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Methadone determination in puparia and its effect on the development of *Lucilia sericata* (Diptera, Calliphoridae)

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ABSTRACT

This paper describes a sensitive UPLC–MS/MS method for quantification of methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in single empty puparial case of *Lucilia sericata*.

Larvae were reared on substrates spiked with different concentrations of methadone (0–4 μg/g). Methadone was quantified in puparia reared on high concentrated substrates (0.8–4 μg/g). The major metabolite of methadone (EDDP) was not detected, confirming rapid elimination of metabolites by the larvae before pupation.

The effects of methadone on the development of *L. sericata* were also investigated. No effect on sex ratio was detected. A significant difference was calculated for emerged adults but no trends could be observed. Concerning the developmental curve, a significant difference was observed between control and high methadone concentrations using the Kolmogorov–Smirnov test.

1. Introduction

Forensic entomotoxicology, a relatively new branch of forensic entomology, studies the use of insects as an alternative toxicological matrix and the effects of drugs on insect physiology [1].

The first interest of this science is the determination of drugs in insect tissue. While detection of drugs in necrophagous species is generally accepted by forensic toxicologists, interpretation is still under discussion. Lack of sufficient entomological knowledge (e.g. metabolism, feeding behaviour), and lack of sensitive equipment are some of the problems leading to unreliable results.

In entomotoxicological studies, larvae are mostly sampled as they are present in a high number and are more visible on-site. However, some studies mention variability of drug concentration in larvae which could be due to the stage of development (first, second or third instar) and/or feeding state of the larvae [2]. After the feeding stage, larvae walk away from the food source, searching for a place to become a pupa enclosed in their last larval cuticles (puparia). Only empty puparial cases remain on or around the corpse after complete development of insects. Pupae and empty puparial cases are more difficult to sample due to their dark colour and localization. However, pupae stop feeding, reducing variability in the analytical toxicological results [3]. In addition, the structure is resistant to chemical attack, microorganisms, and to meteorological factors. Moreover, puparial cases are found long after adult emergence [4].

Inter and intra-site variations of drug concentrations can be very large [5,6]. Therefore, a single specimen must be analyzed to study the source of variability in a pool avoiding different species, developmental stages or feeding activities [3]. Unfortunately, drug concentrations in puparial cases are low [7–12], thus sensitive methods such as liquid chromatography coupled to mass spectrometry (LC–MS) are necessary [8]. While quantification of drugs in insect tissues is possible using sensitive equipment [1,9,12,13], the relationship between the measured drug concentration in an empty puparial case and the related concentration in human or animal tissues is not yet established and remains a major problem.

The second major point of interest is to study drug-induced changes on insect physiology with respect to the post-mortem interval (PMI) estimation. Some physiological parameters such as sex-ratio and mortality are not well investigated [14–16] and could

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affect blowfly development indirectly. Entomotoxicological studies involving drugs and especially opioids (Table 1) seem to indicate different development rates responses in necrophagous flies [14–20]; it can be increased, retarded, or remain unchanged [21].

The aim of this study is, first, to develop and fully validate a simple, reliable and fast UPLC–MS/MS method for quantification of methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in single empty puparial case and, second, to estimate the drug effect on development of *Lucilia sericata*. Methadone was chosen as an example, because in Belgium, methadone is licensed as a substitute for opioid addicts, and it is often co-administered with other psychotropic substances such as other drugs-of-abuse, ethanol or benzodiazepines [22]. Since 2002, a regular increase in these fatal intoxications has been recorded, mainly due to an intensified prescription of this product [22].

2. Materials and methods

2.1. Necrophagous fly experiment

Larvae of the blowfly *L. sericata* were collected from a field in Brussels (National Institute of Criminology and Criminalistics, Brussels, Belgium). The flies were held in an insectarium at 24 ± 2 °C with 70 ± 5% relative humidity (RH) and a photoperiod of LD 16:8 h. About 200 flies were kept in gauze cages (35 cm × 35 cm × 35 cm) and fed ad libitum with water, sugar, powdered milk and brewer's yeast. Flies were also supplied with pig blood to allow ovarian maturation.

