development of herbicides and pesticides, but these had a significant impact on the environment. The corollary was an increase in the area used for field crops for more than 30 years, which ensured food security but massively reduced plant biodiversity and the nectariferous and pollen resources available to insects dependent on them, such as honeybees [1]. In addition, the excessive use of pesticides and herbicides in agriculture led to the debilitation and over-mortality of honeybee colonies in European and French apiaries as from the 1980s. From 1998, these seemingly unusual,

brutal and complete deaths of colonies, reported in the general press [2–5] as well as in scientific journals (Maus et al. 2003), reached their peak in Europe and particularly in France, and were referred to as syndrome called "colony collapse disorder" [6]. This phenomenon has been an important focus for study during the past 20 years by many research laboratories in France and elsewhere in the world (in notably the ANSES laboratory at Sophia-Antipolis and the INRA (*Institut National de Recherche Agronomique*) Joint Research Unit for Bees and the Environment (UMR 406) in Avignon, France). Their work has had a significant economic impact on the beekeeping industry as well as on farming sectors dependent on the pollination services provided by insects such as honeybees. It has been estimated that the disappearance of honeybees could lead to a reduction of about 70% in fruit and vegetable production [7].

Different studies have demonstrated the importance of detoxifying enzymes when assessing the presence of pesticides in honeybees. The detection of detoxification mechanisms, such as the activation of cytochrome P450 and glutathione S-transferase (GST) expression pathways during different phases of the detoxification process offers a potential opportunity to evaluate their impact on the physiological response during pesticide intoxication [8–10]. The action of pesticides

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can also be clarified from the mechanism of action of the pesticide's active ingredient in the honeybee. Organophosphorus compounds such as dimethoate or dichlorvos covalently inhibit acetylcholinesterase (AChE) (BRENDA:EC3.1.1.7), an enzyme that is essential to the functioning of synaptic junctions in the nervous system of animal organisms. The state of modulation of this enzyme could therefore be an excellent biomarker for the presence of pesticide in the honeybee environment, at least with respect to organophosphorus insecticides.

Different methodologies to measure variations in enzymatic activity offer a good means to determine AChE activity [11]. It has been shown that this determination can serve as a qualitative biomarker for the presence of pesticide [8, 11].

Optical techniques such as fluorescence spectroscopy (particularly its "3D" version used to collect emission-excitation matrices to build fluorescence cubes) have been widely developed, particularly in the fields of food quality control and process monitoring in agribusiness and the environmental sector, notably to detect pollutants. 3D fluorescence spectroscopy is also an interesting tool to detect and measure fluorophore concentrations in living biological samples [12–14]. The detection of different fluorophores involved in metabolic and cellular activity makes it possible to identify and monitor physiological changes. Amino acids, cofactors such as NADH or NADPH, proteins or vitamins are good fluorophores and therefore have a characteristic excitation/emission spectrum [14, 15] which can be included in a fingerprinting approach to rapidly differentiate physiological states. However, to our knowledge, fluorescence spectroscopy has not yet been used to study the effects of pesticides on the physiological response of honeybees.

The aim of this study was therefore to detect and monitor the effects of dimethoate[1] in honeybees; this was achieved by observing the fluorophores involved in metabolic and physiological changes with fluorescence spectroscopy and then analyzing the results by means of independent component analysis. Our hypothesis was that the presence of an organophosphorus pesticide would cause a physiological response that could be monitored using 3D front-face fluorescence spectroscopy. Detecting and characterizing the effects of dimethoate in honeybees in this way could open new perspectives for the rapid diagnosis of intoxication.

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[1] Dimethoate was used for this study because it is now banned by the European Union. Its marked effects on bee physiology enabled the development of an indirect 3D fluorescence detection methodology.

Materials and Methods

To investigate the physiology of the honeybee, two types of measurements were performed: measurements using fluorescence spectroscopy in frontal mode that produced a collection of 3D spectra, and determination of the activity of AChE using the Ellman's method and of the total protein content in different compartments of bee samples.

Description of the Intoxication Experiment

Syrup Preparation

Two types of feed were administered, one without pesticide, the other containing pesticide and adapted from that described by Faucon et al. [16]; Pure_Syrup (PS): 1000 g of sucrose +1000 ml water, and Spiked_Syrup (SS): PS plus 1.25 μg l^{-1} dimethoate. These syrups were obtained by mixing with magnetic agitator for 5 h, 1 l of the initial sucrose solution with 1 ml at 1.25 mg l^{-1} dimethoate (to produce a syrup containing 1.25 μg l^{-1} dimethoate). Dimethoate ($C_5H_{12}NO_3PS_2$) was supplied by Cluzeau Info Labo (Sainte-Foy-La-Grande, Gironde, France). From an initial 100 mg dimethoate l^{-1} solution in acetone, we prepared a standard solution at 1.25 mg l^{-1} which was stored at 4 °C and then used extemporaneously to prepare the spiked syrup.

Syrup Feeding and Bee Recovery

Honeybees of the species *Apis mellifera* were collected from the AgroParisTech experimental apiary (Paris, France). The honeybees were divided into 12 boxes of 50 bees. The SS solution was delivered to six of the 12 boxes while the others receiving the PS solution were used as controls. The intoxication phase took place over 5 days, during which the syrup tanks were changed daily and the ingested syrup mass was weighed in order to evaluate the amount of pesticide absorbed by each honeybee (0.6 μg pesticide/honeybee). All dead honeybees were collected, counted, dissected to separate the head, thorax and abdomen and then stored at −80 °C. After 5 days, all the honeybees (both controls and "stressed" i.e. intoxicated) were collected and stored at −80 °C.

