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An international journal dedicated to the applications of medicine and science in the administration of justice

Forensic Science International

An international journal dedicated to the applications of medicine and science in the administration of justice

Forensic Science International is an international journal publishing original contributions in the many different scientific disciplines pertaining to the forensic sciences. Fields include forensic pathology and histochemistry, chemistry, biochemistry and toxicology (including drugs, alcohol, etc.), biology (including the identification of hairs and fibres), serology, odontology, psychiatry, anthropology, the physical sciences, firearms, and document examination, as well as investigations of value to public health in its broadest sense, and the important marginal area where science and medicine interact with the law.

Review Articles and Preliminary Communications (where brief accounts of important new work may be announced with less delay than is inevitable with major papers) may be accepted after correspondence with the appropriate Editor. Case Reports will be accepted only if they contain some important new information for the readers.

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Effects of methamphetamine and its primary human metabolite, *p*-hydroxymethamphetamine, on the development of the Australian blowfly *Calliphora stygia*

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ABSTRACT

The larvae of necrophagous fly species are used as forensic tools for the determination of the minimum postmortem interval (PMI). However, any ingested drugs in corpses may affect larval development, thus leading to incorrect estimates of the period of infestation. This study investigated the effects of methamphetamine and its metabolite, *p*-hydroxymethamphetamine, on the forensically important Australian blowfly *Calliphora stygia*. It was found that the presence of the drugs significantly accelerated larval growth and increased the size of all life stages. Furthermore, drug-exposed samples remained as pupae for up to 78 h longer than controls. These findings suggest that estimates of the minimum PMI of methamphetamine-dosed corpses could be incorrect if the altered growth of *C. stygia* is not considered. Different temperatures, drug concentrations and substrate types are also likely to affect the development of this blowfly. Pending further research, the application of *C. stygia* to the entomological analysis of methamphetamine-related fatalities should be appropriately qualified.

1. Introduction

Analysis of entomological evidence can greatly enhance the scope of death investigations where such evidence is present [1]. A primary objective of forensic examinations upon finding a corpse is the determination of the minimum postmortem interval (PMI). This is often made by studying any infesting insects [2], especially flies (Diptera), and involves an understanding of the biological characteristics of the species present in carrion, including their development [3]. By determining the age of these insects, it is possible to determine their time of colonisation, and hence, the minimum PMI [2]. Although the developmental rates of many forensically important species are known [4,5], bioclimatic influences have been shown to affect growth rates of fly species [6,7]. These factors include temperature, atmospheric humidity, larval density and body location. A notable environmental effect of forensic importance is the presence of drugs in a corpse and their subsequent effects on insect physiology. This is known as forensic entomotoxicology and failure to consider such factors may lead to errors in minimum PMI estimates [8–10]. Preliminary studies have shown that illicit drugs influence the development of various fly species [11–15]. In particular, the larvae of some species have shown accelerated growth upon exposure to stimulants [12,16]. However, few investigations have been undertaken into the physiological effects of drugs of abuse on Australian flies. If these endemic species are to be successfully used to correctly estimate minimum PMI, it is imperative to understand the effect of antemortem drug use and abuse on the growth rate in the postmortem period [17].

The worldwide abundance and social popularity of methamphetamine make it a prime candidate for entomotoxicological analysis. The potential for addiction or overdose for first-time users is one of the highest of all available drugs due to its extreme and enduring physiological effects [18–20]. In previous experiments, whole animals have been used to simulate the pharmacokinetics of methamphetamine in humans [12,20–22], however, there is still some debate as to whether this is optimal as it has been reported that drug metabolism is species-specific [23]. Furthermore, the metabolic breakdown and excretion rates of methamphetamine are also species-dependent. When compared to laboratory rodents, methamphetamine is metabolised at a slower rate and less effectively in humans [23]. As a result, sustained administration of drugs is required to achieve plasma, urine or tissue concentrations comparable to humans, which in turn produce

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more metabolites. As all of these metabolites have the potential to affect larval growth, the effects of metabolites of interest could easily be overrated. The primary metabolic product of methamphetamine degradation in humans is *p*-hydroxymethamphetamine, which accounts for 15–50% of all metabolites excreted in urine [23]. It is itself a potent hallucinogen, and contributes significantly to the half-life of methamphetamine [18]. However, in non-human animals, the primary metabolic products of methamphetamine breakdown have been recorded as *p*-hydroxynorephedrine and *p*-hydroxymethamphetamine (rats), norephedrine and benzoic acid conjugates (guinea pigs), and amphetamine (rabbits) [12,23].

With the exception of amphetamine [24], no published studies have investigated the effects of these compounds on the growth rates of blowfly species. As certain metabolites are always present in overdose victims [20], necrophagous insects feeding on a cadaver will ingest them. The effect of these products on insect metabolic action needs to be substantiated in order to provide a valid minimum PMI estimate for drug-induced fatalities.

The eastern goldenhaired blowfly, *Calliphora stygia* (Fabricius) (Diptera: Calliphoridae), is among the most forensically important species in eastern Australia [25]. It is one of the first species present at a corpse, usually arriving within hours to oviposit. Previous studies on the effects of drugs on this species have focused on morphine [26,27], with no investigation having been done on stimulants or their metabolites. We report here for the first time the effects of methamphetamine and *p*-hydroxymethamphetamine on the growth rate of the blowfly *C. stygia*, with the aim of determining the suitability of this species as a model for estimates of minimum PMI in scenarios in which the corpse is contaminated with these illicit drugs.