The feeding substrate consisted of heart beef (50 g) and a 8% gelatin 200 bloom solution (Louis François, Croissy Beaubourg, France) (50 mL). To this substrate, 5 different methadone concentrations were spiked. Treatments were: T0 control (no methadone); T1 0.2 μg/g methadone; T2 0.4 μg/g methadone; T3 0.8 μg/g methadone; T4 4 μg/g methadone. Spiked concentrations were selected based on reports of human fatalities [23] and on the previous works by Hecht et al. [16] and Strehler et al. [18]. Each meat batch was mixed in a Waring blender (VWR, Leuven, Belgium) for 5 min at ambient temperature to ensure a uniform spread of methadone. Stability and homogeneity of the substrate were controlled by a routine GC–MS analysis.

For oviposition, fresh beef heart was provided. Eggs batches (after 3 h) were collected [24] and deposited on 250 g of beef heart in a plastic box in incubator (Sanyo, Incubator MIR 553) at 25 °C. About 50 newly hatched larvae 1st (approximately 2 h) were deposited with paintbrush in a cup containing 100 g of foodstuff. This low density of larvae allows a reduction of generated heat and degree of competition, which both affect the development rate and survival [25,26]. Each cup was placed in a plastic box containing sand to provide a dry place for pupation. About 1500 specimens in 30 cups were studied for different physiological parameters. Experiments were conducted in incubators (Sanyo, Incubator MIR 553) set at constant temperatures 25 ± 1 °C with a photoperiod of LD 12:12 h. A data logger (Testo, 174) was inserted into the incubators to monitor the temperatures each hour.

The developmental time was monitored every 12 h during photoperiod [27]. Emerged adults were anaesthetized with carbon dioxide (CO_2). The sex was recorded daily and the adults were removed from the box. Empty puparial cases were harvested and stored at −20 °C until analysis.

Statistical analyses were conducted using open source software R-2.8.1. The calculation of Wilcoxon rank sum is used to test homogeneity in the sex-ratio. Emerged blowflies were compared by Kruskal–Wallis rank sum test. The Kolmogorov–Smirnov test compared the fitted curves of the control to the fitted curves of the treatments to determine if there was a difference for each sex between developmental times due to the presence of methadone.

2.2. Analytical method for quantification of methadone

2.2.1. Reagents

Drug standards of methadone and EDDP (1.0 mg/mL methanol) were purchased from LGC Standards (Molsheim, France). A mixed stock solution was prepared (4 μg/mL), which was further diluted with 0.1% aqueous formic acid to yield working solutions at appropriate concentration to prepare calibrator-samples.

Deuterated internal standards, methadone-d_9 and EDDP-d_3 were also obtained from LGC Standards (Molsheim, France) at a concentration of 0.1 mg/mL in methanol. A mixed stock solution was prepared (1 μg/mL) and further diluted with 0.1% aqueous formic acid.

Ammonium chloride buffer (powder) and formic acid (98%) were purchased from Sigma–Aldrich (St. Louis, MO). Chlorobutane (HPLC-grade) was purchased from Merck (Darmstadt, Germany). Water, 0.1% aqueous formic acid (ULC–MS grade) and acetonitrile (ULC–MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands).

2.2.2. Sample preparation

Empty puparial cases were copiously washed with deionized water (2 cycles) and dried using a paper towel to avoid contamination from the food sources. Each single empty puparial case of *L. sericata* was placed in a disposable vial (Precellys kit MK28 with metal beads, France); use of disposable vials prevents cross contamination between samples. Thereafter, samples were grinded to powder for 30 s at 6500 rpm in a tissue homogenizer Precellys 24 (Bertin Technologies, Montigny-Le-Bretonneux, France). The samples were transferred to a glass vial after addition of 600 μL of deionized water and 500 μL of saturated ammonium chloride buffer (pH 9.2). After addition of 4 mL of 1-chlorobutane, mechanical shaking was carried out for 10 min. Following centrifugation at 2333 × *g* for 10 min, the clear organic phase was transferred to a clean vial and evaporated to dryness in a vacuum centrifuge (Jouan, Heverlee, Belgium). Then the samples were reconstituted in 500 μL of aqueous mobile phase and an aliquot of 10 μL was injected into the UPLC–MS/MS system.

2.2.3. Calibrators and quality control samples

Before pulverization, the average weight of an empty puparial case was determined to be 2.5 mg. A series of calibrators (10, 20, 30, 40,100, 250 pg/mg) and quality control (QC) samples (48, 96, 160 pg/mg) were prepared by spiking the pulverized drug-free empty puparial case aliquots with methadone and EDDP standards.