General Procedure for Sample Preparation

Honeybee samples stored at −80 °C were prepared for analysis. Four aliquots per bee compartment were prepared from all the "Control" and "Stressed" bees, producing 48 aliquots. As indicated in Diagram 1, each aliquot was prepared with 10 pooled body-parts (10 heads, 10 thorax and 10 abdomens) and then crushed in liquid nitrogen to completely halt protease activity and thus preserve as much as possible the physiological state of the samples prior to analysis [17, 18].
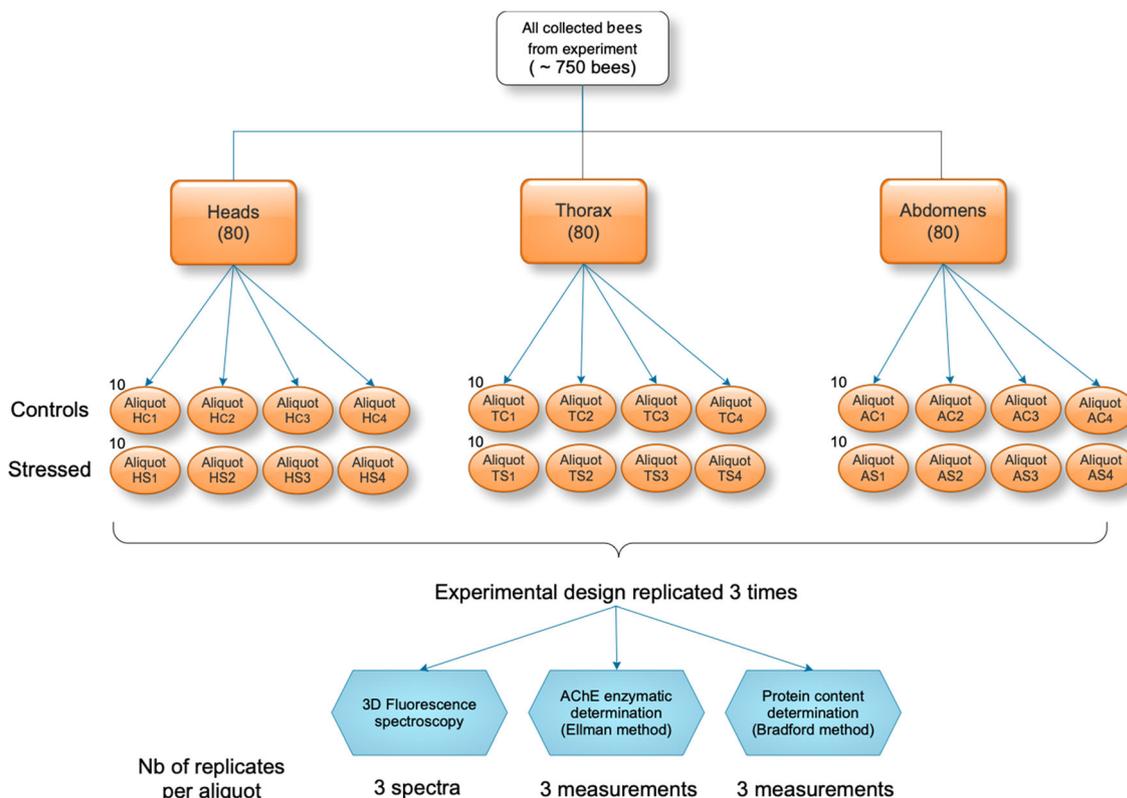
Diagram 1 Experimental design for bee sample preparation and analyses. *HC* head control, *HS* head stressed, *TC* thorax control, *TS* thorax stressed, *AC* abdomen control, *AS* abdomen stressed

Front-Face Fluorescence Spectroscopy

Fingerprinting in front-face fluorescence spectroscopy was performed on a FluoroMax-4 spectrometer (Spex-Jobin Yvon, Longjumeau, France). The angle of incidence for excitation was 56° in order to limit light reflection and dispersion phenomena. Integrity of the system was tested using the Horiba diagnostic procedure to verify the correct parameters to produce the optimum trace. The quality of the xenon lamp was thus confirmed to have a maximum excitation peak at 467 nm, and water Raman spectra were recorded systematically before each series of measurements in order to identify any abnormalities caused by accessory problems or miscalibrations. The wavelength ranges for excitation and emission were 280 nm – 400 nm (increasing by 2 nm increments for emission, 4 nm for excitation, and slits of 3 nm for both excitation and emission) and 300 nm – 550 nm, respectively. Three spectra were recorded for each sample in suprasil quartz cuvettes (Helma, France), using different sample aliquots from both control and intoxicated honeybees. The measurements were carried out at room temperature.

AChE Activity

A semi-qualitative evaluation of acetylcholinesterase activity was achieved using the Ellman method [11, 19, 20] based on measuring thiocholine production from acetylthiocholine (a similar substrate), which interacts with DNTB (5,5′-Dithiobis (2-nitrobenzoic acid)) to produce a yellow compound, measurable at 412 nm with a UV-Visible spectrophotometer. The enzymatic activity of acetylcholinesterase as expressed in μmol/min/mg of protein. The protein concentration was determined in the sample using Bradford's method [21], an assay that required 0.05 g of head, 0.25 g of thorax and 0.3 g of abdomen taken from the preparation described in General Procedure for Sample Preparation previously decribed. Protein extraction was performed in 0.1 M extraction phosphate buffer (10 ml) in 0.01 M NaCl at pH 8, with a sample concentration of 5% *w/v*. The extract was centrifuged at 3000 g (centrifugal Q-sep 3000, Restek Corporation, USA) for 15 min. The reagents for the acetylcholinesterase reaction were: 10 ml of a solution of DNTB at 0.01 M in 0.1 M pH 7 phosphate buffer; and a 0.075 M solution of acetylthiocholine. The reaction was carried out at room temperature in 3 ml of extraction buffer at pH 8, 100 μl DNTB solution, 20 μl acetylthiocholine substrate and 200 μl protein extract over 5 min, with one measurement taken every 30 s. Enzymatic activity could be calculated as follows:

$$\text{Activity} = \frac{\Delta A \cdot V_t \cdot 10^2}{13600 \cdot L \cdot V_S \cdot [P]}$$