2. Materials and methods

Methamphetamine and its primary human metabolite, *p*-hydroxymethamphetamine, were applied to the food source of *C. stygia* larvae to simulate postmortem conditions in methamphetamine overdose victims. Eggs were collected from blowfly cultures and assigned randomly to one of ten groups (nine treatments and a control). *Calliphora stygia* specimens were cultured through a minimum of one generation (maximum of five generations) from pupae obtained from Sheldon's Bait (Parawa, Australia). Flies were kept in plastic cages with mesh lids at 23 °C, and exposed to a photoperiod of 12:12 light:dark. Water and granulated sugar were provided *ad libitum*.

Sheep's liver was cut into 3 cm^3 pieces and supplied to cultures to facilitate ovarian maturation in female flies. The cultures, once sufficiently matured, were presented with fresh liver covered with a thin layer of cotton wool to encourage oviposition. Cages were checked every two hours and eggs transferred to a Petri dish. Eggs were counted into 90 groups of 150 each. Ten experimental groups were established for feeding larvae: (1) control, no methamphetamine (MA) or *p*-hydroxymethamphetamine (*p*-OHMA); (2) 0.1 mg/kg methamphetamine (0.1 MA); (3) 1.0 mg/kg methamphetamine (1.0 MA); (4) 10 mg/kg methamphetamine (10 MA); (5) 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-hydroxymethamphetamine (0.1 MA:0.015 *p*-OHMA); (6) 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-hydroxymethamphetamine (1.0 MA:0.15 *p*-OHMA); (7) 10 mg/kg methamphetamine:1.5 mg/kg *p*-hydroxymethamphetamine (10 MA:1.5 *p*-OHMA); (8) 0.015 mg/kg *p*-hydroxymethamphetamine (0.015 *p*-OHMA); (9) 0.15 mg/kg *p*-hydroxymethamphetamine (0.15 *p*-OHMA); and (10) 1.5 mg/kg *p*-hydroxymethamphetamine (1.5 *p*-OHMA).

The control batch containing no methamphetamine was prepared by mixing 15 mL of distilled water into 1.5 kg of kangaroo mince. Treatments of 10 mg/kg, 1 mg/kg and 0.1 mg/kg methamphetamine were prepared by dissolving 15 mg, 1.5 mg and 0.15 mg, respectively, of (±)-methamphetamine hydrochloride in 15 mL distilled water and mixing into 1.5 kg of kangaroo mince. *p*-Hydroxymethamphetamine concentrations of 1.5 mg/kg, 0.15 mg/kg and 0.015 mg/kg were prepared by dissolving 2.25 mg, 0.225 mg and 0.0225 mg of *p*-hydroxymethamphetamine, respectively, in 15 mL of distilled water and mixing into 1.5 kg of kangaroo mince. Concentrations of each ratio of drug:metabolite were prepared by mixing 2.25 mg, 0.225 mg, and 0.0225 mg of *p*-hydroxymethamphetamine with 1.5 mg, 0.15 mg and 0.015 mg of methamphetamine, respectively, in 15 mL of distilled water and 1.5 kg of kangaroo mince to simulate three postmortem ratios of drug and metabolite concentrations. These concentrations were deemed suitable for investigations based on commonly reported self-administered doses of (±)-methamphetamine hydrochloride that have consequently led to toxic bodily concentrations of the drug and death of methamphetamine users [18,28–30].

Each treatment was mixed by hand for 10 min, followed by 5 min of mixing with a blender to ensure an even distribution of drug, liquid and meat, or liquid and meat only for control batches. To avoid contamination, new gloves and mixing containers were used for each treatment, and the blender head rinsed in ethanol and flushed with near boiling water for 10 min between uses. Meat batches were split into 150 g portions and placed into plastic weigh boats. Batches were stored at −20 °C, and meat portions defrosted as required to replenish the larval food source.

(±)-Methamphetamine hydrochloride (99.7 ± 1.3%) and *p*-hydroxyamphetamine hydrochloride (*p*-OHAM) (99.7 ± 1.3%) were obtained under license from the National Measurement Institute (NSW, Australia). *p*-Hydroxyamphetamine hydrochloride was converted to the primary human metabolite of methamphetamine, *p*-hydroxymethamphetamine hydrochloride, prior to insect studies.

A solution of *p*-hydroxyamphetamine hydrochloride (8.49 mg) in Milli-Q water (1 mL) was adjusted to pH 12 by the drop-wise addition of sodium hydroxide (10%). The solution was then extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic layers concentrated to give *p*-hydroxyamphetamine (3.4 mg), which was used without further purification. Boc_2O (5.4 mg) was added to a solution of *p*-hydroxyamphetamine (3.4 mg) in THF (0.004 mL) and Et_3N (2.7 mg) and the reaction stirred at RT under N_2 for 24 h. CH_2Cl_2 (2 mL) was then added and the mixture sonicated and the organic layer extracted. This process was repeated twice and the combined organic layers dried ($MgSO_4$) and concentrated to give *p*-OHAM-Boc (6.3 mg, 80% yield). Confirmation of the success of the chemical transformation came from mass spectrometric analysis, with the ESI-MS spectrum showing a peak at *m*/*z* 374 ($M+H^+$), assigned to the protonated mass of the *p*-OHAM-Boc.

