Chlorpromazine Impacts on the Length and Width of *Sarcophaga haemorrhoidalis* (Diptera: Sarcophagidae) Larvae: Potential Forensic Implications[1]

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Abstract The effect of chlorpromazine (CPZ) on the larval length and width of *Sarcophaga haemorrhoidalis* (Fallén) (Diptera: Sarcophagidae) was investigated under laboratory conditions for possible implications for forensic investigations. Fresh beef was treated with one of four concentrations of CPZ (0, 0.5, 1.0, and 1.5 μg per g of beef) to simulate postmortem concentrations in CPZ-dosed corpses. Ten replicates of 50 *S. haemorrhoidalis* eggs each were placed on the treated beef and maintained at 27°C. The length and width of 2 d old and 4 d old larvae were measured for all treatments and replicates, and the presence of CPZ in the treated meats was qualitatively verified using liquid chromatography–mass spectrophotometry coupled with electrospray ionization. Pearson's product-moment correlation coefficient revealed significant correlations of CPZ concentration with both larval length and width at the two larval ages ($P < 0.001$). However, the mean length and width of larvae fed on CPZ-treated beef did not differ significantly from the length and width of larvae fed on untreated control meat, except with larvae fed on 1.5 μg CPZ per g meat ($P < 0.001$). We conclude from these laboratory assays that *S. haemorrhoidalis* larvae may prove to be a reliable model to use in the estimation of the minimum postmortem interval in corpses that contain CPZ <1.5 μg/g.

Key Words chlorpromazine, Sarcophagidae, forensic entomology

For insect-infested decomposed bodies, there are two major methods of estimating minimum postmortem interval (minPMI): insect succession patterns and insect growth rates (Amendt et al. 2007, George et al. 2009, Goddard et al. 2015, Goff 1993, Syamsa et al. 2017). Several forensic studies have investigated the effect of drug overdoses on different developmental stages of forensically important blowflies (Diptera: Calliphoridae) and consequent estimation of minPMI (de Carvalho et al. 2012, Fathy et al. 2008, George et al. 2009, Goff and Lord 1994, Verma and Paul 2013). However, the impact of chlorpromazine (CPZ), an antipsychotic medication, on *Sarcophaga haemorrhoidalis* (Fallén) (Diptera: Sarcophagidae) (Zumpt 1965) has not previously reported.

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S. haemorrhoidalis can be used as an indicator species when establishing minPMI in forensic investigations (Byrd and Butler 1998), including estimation of minPMI of severely burnt remains (Showman and Connelly 2014). Adult flies are attracted to corpses at early stages of decomposition to deposit larvae on the decomposing flesh in a process called larviposition where eggs hatch within the female. The species develop into four life stages: egg, larva, pupa, and adult fly (Introna et al. 1998, Showman and Connelly 2014). Oviposition was also reported for the same fly (Archer and Elgar 2003, Shalaby et al. 2000). Larval development duration of *S. haemorrhoidalis* varies with temperature (Byrd and Butler 1998; Lecheta et al. 2015) with an approximate duration of 4 d at 23–28°C (Madubunyi 1986) and 7–9 d at 25°C (Pape 1996, Showman and Connelly 2014).

CPZ is classified as a tranquilizer belonging to the phenothiazine chemical class and is used to treat symptoms of psychiatric and behavioral disorders by blocking the action of the neurotransmitter dopamine in the brain (Dundee 1954, Upfal 2007). Thorazine (Bayer AG, Leverkusen, North Rhine-Westphalia, Germany), a brand name of CPZ, is used in the therapy of schizophrenia, an illness of disturbed thinking and emotions (Feldman et al. 1956, Upfal 2007). In addition to its anti-emetic and anti-anxiety effects, Thorazine has been used to treat chronic hiccups (Dundee 1954).