Where: ΔA = increase in absorbance per minute ([At$_{5min}$ −

A_{t0}) / 5] and Vt = total volume of the reaction. In this case, the volume was 3320 μl; 13,600 was the molecular absorption coefficient of DNTB $M^{-1}.cm^{-1}$; L the length of the cuvette (1 cm), Vs the volume of the sample (200 μl) and [P] the protein concentration (μg/μl). The final result was given in μmol of thiocholine (AchI) per min and per mg of protein, corresponding to specific acetylcholinesterase activity.

Data Processing

Statistical and chemometric processing was carried out under Matlab, version 2012b (Mathworks, France) using the functions of the SAISIR® package [22]. Our goal was to demonstrate that the two conditions studied during the experiment – "Control" and "Stressed" (corresponding respectively to bees having been fed with the PS solution and those receiving the SS solution with pesticide) were statistically different with a p value <0.05 (at a 95% confidence level). Because 3D spectrofluorescence was used to analyze the head samples, thorax samples and abdomen samples separately, the data obtained took the form of three cubes of fluorescence such as $\underline{X}$ [126 × 31 × 24], with 126 emission wavelengths (increasing from 280 nm to 400 nm, at 2 nm increments), 31 excitation wavelengths (increasing from 300 nm to 550 nm at 4 nm increments) and 24 spectra per bee body part. In order to reduce data dimensionality, principal component analysis (PCA) was applied first to each data cube in order to obtain a score matrix. The score matrix calculated from each fluorescence cube was then used as input in a stepwise discriminant analysis according to the method described by Bertrand et al. [23]. The basic idea is to assess a factorial discriminant analysis on the scores of a previous PCA. The criterion for score selection is maximization of the trace of $T^{-1}B$, where T is the total variance-covariance matrix assessed according to T = X^TX, with X the initial data matrix and X^T its transposed form. Here, X was the score matrix given by PCA and B described variations between the groups. We adopted this strategy because some variables might have no discriminant ability so it was worth choosing a relevant subset of the original variables. Romederg [24] had shown that variables could be introduced efficiently one after the other. The criterion he proposed for introducing a new variable was to maximize the trace of the matrix defined by $T^{-1}B$. The second part of data processing consisted in calculating an independent component analysis (ICA) in order to facilitate chemical interpretation of the fluorescence data, as described below.

Independent Component Analysis

Our chemometric strategy was based on independent component analysis (ICA) applying the JADE algorithm [25] to the fluorescence cube (emission-excitation matrix collection). This data cube needed to be rearranged before analysis using

bilinear factorial techniques such as principal component analysis (PCA) or independent component analysis. This pretreatment will not be detailed here but the reader can find the details elsewhere [26].

Independent Component Analysis is a source-separation technique derived from the field of neuromimetic signal processing [27]. In other words, ICA can extract N unknown source signals, at unknown proportions, from a set of P signal mixtures. For ICA, each signal is a mixture of source signals with the underlying assumption of statistical independence. More precisely, ICA makes the hypothesis that these signals have intensities that are distributed in a less Gaussian way than their mixtures, so are related to "non-Gaussianity". ICA therefore seeks to maximize the non-Gaussianity of extracted signals in order to construct new variables (ICs) as linear combinations of the original variables. These ICs do not have orthogonality constraints in the same way as the PCs of Principal Component Analysis (PCA); the consequence is a greater ability to discriminate groups when they exist. The major advantage of ICA over PCA is that the building engine for ICs is not driven by variance, unlike PCA. The general model of ICA is a linear model such as:

$$X = A \cdot S$$

Where X is the matrix of recorded signals, S the matrix of "pure" source signals and A is the mixing matrix corresponding to the equivalent of the PCA scores but in this case referred to as "proportions" of the source signals in each mixture. Unlike PCA, ICA-generated loadings (matrix S) are much more chemically interpretable, which is an additional advantage of the technique. In recent years, the number of applications for ICA has increased considerably in the field of analytical chemistry and particularly in the processing of recorded signals in agribusiness [28–31].

Scores or proportions from the ICA model were used as inputs for one-way ANOVA in order to verify whether the differences observed regarding both fluorescence data and acetylcholinesterase activity measurements were statistically significant between the "Stressed" and "Control" groups. The test would be deemed positive for any p value ≤0.05 between the two groups.