A solution of *p*-OHAMP-diBoc (14.3 mg) in dry THF (0.5 mL) was added drop-wise over 5 min to a mixture of NaH (1.45 mg, 2.42 mg from 60% NaH stock, 1.5 eq) in dry THF (1 mL) in a 5 mL flask under N_2 gas and at 0 °C. MeI (0.08 mL, 17.33 mg, 3 eq) was then added drop-wise into the mixture, which was stirred for 48 h at RT, and the reaction monitored by ESIMS. The reaction was quenched with water (2 mL), and the reaction extracted with CH_2Cl_2, the combined organic layers dried ($MgSO_4$) and then concentrated and dried under vacuum to obtain *N*-methyl-4-hydroxymethamphetamine (14.2 mg, 96 %) as an UV active pale yellow powder; ^{1}H NMR ($CDCl_3$, 500 MHz), 7.14 (d, ^3J = 7.3 Hz, 2H, H2′, H4′), 6.84 (d, ^3J = 7.3 Hz, 2H, H3′ and H5′), 3.78 (s, 3H, N—CH_3), 3.39 (d, ^2J = 15.1 Hz, $H1_A$), 3.33 (m, 1H, H2), 2.80 (d, ^2J = 15.1 Hz, $H1_B$), 1.33 (m, 3H, H3); ^{13}C NMR $CDCl_3$, 125 MHz), 158.8 (C4′), 130.4 (C2′, C6′), 127.8 (C1′), 114.3 (C3′, C5′), 57.4 (C2), 55.3 (N—CH_3), 38.6 (C1), 15.6 (C3); ESIMS, *m*/*z* 166 ($M+H^+$).

Each subset of 150 eggs, collected as above, was placed immediately onto a small piece of cotton wool and allocated to

one of the ten groups (nine replicates of each group in total). Weigh boats containing spiked meat were placed into 850 mL rectangular plastic containers with mesh lids, lined with a shallow layer (5 mm deep) of wheaten chaff. The chaff provided a medium for the larvae to crawl into when they had finished feeding. All larvae were reared at 23 °C in a temperature-controlled incubator (Thermoline Scientific, Australia) and exposed to a photoperiod of 12:12 light:dark. The day on which eggs were laid, collected and distributed to an experimental group was designated as day 0. The sampling design of our study followed that of George et al. [27], in order to facilitate comparison with the previous toxicological work on this blowfly. Samples were therefore compared at four time points. The first two comparison stages took place on days 4 and 7 of experimentation, during the larval stage. Three replicates of each group were removed at the day 4 and 7 comparison interval. Larvae were extracted from the meat and starved for 4 h to encourage expulsion of the contents of the crop. Larvae were killed and fixed by placing in boiling water for 60 s and individually washed in near-boiling water for 30 s to ensure that all adhesive substrate was removed. Larvae were then stored in 80% ethanol, after which their length, width and weight were measured. The posterior spiracles of each specimen were also inspected to record developmental instar. To determine the persistence of methamphetamine and *p*-hydroxymethamphetamine in the larval food source, 1 g samples of kangaroo mince were taken from each group for later analysis using high performance liquid chromatography.

The third comparison, incorporating a third set of three replicates per experimental group, was made during the pupal stage. Observations were made every 4 h to accurately record the day and hour (where suitable) at which pupariation (the process of puparium formation) began and concluded in each replicate container. Commencement of pupariation was recorded at the first indication of colour change of the prepupa from white to orange. Conclusion of pupariation was denoted by all samples having progressed from orange to dark brown, whereupon the length, width and weight of all samples were measured. Following measurement, pupae were returned to their original containers for eclosion.

The fourth comparison was made once adults had emerged. Observations were made every 4 h to record as accurately as possible when emergence began and concluded. The day and average hour of initial eclosion were recorded, as were the day and hour of average eclosion (P50 value). Adults were removed from plastic containers and placed in a freezer for 5 min to slow their movement. Flies were then killed by asphyxiation with ethyl acetate and stored in 80% ethanol. Measurements of adult weight were made within 4 h of collection. The left wing and rear left leg were then removed for later analysis.

Larvae and pupae were viewed under a dissecting microscope (MZ16A, Leica Microsystems, Germany). A fibre optic light source (CLS150X, Leica Microsystems, Germany) was used to illuminate samples on a contrasting background to assist analysis. Each specimen was photographed with a digital camera (DFC259, Leica Microsystems, Germany) and Leica Application Suite V3.8 software (Leica Microsystems, Germany) employed to measure length and width parameters to the nearest 0.001 mm. Larvae were viewed laterally, and their lengths measured between the most distal point of the head and the most posterior abdominal segment (Fig. 1(a)). Larval width was measured across the intersection of the fifth and sixth abdominal segments. Ultrasensitive scales (ML204, Mettler Toledo, Switzerland) and LabX direct balance 2.1 software (Mettler Toledo, USA) were utilised to record the weights of each sample to the closest 0.1 mg. Pupae were viewed ventrally and their lengths measured between the most posterior to most anterior points. Width measurements were obtained by measuring samples across the intersection of the first and second abdominal segments (Fig. 1(b)). Adults were removed from ethanol and allowed to dry for 10 min before being weighed. Wing and leg samples were viewed under the dissecting microscope and photographed. For each sample, the length of the costa (one of the peripheral wing veins) and tibia (one of the sections of the leg) were measured to give an indication of adult size and to determine if any differences existed between treatments due to drug exposure during earlier life stages (Fig. 1(c,d)).

Following measurement, maggots, puparia and adults were individually ground using a mortar and pestle and combined with 800 μL of distilled water (maggots and adults were allowed to dry for 24 h prior to homogenisation). Cellular debris was removed by precipitation in methanol (Ajax Finechem, Australia) and centrifugation (Model 5412D, Eppendorf South Pacific, Australia). The solution was run through filter paper to ensure that all large contaminants were removed. Smaller contaminants were removed by HPLC specific 4 mm syringe filters with 0.45 μm polytetrafluoroethylene membranes. Sampled meat was ground in a mortar and pestle and combined with 1000 μL of distilled water. Cellular debris was removed by precipitation in methanol and centrifugation and filtered as for larvae, puparial and adult samples. As little is known about the pharmacokinetics of drugs of abuse ingested by insects, two standard solutions of methamphetamine were prepared before any analysis of samples was attempted in order to determine which form of methamphetamine, if any, was present