Autopsies have shown that CPZ is detected in femoral blood and urine samples (Kinoshita et al. 2008, Takase et al. 2010, Tanaka et al. 2011, 2012). In cases of accidental or intentional exposure, the reported levels of CPZ range from 0.1–0.2 mg/dl blood (Gossel and Bricker 1994). Some commonly reported CPZ side effects include: pyrexia, tachycardia, drowsiness, coma, hypotension, muscular tremors, and convulsions (Upfal 2007). In fact, patients with schizophrenia attempt suicide three times greater than other psychiatric patients (Siris 2001). A retrospective study conducted in Japan from 2003 to 2006 ranked CPZ as third among the 22 most frequently detected compounds in 535 poisoning cases and found that the sales of CPZ correlated with associated mortality rates (Kudo et al. 2010). For forensic entomologists, encountering corpses containing CPZ may alter their estimation of minPMI, which is used to clarify timelines in both suspicious and non-suspicious deaths (including suicides).

This study was designed to examine the effect of CPZ-treated meat on the development of *S. haemorrhoidalis* larvae to determine the reliability of using *S. haemorrhoidalis* to estimate minPMI of victims with CPZ in their systems. This laboratory study provides some foundation for possible impact on forensic investigations, but it is only an initial step.

Materials and Methods

S. haemorrhoidalis colonies were obtained from the Laboratory of Forensic Science at Jordan University of Science and Technology. Adult flies were kept in 30-liter clear, cylindrical plastic containers (Dhilal Plastic, Amman, Jordan). The containers were modified according to methods of George et al. (2009) with openings cut in two sides (160 $\times$ 120 mm) and in the lid (230 $\times$ 260 mm) of the containers and covered with organza for ventilation. A 130-mm diameter opening was cut in the front of the container to attach an organza sleeve for accessing the

interior. Sugar cubes, sheep liver (to allow ovarian maturation), and water were provided to adult flies *ad libitum*. All experiments were conducted in a ventilated rearing room at 27 ± 2°C with 60% relative humidity on a 14:10 h light:dark cycle; these conditions were modified from previous studies with *S. haemorrhoidalis* (Byrd and Butler 1998, Madubunyi 1986).

Local fresh beef (never frozen, no spices or additives) was treated with four concentrations of CPZ (0, 0.5, 1.0, and 1.5 μg per g meat). These concentrations were determined on the basis of concentrations found in femoral blood of reported human fatalities (Kinoshita et al. 2008, Takase et al. 2010, Tanaka et al. 2011, 2012). Furthermore, a range of 0.5–2.0 μg CPZ/ml was reported as the toxic range in postmortem heart blood (Takase et al. 2010). Treated meats were prepared using a 1×10^{-2} M stock solution of CPZ (Sigma-Aldrich, St. Louis, MO) that was serially diluted to the target concentrations. Beef (1 kg each) was placed in 25 ml of each treatment solutions and controls were treated with 25 ml deionized water. To ensure uniform distribution of CPZ, each meat batch was mixed thoroughly by hand for 5 min. Then, the batches were divided into 20 × 50-g portions, stored in 250-ml plastic bags (Crystal Plastic Factories, Amman, Jordan) at − 20°C, and thawed before use. Indeed, CPZ was found to be stable in freeze–thaw stability tests in rat plasma and brain homogenates for 3 freeze–thaw cycles (Zhang et al. 2007).

The colonies of *S. haemorrhoidalis* were checked hourly during light phases to collect freshly deposited eggs. The eggs were transferred to 9-cm diameter petri dishes, each prefilled with 50 g of meat prepared as previously described, and covered with 0.5-cm thick, wet cotton to prevent desiccation. These were placed in 2-liter rectangular, clear plastic containers (180 × 120 × 70 mm) (Diamond Plastic Factory, Amman, Jordan). Fifty eggs per replicate were placed on the meat in each of the 4 treatments with 10 replicates per treatment. White, woven wire mesh (0.41 mm) (The Mesh Company Ltd., Warrington, United Kingdom) was used to cover the containers. Treatments were checked hourly to monitor for egg hatch. At that time, each replicate was assigned a start time for measurements of larval size at 2 and 4 d after hatch.