Results

Fluorescence Spectroscopy

Examples of the front-face fluorescence spectra acquired on head samples after Rayleigh correction are shown in Fig. 1a, b. Two main signals were visible in the spectral regions around 290/335 nm (exc/em) for signal1 and 350/410 nm (exc/em) for signal2, with both the "Control" and

Fig. 1 Example of raw data. **a** Spectra acquired for the heads of honeybees nourished with syrup (Control) and **b** honeybees nourished with syrup + pesticide (stressed)
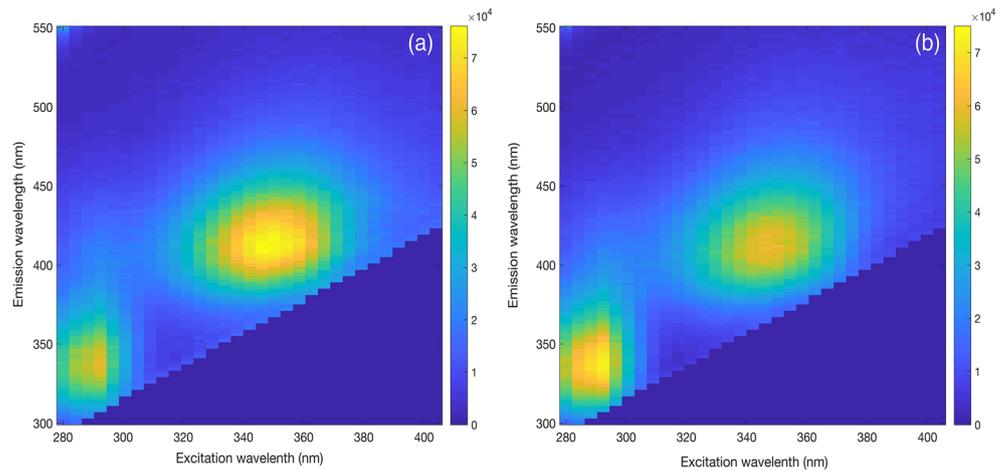
"Stressed" samples. The visual difference between the two conditions concerned the intensity of the signals, which increased for signal1 and decreased for signal2 in the "Control" and "Stressed" bees, respectively. Before extracting information from these spectra using Independent Component Analysis (ICA), we found a multivariate plan that was able to discriminate between the two groups of samples. Stepwise discriminant analysis was therefore performed on the PC scores and the results are presented in Fig. 2 for the head samples. The best discriminant model (i.e. that with the highest classification rate) was found with nine or more components, as illustrated on the left-hand side of the graph in Fig. 2. Using this model, the resulting discriminant function displayed very good discrimination between the

"Control" and "Stressed" samples as shown in the graph at the center of Fig. 2. (Score values on the discriminant function vs # sample in the matrix). The quality of this discrimination was evaluated using one-way ANOVA on the scores calculated for the discriminant function, and a p value (95% confidence level) equal to $4.55E^{-9}$ was found, indicating a significant difference between the average values in the groups compared. This one-way ANOVA made it possible to determine whether the differences observed between "Control" and "Stressed" samples were statistically significant or not (the differences being significant when $p \leq 0.05$). The ANOVA results are presented with Box & Whisker plots showing the median, quartiles, and atypical and extreme values. The same procedure was
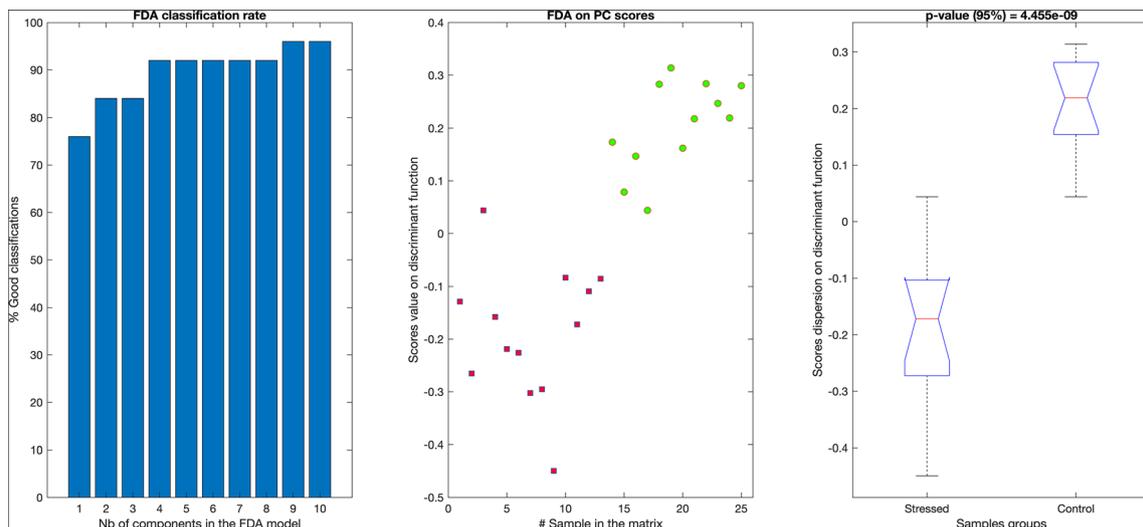

Fig. 2 Stepwise discriminant analysis on PC scores. Determination of a multivariate discriminant function able to statistically separate "Control" samples from "Stressed" samples with a significant p value. **a** Histogram of the classification rate vs the number of latent variables integrated in the FDA model; **b** Discriminant function scores calculated from the FDA model; "Controls" are shown as *circles* and "Stressed" samples are shown as *squares*; **c** ANOVA test on FDA scores indicating the significant difference between the groups thus compared

applied to the abdomen samples and thorax samples, with p-values of $1.79E^{-8}$ and $2.54E^{-10}$, respectively, indicating a significant difference between the fluorescence signals of the studied groups.

In order to extract the source signals reflecting physiological changes due to dimethoate in the different honeybee compartments (head, thorax and abdomen), the data were subjected to Independent Component Analysis (ICA) after unfolding the cube of fluorescence. The ICA model thus calculated had four independent components (4 ICs). Figures 3a–d, 4a–d and 5a–f show the source signals extracted from the head, thorax and abdomen groups, respectively. For each of these IC, Table 1 summarizes the results and specifies for each compartment which fluorophores were involved in the differentiation of "Control/Stress" and the probability p associated with the test.