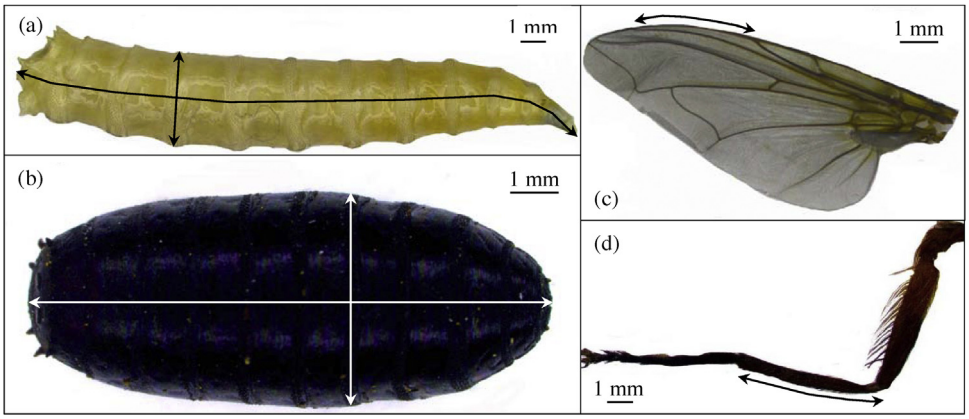

Fig. 1. (a) Larvae of *Calliphora stygia*, showing length and width measurements; (b) *C. stygia* pupa, showing length and width measurements; (c) left wing of *C. stygia*, showing costal length measurement; (d) rear left leg of *C. stygia*, showing tibial length measurement.

in *C. stygia* samples. The first was prepared by dissolving 7 mg of (±)-methamphetamine hydrochloride in 5 mL of distilled water, thus leaving the hydrochloride salt intact. The second was prepared by neutralising 7 mg of (±)-methamphetamine hydrochloride with sodium hydroxide. The solution was then filtered with an HPLC specific syringe filter, and mixed with 5 mL methanol. Standards for *p*-hydroxymethamphetamine hydrochloride were prepared in the same manner. The presence or absence of methamphetamine was confirmed by high performance liquid chromatography (HPLC) under isocratic conditions. Solvents were acetonitrile (190 grade) and water and ethylamine. Solvents were sonicated in an ultrasonic cleaner (Ultrasonics, Australia) for 20 min prior to use. Analytes were detected by UV light at 254 nm. The presence or absence of methamphetamine or *p*-hydroxymethamphetamine was recorded for each sample.

Statistical analysis was undertaken using JMP v7 for Windows (SAS, USA). Normality of the data was evaluated using *Q*–*Q* plots and Kolmogorov–Smirnov normality tests. Measured sample parameters (length, width and weight) were analysed using nested ANOVAs, which allowed identification of significance both between groups, and between the replicates of groups. Survivorship was investigated at the larval and adult comparison stages. Kruskal-Wallis tests were used to determine whether there had been any appreciable loss in sample numbers as a result of their exposure to drug compounds. Significant results were further examined with a Tukey–Kramer test to assess where any differences lay.

3. Results

HPLC chromatograms qualitatively determined the absence of methamphetamine compounds in the control meat. The presence of methamphetamine and/or *p*-hydroxymethamphetamine in the treatment groups was confirmed by HPLC–UV analysis. These compounds would therefore have been ingested by feeding larvae.

Development rates of larvae were determined by increases in the length and width of sampled specimens. Weight measurements were also taken to identify any unusual growth patterns. No obvious differences in average temperature were noted between groups or replicates.

3.1. Comparison of day 4 larvae

A nested ANOVA of mean larval length after four days of growth identified significant differences between treatment types ($F_{9,\ 3485} = 40.75$, $p < 0.0001$) (Fig. 2(a)).

A *post hoc* Tukey–Kramer test determined that the mean lengths of each of the pure methamphetamine treatments (0.1 MA, 1.0 MA and 10 MA) were significantly greater than the control group, as was the mean length of larvae exposed to the two lower concentrations of pure metabolite (0.015 *p*-OHMA and 0.15 *p*-OHMA). Similarly, the two lower methamphetamine:metabolite ratios (0.01 MA:0.015 *p*-OHMA and 1.0 MA:0.15 *p*-OHMA) produced larvae of significantly greater length than the control. The treatment with the highest concentration of *p*-hydroxymethamphetamine (1.5 *p*-OHMA) and the intermediate ratio of methamphetamine:*p*-hydroxymethamphetamine (1.0 MA:1.5 *p*-OHMA) were not significantly different in mean length from the control group. Significant differences were also seen between replicates within groups ($F_{20,\ 3485} = 16.96$, $p < 0.0001$). A *post hoc* Tukey–Kramer test revealed there was no significant difference in mean length between replicates within each group except for in the control and 0.15 *p*-OHMA treatment, for which all replicates were significantly different from each other.