2.2.4. UPLC–MS/MS

Analytes were separated using an Acquity UPLC HSS C18 column (2.1 × 100 mm, 1.8 μm) (Waters, Milford, MA, US) set at 30 °C, using a flow rate of 0.35 mL/min in gradient mode. All aspects of system operation and data acquisition were controlled using MassLynx V4.1 SCN627 software (Waters). The aqueous solution A consisted of 0.1% formic acid, and solvent B was acetonitrile. The gradient was carried out starting from 20% B to be linearly increased to 40% B over the first 3 min. At 4 min B was set to 95% for 1 min returning then to the initial conditions and equilibrating for 1.5 min, resulting in a total run time of 6.5 min. The mobile phase flow was set to 0.35 mL/min during the whole run.

A Quattro Ultima triple-quadrupole MS (Waters) fitted with a Z-Spray ion interface was used. Ionization was achieved using electrospray in positive ionization mode (ES+). The following conditions were optimal for the analysis of methadone and EDDP: capillary voltage, 1.0 kV; source block temperature, 120 °C; desolvation gas (nitrogen) heated to 350 °C and delivered at a flow rate of 800 L/h. In order to establish appropriate multiple reaction monitoring (MRM) conditions for the methadone, EDDP and their respective deuterated analogues (solutions of the standards in 50 ng/mL 50:50 (0.1% aqueous formic acid: methanol, v/v)) were individually infused into the MS and the cone voltage (CV) was adjusted to maximize the intensity of the protonated molecular species $[M+H]^+$. Collision induced dissociation (CID) of each protonated molecule was performed. Collision gas (argon) pressure was maintained at 2.5×10^{-3} mbar and the collision energy (eV) adjusted to optimize the signal for the most abundant product (quantifier) and

Table 1
Summary of studies about effects of opioids on adult physiology of blowflies.

Opioids	Species	Substrate	Effect on adult physiology			References
			Developmental time	Sex-ratio	Adult emergence	
Codeine	*Lucilia sericata*	Artificial	Yes	/	/	[17]
Heroin	*Boettcherisca peregrina*	Rabbit	Yes	/	No	[15]
Methadone	*Calliphora vicina*	Artificial	Yes	/	/	[18]
	Lucilia sericata	/	Yes	/	No	[16]
Morphine	*Lucilia sericata*	Rabbit	No	/	/	[19]
	Calliphora stygia	Artificial	No	/	No	[14]
	Calliphora vicina	Rabbit	No	/	/	[20]
Oxycodone	*Phormia regina*	Pig	No	No	/	[21]

for a lower product (qualifier). MS spectra and MS/MS spectra were collected during infusion experiments. Quantifier and qualifiers transitions of methadone and EDDP are shown in Table 2. For the corresponding deuterated analogues, only one transition was monitored.

2.2.5. Method validation

Validation was performed based on the FDA guidelines [28] and recent publications concerning validation of bioanalytical methods [29,30].

The selectivity of the method was verified by examination of the chromatograms obtained after the extraction of nine blank empty puparial cases from a different source. Moreover, blank empty puparial cases ($n = 3$) spiked with 27 benzodiazepines, benzoylecgonine, codeine, cocaine, 6-MAM, morphine, THC, 11-OH-THC, THC-COOH, zolpidem, zopiclone and zaleplon were also analyzed to check for interferences.

Standard curves, freshly prepared with each batch of QC samples and authentic samples, were generated using a least-squares linear regression, with a $1/x$-weighting factor for all compounds.

The limit of detection (LOD) was determined from blank empty puparial cases, spiked with decreasing concentrations of the analytes of interest. It was defined as the concentration for which the response of the qualitative ion could reliably be differentiated from background noise, i.e. signal-to-noise ratio (S/N) equal to or greater than 3:1. The acceptance criteria for ion ratios should be equal to or lower than 20% and retention time deviations lower than 3.5% relative to that of the corresponding control or calibrator.

The limit of quantification (LOQ) was estimated by duplicate analysis over 8 different days and was defined as the lowest analyte concentration with a bias and imprecision less than 20%.