The width and length of 20 randomly selected larvae from each replicate in the designated four treatments were measured on day 2 (48 h after hatching) and on day 4 (96 h after hatching). *S. haemorrhoidalis* larvae require 48 h as a period of maximum growth, whereas 96 h is the period before starting active migration and abandoning food substrate for pupation (Madubunyi 1986). Those larvae collected for measurement were fixed in boiling water for 1 min and patted dry against paper towels. Then, the larvae were photographed with a digital camera (Model PC 1256, Canon, Tokyo, Japan) to be analyzed later by Image J v1.37 software (National Institutes of Health, Bethesda, MD), with an image scale set to 1 mm. Larval length and width were measured according to a method used by Day and Wallman (2006) in which the length was measured between the most distal parts of the head and the eighth abdominal segment, and the width was measured at the junction of the fifth abdominal segments.

Electrospray ionization mass spectrometry coupled to liquid chromatography was conducted at the Pharmaceutical Research Center at Jordan University of Science and Technology. Methods from Zhang et al. (2007) were used for qualitative detection of CPZ in the meat to ensure continuous presence of the chemical during the test. Aliquots of meat samples were analyzed daily until the end

of day 4 from all replicate containers of the four treatments. The samples were separated using Waters Atlantis TM dC-18 (30 × 2.1 mm internal diameter, 3 μm) column with a 4.0 × 2.0 mm Phenomenex Security Guard C8 column. Mobile phase A contained 20 mM ammonium formate (Fisher Scientific, Shanghai, China) in water. The pH was adjusted to 4 by using formic acid (Fisher Scientific, Shanghai, China). Mobile phase B was acetonitrile (Fluka, Buchs, Switzerland). The flow rate was set to 0.3 ml/min. The injection volume of each sample was 10 μl. The samples were separated and eluted using the following gradient for minutes, mobile phase B percentage, and flow rate in ml/min: (0, 15, 0.3) (5, 70, 0.3) (7.5, 77, 0.3) (7.6, 90, 0.6) (8.8, 90, 0.6) (8.9, 15, 0.3) (12.5, 15, 0.3). To remove the remaining meat extract residues from the column following each injection, acetonitrile (90%) was used to wash the column at a flow rate of 0.6 ml/min; without that step, ion suppression from the meat extract residues of the previous injection would cause decreased response of CPZ in next injection. The column temperature was maintained at 25°C. The auto-sampler needle was washed with methanol (Fluka) to reduce carry-over after each injection.

Data were analyzed using the package "Rcmdr" within R software, version R 3.2.3 (R Development Core Team, 2015); $P \leq 0.05$ was considered significant for all of the analyses. Normality was tested using Shapiro–Wilk normality tests, and homogeneity of variance was determined by using Levene's test (George et al. 2009). The means of treated groups were compared to the control by using one-way analysis of variance with Tukey contrasts for specific comparison of means (de Carvalho et al. 2012, George et al. 2009). The correlation of CPZ concentrations with both larval length and width was examined using Pearson's product-moment correlation coefficient.

Results

The continuous presence of CPZ in the beef samples was confirmed by liquid chromatography–mass spectrophotometry chromatograms, whereas no CPZ was detected in the control meat. Samples of the meat were obtained from different sites assuming that the feeding action of larvae mixed the meat and kept the chemical content homogenous (George et al. 2009). These results concur with findings by Saar et al. (2012), who detected and measured CPZ up to 9 d postmortem in 273 autopsy blood samples.

Two-day-old larvae exposed to 0, 0.5, 1.0, and 1.5 μg CPZ per g of meat had a mean (± SD) length of 9.89 (± 2.52), 10.67 (± 2.10), 10.77 (± 2.12), and 12.46 (± 1.86) mm, respectively, and the mean (± SD) width was 2.07 (± 0.41), 2.08 (± 0.27), 2.04 (± 0.33), and 2.32 (± 0.31) mm, respectively. At 4 days after hatch, the mean (± SD) length was 11.96 (± 1.78), 11.87 (± 1.40), 12.43 (± 1.77), and 13.33 (± 1.31) mm, whereas the mean width was (± SD) 2.33 (± 0.44), 2.46 (± 0.30), 2.40 (± 0.28), and 2.58 (± 0.29) mm for the respective CPZ concentrations. The mean length and width of larvae fed on the highest concentration of CPZ were significantly greater than those fed the control and lower concentrations of CPZ at day 2 and day 4 (Fig. 1) (day 2: length: $F = 31$; $df = 3$; $P < 0.001$, and width: $F = 11$; $df = 3$; $P = 0.001$; day 4: length: $F = 21$; $df = 3$; $P < 0.001$, and width: $F = 10$; $df = 3$; $P = 0.002$). Pearson's product-moment correlation coefficient revealed a significant