Similar trends were observed in analyses of mean replicate width (Fig. 2(b)). A nested ANOVA showed significant differences between groups ($F_{9,\ 3485} = 136.29$, $p < 0.0001$). The mean width of larvae exposed to methamphetamine (0.1 MA, 1.0 MA and 10 MA) was

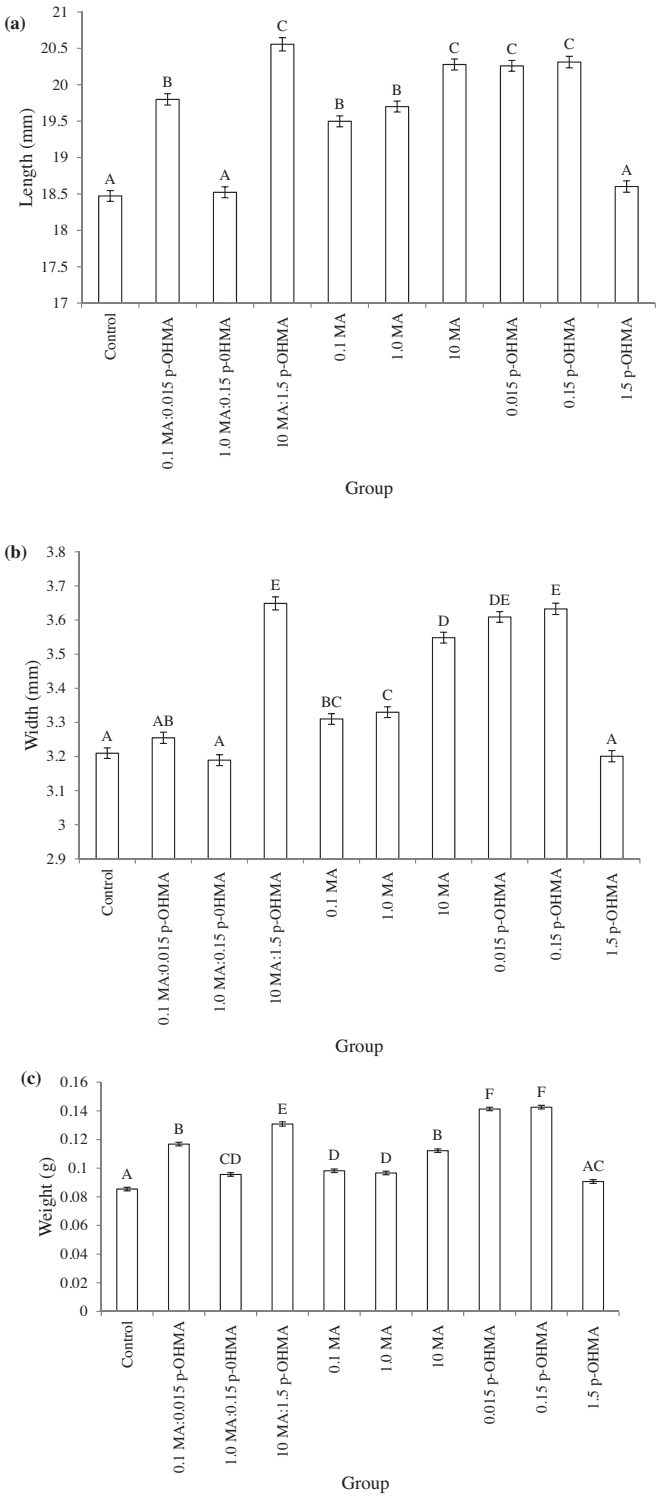

Fig. 2. Mean length (a), width (b) and weight (c) (±SE) of day 4 larvae, exposed during development to different concentrations of methamphetamine and/or *p*-hydroxymethamphetamine. Experimental groups are control; 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (0.1 MA:0.015 *p*-OHMA); 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-OHMA (1.0 MA:0.15 *p*-OHMA); 10 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (10 MA:1.5 *p*-OHMA); 0.1 mg/kg methamphetamine (0.1 MA); 1.0 mg/kg methamphetamine (1.0 MA); 10 mg/kg methamphetamine (10 MA); 0.015 mg/kg *p*-OHMA (0.015 *p*-OHMA); 0.15 mg/kg *p*-OHMA (0.15 *p*-OHMA); 1.5 mg/kg *p*-OHMA (1.5 *p*-OHMA). Groups not connected by the same letter are significantly different.