Repeatability and intermediate precision were evaluated by replicate ($n = 2$) analysis of the QC samples performed over eight different days. Imprecision (expressed as $\%RSD_r$ for repeatability and $\%RSD_t$ for intermediate precision) was determined by performing the analysis of variance: a 'single factor' ANOVA test (significance level (α) of 0.05) [29]. Bias of the method was determined by comparison of the mean of calculated concentrations of QC samples to their respective nominal values.

The stability of the processed sample, when placed in the auto-sampler (maintained at 6 ± 2 °C), was checked by repeated injections of two extracted QC samples at 24 and 160 pg/mg empty puparial case ($n = 6$). Six extracted samples at each concentration were analyzed directly ($T = 0$) and after 96 h. The internal standard (IS) was always spiked just before analysis and the stabilities were estimated by comparing the peak response ratios at each concentration [30].

To assess any potential matrix-effect (ME) and to determine the recovery of the liquid–liquid extraction (LLE), the experiment described by Matuszewski et al. [30] was performed. ME was determined by comparison of the peak responses of the analytes spiked at 48 and 160 pg/mg ($n = 6$) to blank empty puparial case extracts with those obtained after being spiked in the mobile phase at the same concentration levels. Extraction recoveries were determined at a concentration of 24 and 160 pg/mg empty puparial case corresponding respectively at QC low and high ($n = 6$). Therefore, standard working solutions were spiked in blank matrix samples (extraction samples) before or after sample preparation (control samples).

Carryover was evaluated by the analysis of blank empty puparial cases spiked with the IS after the analysis of the upper calibrator (200 pg/mg of puparia, $n = 8$), and after the analysis of experimental puparia samples ($n = 16$).

3. Results and discussion

3.1. Validation of the method

The applied chromatographic method ensured the elution of all the compounds within 6.5 min and produced peaks of acceptable symmetry. The selectivity of the method was acceptable in terms of absence of interferences in the nine analyzed blank samples. No interferences were observed after the analysis of blank larvae spiked with co-administrated drugs, ensuring the selectivity of the method.

The linearity was tested from 10 to 200 pg/mg puparial case and an $r^2 > 0.99$ for all the compounds was observed. Back-calculated concentrations did not deviate more than ±15% of the nominal calibration values of the weighed ($1/x$) linear regressions for the selected range during 8 different days.

The LOD and LOQ were set respectively at 2 pg/mg and 10 pg/mg puparial case for all the compounds.

Fig. 1 shows the MRM chromatograms obtained following the analysis of spiked with the lowest calibrator. Bias and imprecision were satisfactory with all RSDr and RSDt lower than 10%. The results indicated that the bias of the assay was lower than 5% for the compounds.

The autosampler stability samples were tested against the mean value of the stability samples (=control samples). The samples should fall within 90–110% of the mean of the control samples and the 95% confidence interval of the stability samples should be included in the interval 80–120% of the mean of the control samples. No instability was observed after 96 h at 4 °C. Freeze-thaw stability was not evaluated due to our experimental protocol; samples were analyzed directly and in total after storage in freezer [3].

No significant matrix effect was observed with this sample clean up procedure. High and reproducible recoveries were obtained with this LLE procedure for all analytes (>86% for all the compounds).

No carryover was observed in a blank sample injected after the analysis of the upper calibrator (200 pg/mg puparial case) and after the analysis of experimental puparial case samples. The wash procedure was optimized to diminish carryover; a strong wash solvent of methanol, acetonitrile, water, isopropanol (25:25:25:25) with 0.1% formic acid was used.

3.2. Quantitative analysis of pupae reared on methadone spiked substrate

Accumulation of methadone was only measured at treatment T3 and T4 but not at T0, 1, 2 for single puparial cases (Fig. 2). This result confirms that while methadone is excreted by larval organs (via Malpighian tubules or nephrocytes), a quantity of drug is included into the cuticle of the puparium [12]. A drug can only be detected from insects when its rate of absorption exceeds the rate of elimination [1]. For methadone, ratio elimination/accumulation is superior to 1 below T3 and thus no bio-accumulation in the cuticle was observed. Concentrations in puparial cases seem to be stable between treatments (T3, 4). However, methadone has not been quantified in all specimens (only 12 and 15 specimens), confirming variability within a pool of puparial cases. This variability, already observed in larvae [8,11], suggests that absence of a drug from the larvae or puparial case is not necessarily an indication that it was not present in the corpse. In addition, we must be aware that methadone puparial case concentrations are 60 times lower than methadone concentration in 3rd instar larvae [3].