Acetylcholinesterase Activity

Acetylcholinesterase is a biomarker of neurotoxicity that is widely used to identify exposure to certain organophosphorus pesticides, pyrethroids and carbamates [20, 32]. The presence of chemical agents in the hive introduced by foragers during

harvesting therefore has a major impact on the physiology of the honeybee and on every part of its body (head, thorax, and abdomen). The results concerning acetylcholinesterase activity (see Table 2) revealed significant differences between the "Control" and "Stressed" samples, particularly in the "Thorax".

Discussion

The extraction of source signals by independent component analysis made it possible to propose a biochemical interpretation of the honeybee stress response. All things being equal in the experiment, the variations in fluorescence in the "Stressed" samples versus the "Controls" in a specific area of the spectrum were necessarily related to the presence of dimethoate and its effects on cell physiology. Moreover, it is known that dimethoate in insects, and particularly in honeybees, causes stresses on the body and induces metabolic disorders [8, 33]. Fluorophore compounds whose concentrations rise or fall in the presence of dimethoate are potentially good biomarkers of intoxication. However, it cannot be excluded that some of the fluorescence signal recorded integrated other
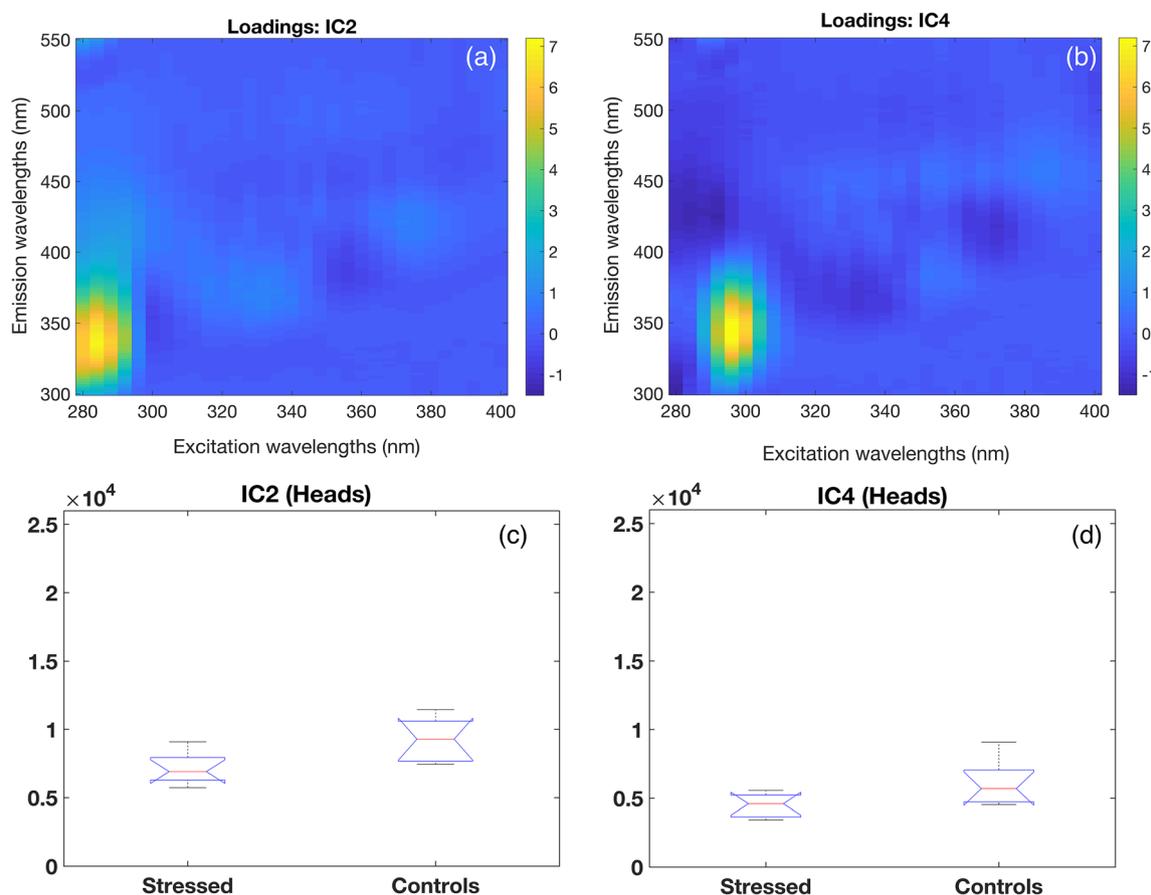