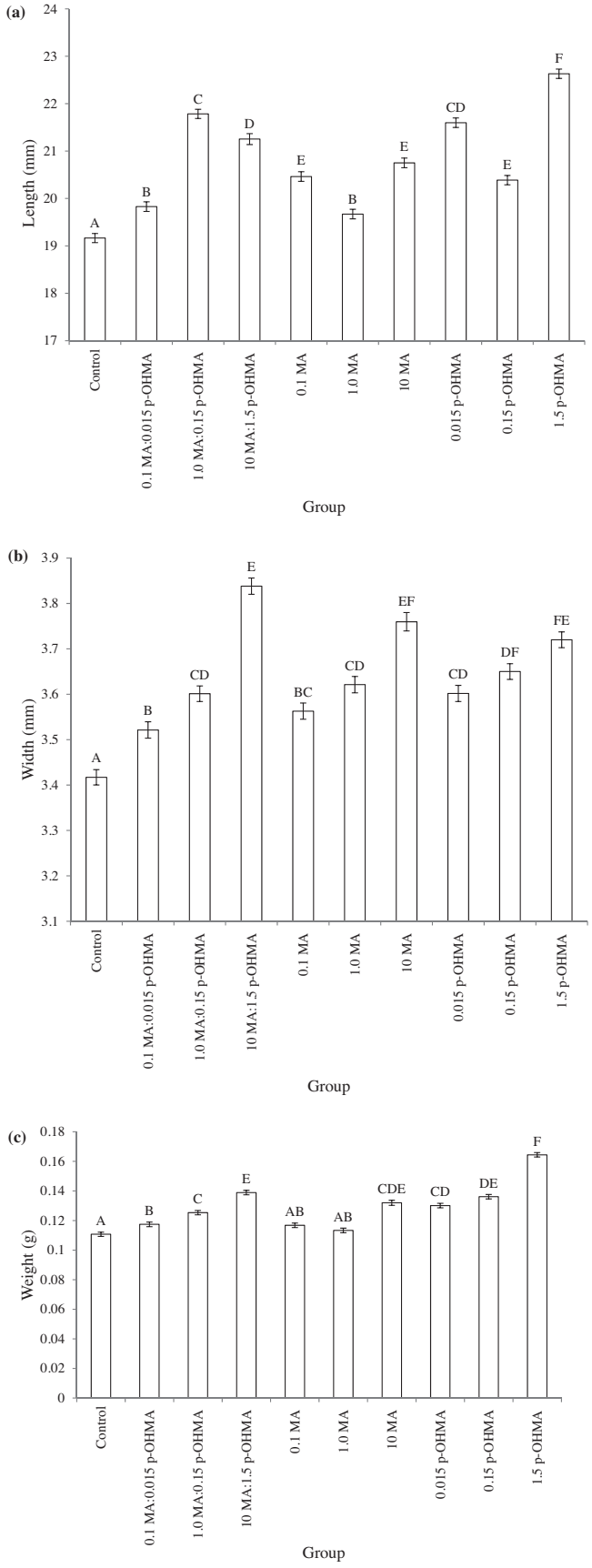

Fig. 3. Mean length (a), width (b) and weight (c) (±SE) of day 7 larvae, exposed during development to different concentrations of methamphetamine and/or *p*-hydroxymethamphetamine. Experimental groups are control; 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (0.1 MA:0.015 *p*-OHMA); 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-OHMA (1.0 MA:0.15 *p*-OHMA); 10 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (10 MA:1.5 *p*-OHMA); 0.1 mg/kg methamphetamine (0.1 MA); 1.0 mg/kg methamphetamine (1.0 MA); 10 mg/kg methamphetamine (10 MA); 0.015 mg/kg *p*-OHMA (0.015 *p*-OHMA); 0.15 mg/kg *p*-OHMA (0.15 *p*-OHMA); 1.5 mg/kg *p*-OHMA (1.5 *p*-OHMA). Groups not connected by the same letter are significantly different.

significantly greater than the control. Similarly, the mean widths of larvae in the highest ratio treatment (10 MA:1.5 *p*-OHMA) and the low and intermediate *p*-hydroxymethamphetamine concentrations (0.015 *p*-OHMA and 0.15 *p*-OHMA) were significantly greater than the control group. By contrast, the mean widths of the lower ratio treatments (0.1 MA:0.015 *p*-OHMA and 1.0 MA:0.15 *p*-OHMA) and the highest *p*-hydroxymethamphetamine treatment (1.5 *p*-OHMA) did not differ significantly from the control. With the exception of the control and 0.15 *p*-OHMA groups, where one replicate differed significantly from the other two ($F_{20,\ 3485} = 54.07$, $p < 0.0001$), a *post hoc* Tukey–Kramer test did not identify significant differences between replicates within each group.

A nested ANOVA analysis identified significant differences between the larval groups for mean weight ($F_{9,\ 3485} = 100.21$, $p < 0.0001$). The *post hoc* Tukey–Kramer test revealed similar trends to length and width comparison. Average larval weights of the methamphetamine (0.1 MA, 1.0 MA and 10 MA) and ratio treatments (0.1 MA:0.015 *p*-OHMA, 1.0 MA:0.15 *p*-OHMA and 10 MA:1.5 *p*-OHMA) were significantly greater than the control. The mean weights of the two lower *p*-hydroxymethamphetamine treatments (0.015 *p*-OHMA and 0.15 *p*-OHMA) were also significantly greater than the control. By contrast, the mean larval weight of the highest *p*-hydroxymethamphetamine treatment (1.5*p*-OHMA) did not differ significantly from the control (Fig. 2(c)). Significant differences between replicates within treatments($F_{20,\ 3485} = 102.67$, $p < 0.0001$) were identified by a *post hoc* Tukey–Kramer test. However, only one replicate of each of the control and 0.1 MA groups differed significantly from the other two replicates within the group.

3.2. Comparison of day 7 larvae

A nested ANOVA of mean length identified significant differences between treatments ($F_{9,\ 3485} = 48.77$, $p < 0.001$ The *post hoc* Tukey–Kramer test revealed that the mean lengths of larvae exposed to any drug compound were significantly greater than the control (Fig. 3(a)). However, significant variation between replicates within groups was also detected ($F_{20,\ 3485} = 51.05$, $p < 0.001$). One replicate of each of the control, intermediate methamphetamine concentration (1.0 MA) and the highest *p*-hydroxymethamphetamine concentration (1.5 *p*-OHMA) was significantly different from the other two within each group.

Substantial variation was also seen between the mean replicate widths of day 7 larvae ($F_{9,\ 3080} = 19.28$, $p < 0.001$) (Fig. 3(b)). The mean widths of larvae exposed to any drug treatment were significantly greater than the control group. A nested ANOVA of replicate within treatment showed that one replicate of the 1.5 *p*-OHMA group differed significantly from the other replicates within the treatment ($F_{20,\ 3080} = 19.10$, $p < 0.001$).

Similarly, nested ANOVA determined that the larvae exposed to methamphetamine compounds were significantly heavier on average than control samples ($F_{9,\ 3080} = 65.45$, $p < 0.001$) (Fig. 3(c)). Significant differences were also detected between replicates within groups ($F_{20,\ 3080} = 50.37$, $p < 0.001$). One replicate of each of the 1.5 *p*-OHMA and 1.0 MA:0.15 *p*-OHMA treatment groups, and the intermediate methamphetamine group (1.0 MA) were significantly different from the other two replicates within their treatments. Furthermore, each replicate of the control was significantly different from every other.

3.3. Comparison of pupae

Statistical significance between the mean pupal lengths of each treatment was detected by a nested ANOVA ($F_{9,\ 3022} = 87.59$, $p < 0.0001$). A *post hoc* Tukey–Kramer test revealed that the mean lengths of pupae exposed to any drug compound were significantly longer than the control (Fig. 4(a)). Significant variation was also observed between replicates within each of the groups ($F_{20,\ 3022} = 20.10$, $p < 0.0001$). One replicate of the control and each pure methamphetamine treatment (0.1 MA, 1.0 MA, 10 MA) varied significantly from the others within each group.