EDDP is only quantified in a few specimens at T3. In a previous study by our group, it was clear that in post-feeding larvae the

Table 2
MRM transitions and conditions for the measurement of methadone and EDDP and their respective deuterated analogues.

Compound	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Methadone	310.3	105.1	40	30
	310.3	265.2	40	15
Methadone-d_9	319.4	268.3	50	15
EDDP	278.3	234.2	40	30
	278.3	249.3	40	20
EDDP-d_3	281.2	234.0	30	30

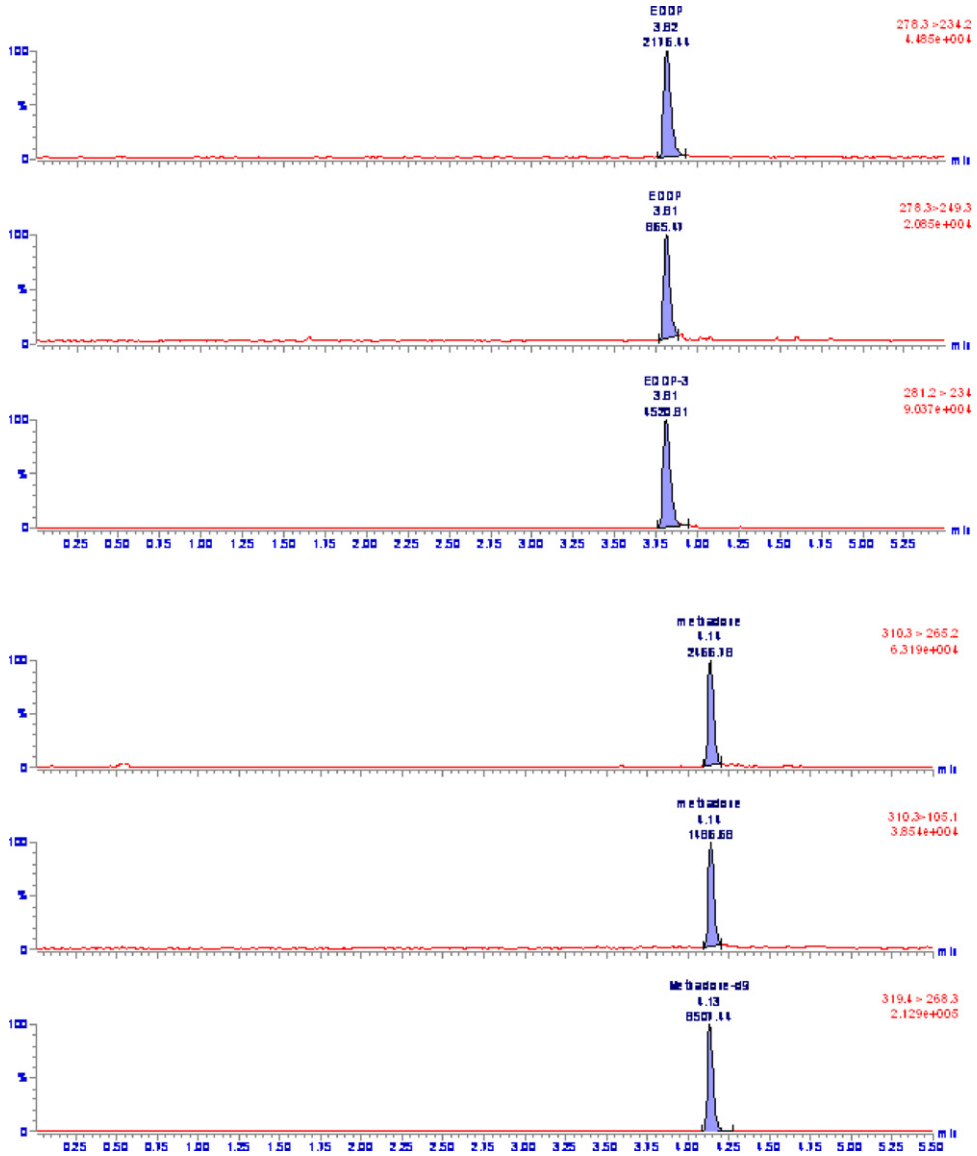

Fig. 1. MRM chromatograms (LOQ) for (top to bottom): methadone (quantifier), methadone (qualifier), methadone-d_9, EDDP (quantifier), EDDP (qualifier), EDDP-d_3 in a single empty puparial case. Detected peaks are annotated with the integrate area under the MRM chromatogram. Peak intensity and MRM's are shown on the top right-hand corner of each trace.