Fig. 3 Top **a** and **b**: Extraction of source signals using Independent Component Analysis on "Heads". Protein (tryptophan) signals on IC2 loadings and neurotransmitter signals on IC4. Bottom **c** and **d**: ANOVA test on IC scores for IC2 (p value = 0.0069) and IC4 (p value = 0.0196)
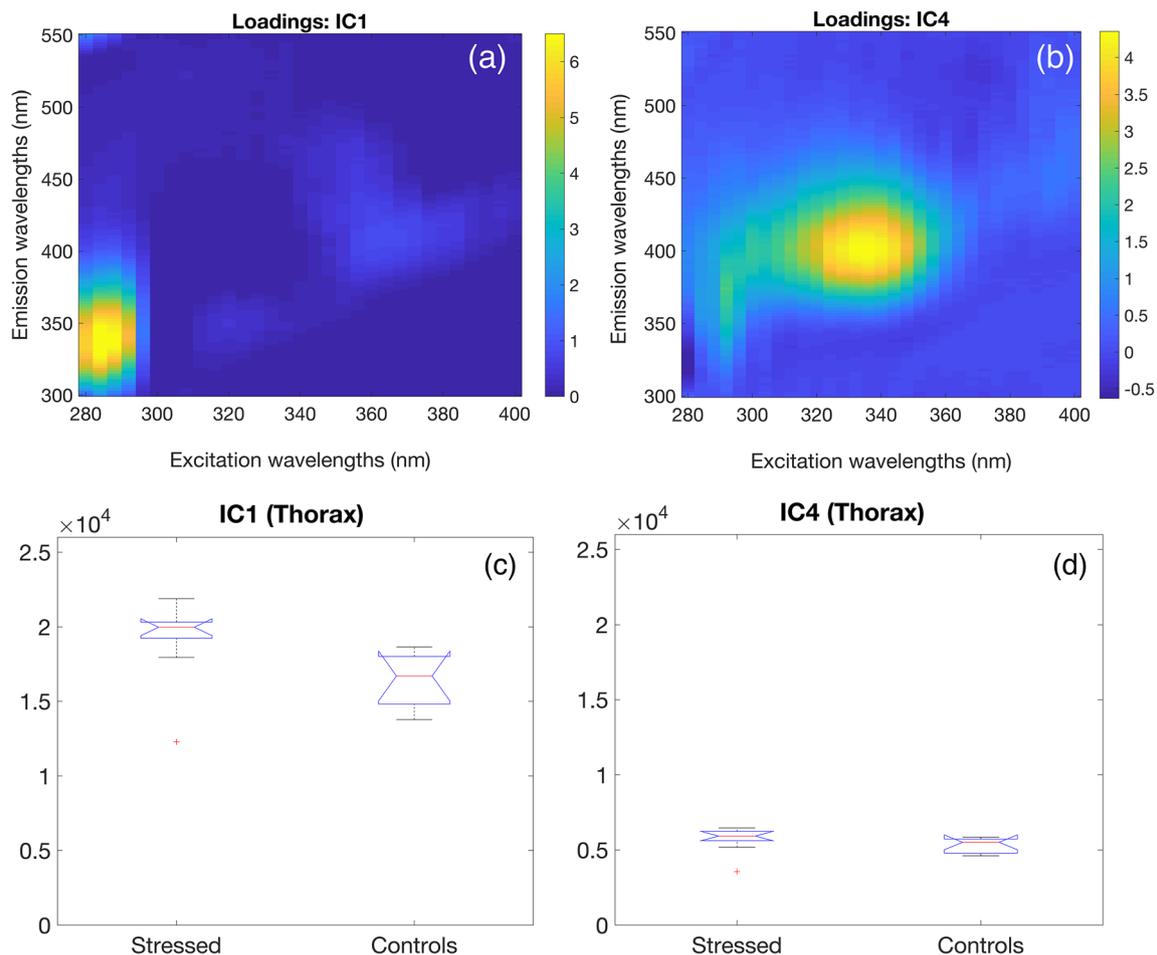
Fig. 4 Top **a** and **b**: Extraction of source signals using Independent Component Analysis on "Thorax". Protein (tryptophan) signals on IC1 loadings and neurotransmitter signals on IC4. Bottom **c** and **d**: ANOVA test on IC scores for IC1 (p value = 0.0302) and IC4 (p value = 0.2104)

fluorescent neuromodulators or unknown molecules that we cannot present or discuss here because of a lack of information in the scientific literature. Our interpretation is therefore based on the available and accessible literature and knowledge of the acute stress response in insects and particularly honeybees.

Head

Both a decrease and increase in the proportions of the fluorescence signal were observed simultaneously for the IC scores at $280_{ex}/330_{em}$ nm, respectively, in the head and thorax of honeybees (See Table 1, Figs. 3a, b and 4a, b). The signal detected corresponded to the fluorescence emitted by amino acids such as tryptophan which make up the residual chains of proteins [14, 15]. On the other hand, protein concentrations (determined according to Bradford's method) did not display any significant differences between "Controls" and "Stress" in the head samples, whereas they were significant in the thorax and abdomen (see Table 2). The presence of numerous pesticides increases oxidative cell stress, which also affects neurons [34]. The replacement of damaged tissues by scar tissues [35]