A nested ANOVA of width showed significant differences between groups at the pupal stage ($F_{9,\ 3022} = 94.23$, $p < 0.001$) (Fig. 4(b)). The mean widths of pupae exposed to any methamphetamine and/or metabolite treatment during the larval stage were significant greater than the control samples. Furthermore, nested ANOVA analysis identified significant variability between the mean widths of individual replicates within groups ($F_{20,\ 3022} = 97.87$, $p < 0.001$). With the exception of the ratio treatments (0.1 MA:0.015 *p*-OHMA, 1.0 MA:0.15 *p*-OHMA and 10 MA:1.5 *p*-OHMA), one replicate in each treatment was significantly different from the other two. Each replicate of the control was significantly different from the remaining two replicates.

When average weight was compared, significant differences were detected between treatments using a nested ANOVA ($F_{9,\ 3022} = 3.74$, $p < 0.001$) (Fig. 4(c)). A *post hoc* Tukey–Kramer test of mean pupal weight showed that all pure methamphetamine treatments (0.1 MA, 1.0 MA and 10 MA) and the two lower ratio treatments (0.1 MA:0.015 *p*-OHMA and 1.0 MA:0.15 *p*-OHMA) were significantly heavier than the control group. Furthermore, significant differences between replicates within groups were also identified ($F_{20,\ 3022} = 3.68$, $p < 0.001$). The highest methamphetamine treatment (10 MA) had one replicate that differed significantly from the other two within the treatment.

3.4. Comparison of adults

Once adult flies emerged from the puparium, the left wing and rear left leg were analysed to assess overall fly size. The costal vein of the wing and the tibia of the leg were measured. A nested ANOVA showed that there were significant differences between the mean costal length in each group ($F_{9,\ 2755} = 15.17$, $p < 0.001$) (Fig. 5(a)). A *post hoc* Tukey–Kramer test identified that the pure methamphetamine treatments (0.1 MA, 1.0 MA and 10 MA), and the low and high ratio treatments (0.1 MA:0.015 *p*-OHMA and 10 MA:1.5 *p*-OHMA) varied significantly from the control. No significant difference was seen between the mean costal length of replicates within groups ($F_{20,\ 2755} = 0.14$, $p = 0.87$).

Significant differences between mean tibial lengths were also detected by a nested ANOVA ($F_{9,\ 2755} = 40.22$, $p < 0.001$) (Fig. 5(b)). A *post hoc* Tukey–Kramer test showed that all treatments containing drug compounds produced flies with significantly greater mean tibial lengths than the control group. A further nested ANOVA showed a significant effect of replicate within group ($F_{20,\ 2755} = 3.84$, $p = 0.021$) with a *post hoc* Tukey–Kramer revealing one replicate of each of the lowest methamphetamine treatment (0.1 MA) and the control group differing significantly from the other two replicates within these groups.

The average weights of adult flies were determined to be statistically different by a nested ANOVA ($F_{9,\ 2755} = 244.43$, $p < 0.001$) (Fig. 5(c)). A *post hoc* Tukey–Kramer test showed that all flies exposed to methamphetamine or *p*-hydroxymethamphetamine as larvae were significantly heavier than the control samples. A nested ANOVA was used to investigate variability within treatments between replicates. Significant differences were identified ($F_{20,\ 2755} = 11.22$, $p < 0.001$) and identified by a *post hoc* Tukey–Kramer to be between