elimination was already stronger than for methadone [3]. In fly larvae, water-soluble molecules are generally excreted from the haemolymph by Malpighian tubules [31]. More hydrophobic toxins, become more hydrophilic by biotransformation in insects [32]. These less-soluble substances are deposited in close proximity to pore canals in the cuticular matrix [31]. The elimination of EDDP confirms that the metabolite, more hydrophilic, is not accumulated in insect tissue. Moreover, larvae metabolize and eliminate drugs with varying levels of efficiency [11]. Elimination of a compound will differ according to their chemical complexities and lipid/water solubility [8].

During pupariation, an opaque layer of fat bodies is deposited under the cuticle [33]. In this respect, the cuticle acts as storage organ, similar to adipocytes and pericardial nephrocytes [31]. Methadone is probably deposited in this layer. However, immunohistochemical studies on methadone localization must be conducted in the near future to confirm this hypothesis. In [9,15], the parent drug concentration is superior to metabolite concentration in all puparial cases. The parent/metabolite ratio is the inverse for nordiazepam [13] and for codeine in pupae [17].

Few studies [12,17,20,34,35] demonstrate a correlation between drug concentrations in feeding larvae and their substrate. However, several authors are skeptical concerning these correlations [5,6] due to inter-larvae variations. In this study, no correlation between methadone concentration in a single puparial case and in its substrate is observed. No significant difference between methadone concentrations in T3, 4 were measured ($W = 83$, p-value = 0.5617). This threshold (T3) corresponds to lethal concentrations found in different organs (brain, kidney and liver) sampled on humans intoxicated by methadone. The threshold of lethality (minimum) ranged from 0.5 μg/g (brain) to 1.8 μg/g (liver) with a maximum average at 3.8 μg/g [23]. This cut-off (T3) could be used as threshold for human fatality but should be confirmed in future studies.

3.3. Effects of methadone on physiological parameters of L. sericata

3.3.1. Sex-ratio

All of the treatments demonstrate that emerged flies were not biased towards one sex ($W = 19$, p-value = 0.94). The results of sex-ratio are presented in Table 3. According to Monthei [21], the sex-

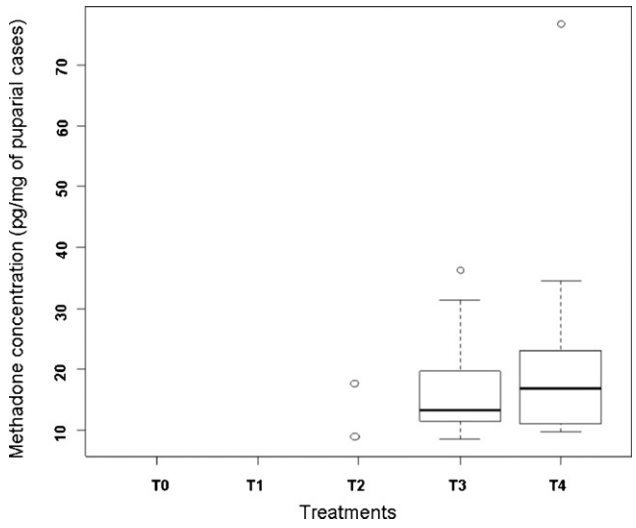

Fig. 2. Boxplots of methadone concentrations (pg/mg puparial cases) in single empty puparial case of *Lucilia sericata* for different treatments (T0, 1, 2, 3, 4). Number of specimens used in this analysis is respectively for T2 (2), T3 (15) and T4 (12). No traces were detected in T0, 1.

ratio is biased towards one sex (males) by 2:1 in the untreated tissue but not for the adults that emerged from the oxycodone treated loin, liver tissue and control liver.

It is difficult to generalize the methadone influence on sex-ratio for all opioids. Use of different drugs, insects species or experimental conditions such as temperature or food can also affect sex-ratio [21,36]. Sex-ratio distortion towards one sex due to presence of toxic substances could modify blowfly development if correlation between sex and developmental time is confirmed. Experiments testing this hypothesis should be performed for opioids in the future.