enriched in collagen fibers (mainly composed of glycine, proline and lysine) is difficult to detect using fluorescence because of a lack of aromatic groups in this type of tissue. The ANOVA performed on the ICA scores for heads showed a decrease in stressed honeybees compared to controls in the spectral zone around $295_{ex}/340_{em}$ nm (see Table 1 and Fig. 3b). Biogenic amine neurotransmitters or neuromodulators may produce fluorescence in the same spectral region [13, 15] (e.g. serotonin, dopamine and octopamine). The reduced signal associated with these neurotransmitters could be explained by the fact that these molecules are neuromodulators of stress and participate in the neurohormonal response. The consequence is a displacement of some of these neurohormonal compounds within the body of honeybees, leading to lower concentrations in the head but higher levels in the organs, tissues or glands. This tends to illustrate the expression of a dynamic response to stress [36–39]. The fluorophore most likely to be detected in this spectral region is dopamine, probably associated with octopamine. Dopamine is
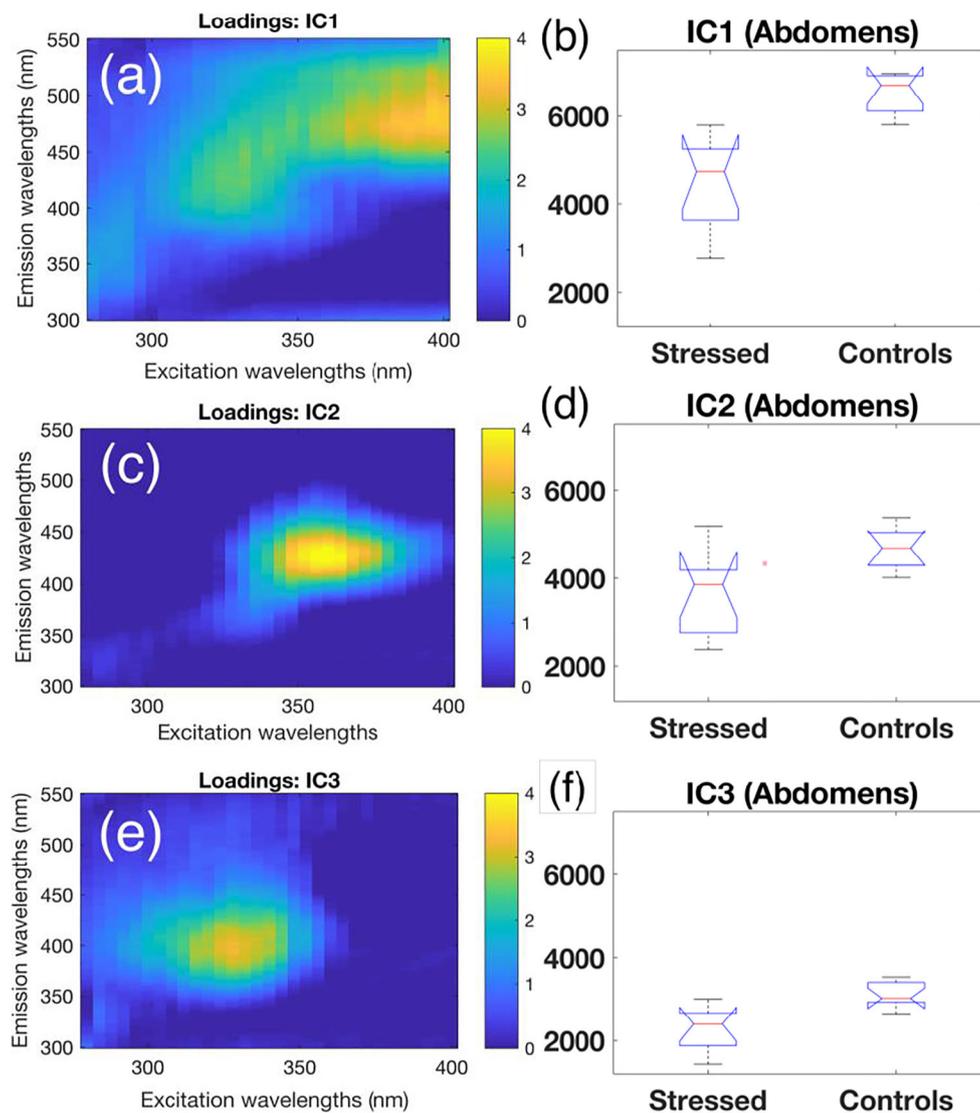
Fig. 5 Left **a**, **c** and **e**: extraction of source signals using Independent Component Analysis on "Abdomens". FAD signals on IC1 loadings **a**, vitamin A on IC2 **c** and NAP(P)H of cell signals on IC3 **e**. Right **b**, **d** and **f**: ANOVA test on IC scores for IC1, p value = 6,1E^{-5} **b**; IC2, p value = 0.0069 **d**; IC3, p value = 0.0009 **f**

Table 1 ANOVA on IC scores – Comparison between "Control" and "Stressed" samples

Physiological compartment	IC	Fluorophore	n	ANOVA		
				df (degree of freedom)	F	Probability
Head	IC2	Proteins	280/330	17	9,62	0,0069*
	IC4	Neurotransmitters	295/340	17	6,73	0,0196*
Thorax	IC1	Proteins	280/330	17	5,66	0,0302*
	IC4	NADH	340/400	17	1.70	0.2104
Abdomen	IC1	FAD$^+$/FAD	>400/500	17	28,97	6,1E-05*
	IC2	Vitamin A	360/440	17	9,6	0,0069*
	IC3	Cellular NADH	330/440	17	16,53	0,0009*

*Statistically significant differences with a 95% level of confidence

Table 2 Determination of the enzymatic activity of acetylcholinesterase and ANOVA

Sample	n^a	Protein Concentration (µg/ml)	Acetylcholinesterase Activity (µmol Thiocholine/min·mg protein)	ANOVA 1 Factor Protein concentration			ANOVA 1 Factor Effect of dimethoate on acetylcholinesterase activity		
				DF	F	Probability	DF	F	Probability
Head C	3	1.279 ± 0.130	0.036 ± 0.010	5	2.4098	0.1955	5	2.9816	0.1593
Head S	3	1.462 ± 0.475	0.028 ± 0.010						
Thorax C	3	1.354 ± 0.520	0.004 ± 0.001	5	15.5263	0.0170*	5	51.2902	0.0020*
Thorax S	3	0.854 ± 0.109	0.009 ± 0.002						
Abdomen C	3	0.895 ± 0.173	0.007 ± 0.001	5	19.1566	0.0119*	5	2.9892	0.1589
Abdomen S	3	0.581 ± 0.246	0.009 ± 0.004						

[a] Number of true samples. Each sample analyzed consisted of a sample of 10 heads, 10 thoraxes or 10 abdomens that had been pooled to form one sample in which enzymatic activity was determined Probabilities are significant when <0.05

known to trigger a cascading neurohormonal response, and its levels always rise initially before declining rapidly when exposure to stress is sustained for a longer period [37].