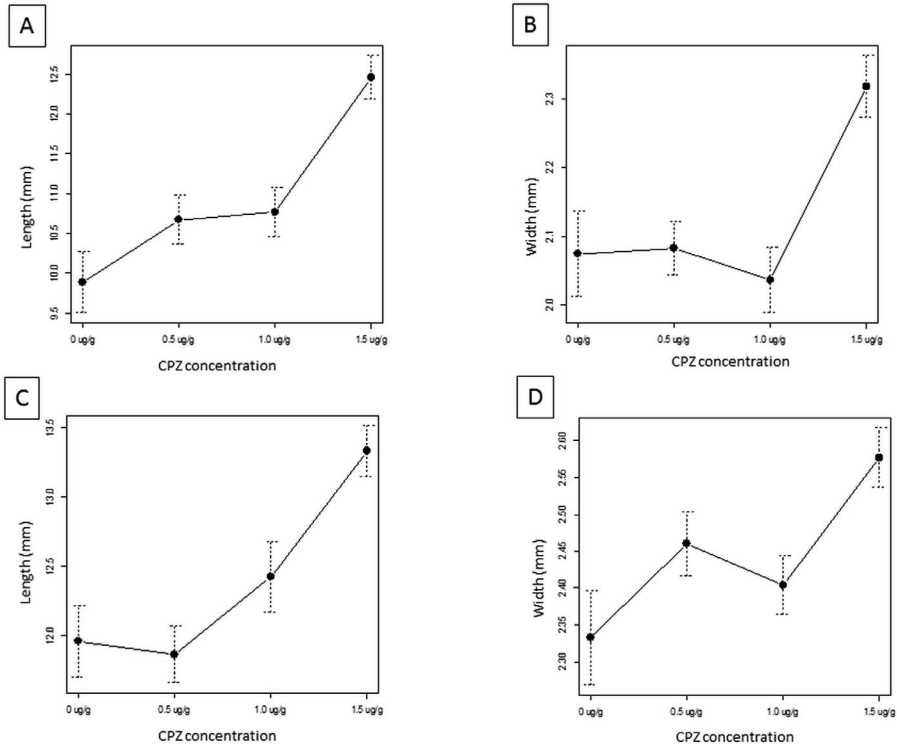

Fig. 1. Response of *Sarcophaga haemorrhoidalis* larval growth rates to chlorpromazine in diet with 2 d old length (A), 2 d old width (B), 4 d old length (C), and 4 d old width (D). Bars are standard deviation about the respective means.

correlation of CPZ concentration with both of length and width variables. The strongest positive correlation was observed at day 2 ($r = 0.7$; $P = 0.001$) (Table 1).

Discussion

CPZ was successfully added to beef in this study to simulate postmortem conditions and concentrations of the chemical. This approach was taken to avoid overdosing live animals for the study material and due to the knowledge that CPZ metabolism varies among animals. For example, the CPZ-metabolizing activity of the rat lung was found to be 10 times higher than that of the rabbit lung, with the principal metabolic pathway of N-oxidation in the rat lung and N-demethylation in the rabbit lung (Yoshio and Mehendale 1982). There are well-known examples of differences in metabolism of chemicals between humans and commonly used laboratory mammals, including nicotine in rabbits (Hucker et al. 1960) and paracetamol in cats (Court and Greenblatt 1997). Therefore, using another

Table 1. Linear regression statistics of the responses of 2 and 4 d old *Sarcophaga haemorrhoidalis* larval length and width to concentrations of chlorpromazine in diet.