3.3.2. Percentage of emerged blowflies

A significant difference was detected in the number of adults that emerged between all treatments (Kruskal–Wallis chi-squared = 12.88, df = 5, p-value = 0.02) but there is no trend towards high mortality with methadone treatment. Emerged blowflies (%) are presented in Table 3. In Hecht et al. [16], the percentage of emergence rises with methadone concentration, but the maximal concentration is below the lethal concentration in human organs and thus does not reflect reality. We suggest that the intrinsic variability is of more importance and does not reflect a clear methadone effect. Few studies deal with insect mortality due to opioids. Previously published work [14–16] indicates that high drug concentrations do not tend to result in high mortality, as one might expect.

3.3.3. Development

The developmental time is similar to previous studies [16,18,37]. In our study, the window of development is similar for all treatments. However, there is a shift for median interval time (14 days) in T4.

Table 3
Sex-ratio and emerged adults for each treatment.

Treatments	Adults (N)		Emerged adults (%)
	Males	Females	
BL	107	98	82.0 ± 5.5
T0	95	87	72.8 ± 6.4
T1	88	101	75.6 ± 5.2
T2	67	85	76.0 ± 9.9
T3	91	82	69.2 ± 6.7
T4	102	114	86.4 ± 7.3

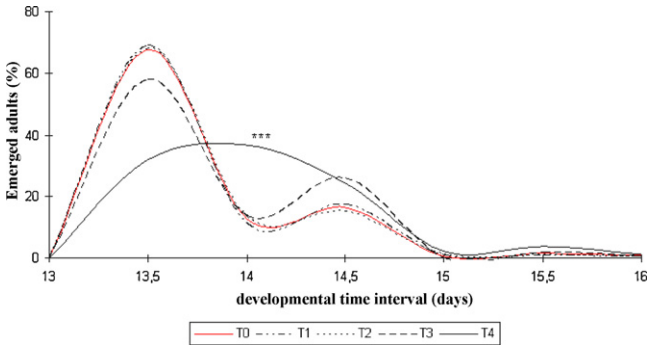

Fig. 3. Emerged adults of *Lucilia sericata* (%) at different treatments. Asterisks (Kolmogorov–Smirnov $^{***}p < 0.001$) indicate emergence profile that differ statically from T0.

When comparing developmental curves (Fig. 3), a significant difference was observed at $\alpha = 0.001$ using the Kolmogorov–Smirnov test in development on T4 (respectively $D = 0.36$). No significant differences were detected between T1, T2 and T3 (respectively $D = 0.01$, 0.01, 0.1) and T0. So, development of *L. sericata* seemed to be slightly decelerated in presence of high methadone concentration (T4). However, because of the lack of differences for firstly emerged blowflies, there should not be any alteration of PMI estimation due to methadone when the minimum developmental time data of *L. sericata* are used [37].

This modification of development could be due to the drug detoxification process, with a mobilization of reserve in the fat-body for the energy production [38]. For T1, 2, rapid elimination of parent drugs and metabolites result in no alteration of insect physiology. From T3, methadone accumulates in the insects' remains (cuticle in puparia and meconium in adults). In T4, accumulation in the cuticle is no longer possible and methadone is stocked in fat bodies. Previous studies have shown that drugs are accumulated in adipocytes [31], and that there is a possible alteration of fat bodies in these reserves [38] which could act on insect physiology. A more detailed study on methadone localization and alterations of fat bodies must be realized in near future to better understand these processes.

Our results are similar to those from previous comparative studies performed on methadone spiked substrates [16], but a high variability between treatments in these studies did not allow a generalization of methadone effect. This variability could be due to a species effect (*Calliphora vicina* on the former study, *L. sericata* in the present one) or to the different experimental design.

4. Conclusion

The presented method was validated based on the FDA guidelines and is 'fit for purpose'. The quantification of methadone in a single empty puparial case from a methadone spiked substrate was successful. However, no linear relationship between the methadone concentration in the substrate and an empty puparial case could be established. EDDP, the primary metabolite of methadone, is completely eliminated by larvae and only few traces of it were detected in the examined puparial cases.

No methadone effect on sex-ratio was measured, but a significant difference was observed for mortality and insect development.

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