Thorax

On the other hand, in the spectral zone ($280_{ex}/330_{em}$ nm), analysis of the thorax (Fig. 4a) showed a significant increase in fluorescence intensity and AChE activity in the "Stressed" honeybees versus the "Controls", while protein levels fell significantly. All of these variations constituted the physiological response of honeybees to stress. Determination of the protein content in the thorax indicated a reduction in the total protein concentration. This could be explained by the fact that in a stress situation, the mobilization of resources to overcome the damage caused (to the neurons and musculoskeletal system) results in a lowering of protein levels in the thorax and abdomen (displacement of resources towards the locomotor system, particularly to generate additional energy) [38, 40]. The amino acid composition of major muscle protein sequences (myosin, tropomyosin and actin) is poor in aromatic amino acids [41] so that muscle breakdown is a phenomenon that is not significant in fluorescence. Nevertheless, the production of enzymes and proteins such as Heat Shock Proteins (HSPs such as HSP70 or HSP90) in response to chemical stress initiates the detoxification mechanism in honeybees that leads to the production of cytochrome P450 and Glutathione S-Transferase (GSTs) [9, 10, 36], globular protein complexes that can be traced using fluorescence spectroscopy [42]. The increase in the signal proportions on IC1 (see Fig. 4a) in the thorax could be explained by the expression of globular proteins in response to abiotic stress. This observation was supported by the acetylcholinesterase assay, whose measurements were only significant in the thorax ($p = 0.0020$, $df = 5$). This result was logical because acetylcholine is a fast neurotransmitter, its distribution mainly being oriented to the motor

(spinal) nervous system located in the thorax [43]. In addition, our honeybee samples had been exposed to the pesticide for a prolonged period (5 days), which induced an increase in the gene expression of complementary cholinesterases in response to the blockage of acetylcholinesterase, mainly in the thorax [20, 32]. The effect of the pesticide on AChE was also less marked in the head ($p = 0.1593$, $df = 5$) and the abdomen ($p = 0.1589$, $df = 5$), and the analysis of variance was not significant (Table 2). The main reason was an increased expression of other cholinesterases [44] that could catalyze the substrate (in this case acetylthiocholine) and thus interfere with the results during prolonged exposure to the pesticide. Finally, the increase in the fluorescence signal on IC4 (Fig. 4b) in the area around $330_{ex}/440_{em}$ nm was related to an increase in the levels of NADH, a biomarker of cellular activity [45], because of the stress experienced by the honeybee.

Abdomen

As for the abdomen, the fluorescence spectrum around the fluorescence maximum $>400_{ex}/500_{em}$ nm (Table 1 and Fig. 5a), and the ANOVA performed on the IC1 scores (Fig. 5b) showed a significant reduction ($p = 6.10E-5$, $df = 5$) in this signal in "Stressed" honeybees compared to the "Controls". This fluorescence zone corresponds to Flavin Adenine Dinucleotide (FAD), which is directly involved in the mechanisms for oxidation and energy production in mitochondria [14, 46]. The lowering of ICA scores related to the course of this cellular cofactor in stressed honeybees revealed the existence of a metabolic regulation of abdominal cells which resulted in energy saving in this physiological compartment to the benefit of other compartments (head, thorax). During this process, most available nutrients are diverted to the head and abdomen, and this stress response mechanism is commonly referred to as a "Fight or Flight" reflex [37, 39, 40]. Table 2 also shows the variation in protein levels in the thorax, which tended to confirm this assumption of a distribution of

resources to other compartments of the honeybee. The analysis of variance applied to IC2 scores (Fig. 5c, d) showed significant variations in fluorescence ($p = 0.0069$, $df = 17$) in the abdomen at around the $360_{ex}/440_{em}$ nm wavelength couple (See Table 1), with a significant difference in the reduction of the signal between stressed and control honeybees. This fluorescence was similar in terms of intensity and variation to that seen at around $330_{ex}/440_{em}$ nm. Some of the latter was probably due to NADH in microorganisms (lactic acid bacteria, enterobacteria, etc.) from the digestive tract of the honeybees, as has previously been suggested by other authors. Xenobiotics, and particularly pesticides such as dimethoate, can also affect the microbiota of the honeybee digestive tract by changing the composition of the microbial flora [47, 48], and this can significantly affect fluorescence. The ANOVA applied to the IC3 scores (Fig. 5e, f) showed a significant reduction in fluorescence in the abdomen of stressed honeybees, in the spectral zone centered on $330_{ex}/440_{em}$ nm (see Table 1). This spectral zone may correspond to lipid-soluble vitamin A [14] from the adipose tissue of the honeybee that is mainly located in the abdomen, and is known to be involved in immunity and the stress response [49]. Variations in vitamin A may also be related to resource consumption, decreased adipose tissue and the effects of possible abiotic factors such as xenobiotics that can trigger stress in the honeybee [14, 35, 40].

Conclusion

Fluorescence spectroscopy measurements of certain fluorophores that are endogenous in honeybees enabled study of the metabolic effects of dimethoate on these animals. The fluorescence signals we recorded formed a non-specific fingerprint of this pesticide and could be considered as a general intoxication response. Moreover, biochemical determinations such as AChE activity and total protein content provided qualitative and quantitative information on the presence of the pesticide in the honeybees and allowed us to highlight the cellular stress generated by this substance. Physiological changes in response to this stress include an increased expression of cholinesterases, anti-stress heat-shock proteins and detoxification proteins (P450 or GST), the relocation of resources to major organs, or the consumption of proteins, etc. Independent component analysis enable the efficient extraction of source signals reflecting a physiological response altered by the presence of the dimethoate and visualized the overall response to stress in the head, thorax and abdomen. Lastly, the separation of fluorophore signals such as proteins or metabolism cofactors may open the way towards systematic monitoring and global quantification of these fluorophores in honeybees and suggests that a rapid method to monitor the health of honeybee colonies might be possible.

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