Variable	*r*	R^2	Regression line	F value	*P* value
CI 1 Length	0.4	0.14	$8.11x + 97$	31	<0.001
CI 1 Width	0.2	0.05	$0.7x + 20$	11	0.001
CI 2 Length	0.3	0.09	$4.69x + 117$	21	<0.001
CI 2 Width	0.2	0.05	$0.68x + 23$	10	0.002

CI = Comparison Interval.

mammal as a model for such investigations is not guaranteed to simulate CPZ in human corpses.

It is also important to isolate the effects of CPZ from the potential confounding factors of secondary metabolites, especially when considering that metabolites could differ among species. In fact, CPZ metabolism differs among species and even among tissues within the same species. Guinea pig hepatic tissue produced N-oxides metabolites, whereas human hepatic tissue produced 7-hydroxyl metabolites with a minimal amount of N-oxides (Hartmann et al. 1983). In the same study, the guinea pig intestinal tissue formed desmethyl products of CPZ, whereas the human produced S-oxide products (Hartmann et al. 1983). A previous study emphasized that the interaction between hepatic microsomal enzymes and endogenous molecules could play a pivotal role in inhibiting CPZ metabolism in different species (Hartmann et al. 1983). In addition, there are external conditions that affect living organisms and alter their metabolism of chemicals, such as inflammation that is a major component of idiosyncratic drug reactions (Gandhi et al. 2013).

Any of the aforementioned conditions could impact blowfly larval growth that could, in turn, influence the interpretation of the observed outcomes. Considering that metabolism of CPZ is very complex (Hartmann et al. 1983), it is well known that the rates of metabolite formation and excretion differ considerably between humans and other animals for many drugs. Additionally, it was shown that metabolites could increase the effect of CPZ. For example, the demethylated metabolites, mono- and di-desmethyl-CPZ, were able to release aspartate aminotransferase in rat hepatocytes from 3 to 6 times more potent than CPZ (Abernathy et al. 1977), whereas 7-hydroxyhchlorpromazine was equally potent (Manian et al. 1965).

Compared to our untreated control, larval length and width on day 2 and day 4 were not significantly different at all concentrations of CPZ, except with those larvae exposed to the highest concentration, thus indicating that CPZ can affect *S. haemorrhoidalis* larval size at concentrations $\geq$1.5 μg/g. There may be several plausible explanations for the observation including, but not restricted to, (1) acting as a feeding stimulant by antagonizing α_1-adrenergic receptors (Fernández et al. 1986); (2) inhibiting juvenile hormone III production from corpus allata (Yang et al. 2014); (3) antagonizing dopamine receptors (Faeder et al. 1974); (4) activating the

cytochrome P450 enzyme that inactivates the juvenile hormone, JH III, by hydroxylation (Wu et al. 2016); (5) inducing anabolic or catabolic reactions including the Krebs cycle that enhanced cell generation necessary for growth (Gilbert 2009); (6) inhibiting apoptosis (Minamitani et al. 1989); and (7) enhancing the production of biomolecules (i.e., phospholipids, proteins, RNA) essential for development (Wu et al. 2016).

As a continuum to previous studies focused on larval and pupal growth to estimate minPMI in forensic investigations (de Carvalho et al. 2012, Fathy et al. 2008, George et al. 2009, Goff and Lord 1994, Verma and Paul 2013), our study reports significantly larger body length and width of 2 and 4 d old *S. haemorrhoidalis* larvae exposed to CPZ at $\geq$1.5 μg/g; no significant differences in larval size were noted in larvae exposed to $<$1.5 μg/g. The results of our laboratory investigation suggest that *S. haemorrhoidalis* larvae are reliable models to estimate minPMI in forensic investigations involving corpses with CPZ levels $<$1.5 μg/g. However, further research is needed to ascertain these responses in non-laboratory conditions and to further elucidate mechanisms that could have impacted the observed response of larval growth to CPZ.

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