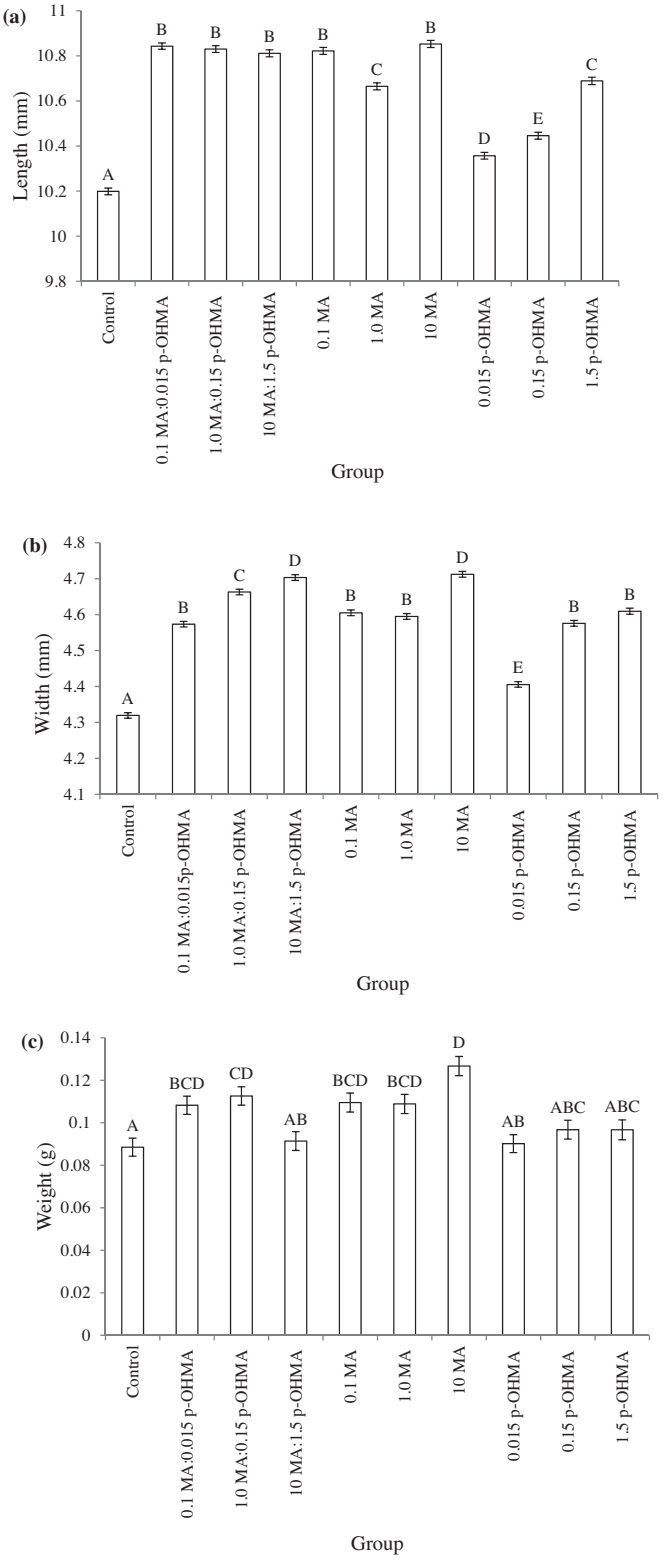

Fig. 4. Mean length (a), width (b) and weight (c) (±SE) of pupae, exposed during development to different concentrations of methamphetamine and/or *p*-hydroxymethamphetamine. Experimental groups are control; 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (0.1 MA:0.015 *p*-OHMA); 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-OHMA (1.0 MA:0.15 *p*-OHMA); 10 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (10 MA:1.5 *p*-OHMA); 0.1 mg/kg methamphetamine (0.1 MA); 1.0 mg/kg methamphetamine (1.0 MA); 10 mg/kg methamphetamine (10 MA); 0.015 mg/kg *p*-OHMA (0.015 *p*-OHMA); 0.15 mg/kg *p*-OHMA (0.15 *p*-OHMA); 1.5 mg/kg *p*-OHMA (1.5 *p*-OHMA). Groups not connected by the same letter are significantly different.

the replicates of the control, 0.1 MA, 1.0 MA, 10 MA and 0.15 *p*-OHMA groups. Within the control and 10 MA groups, the average weight of each replicate was statistically independent. For 0.1 M, 1.0 MA and 0.15 *p*-OHMA treatments, only one replicate was statistically significant from the other two replicates of the treatment.

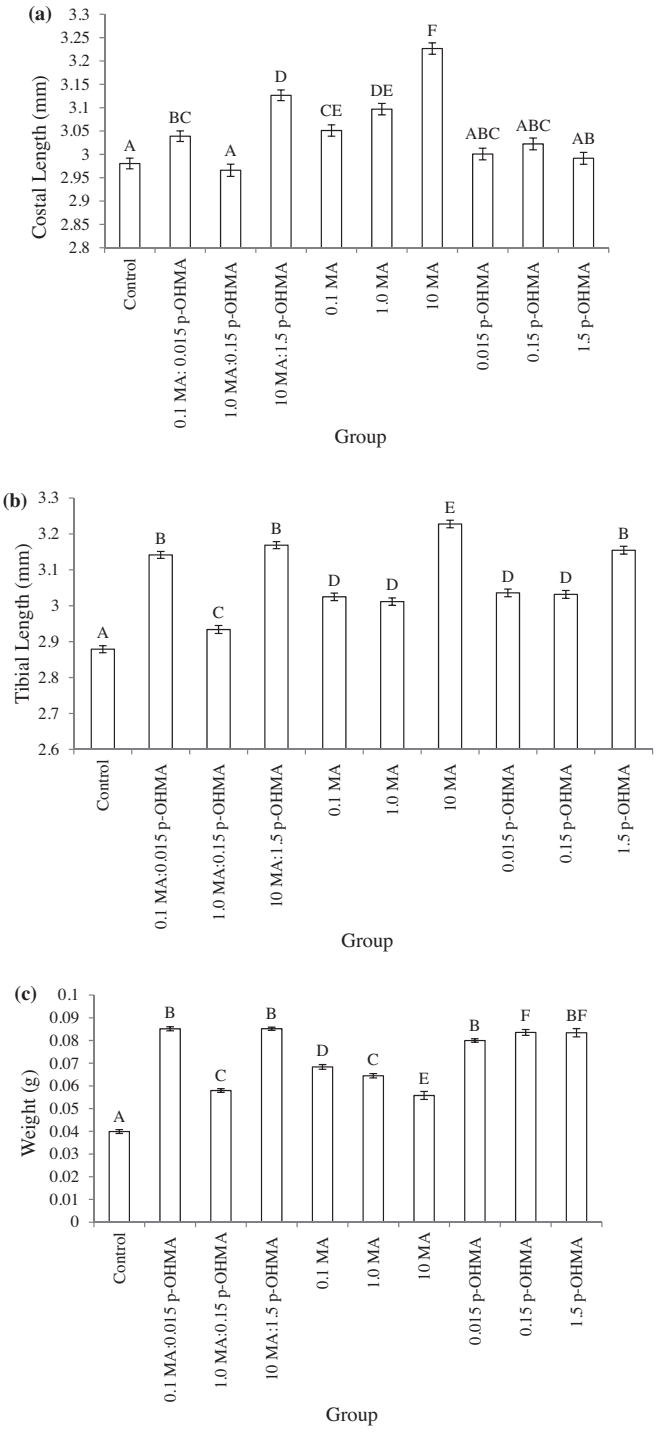

Fig. 5. Mean costal length (a), tibial length (b) and weight (c) (±SE) of adults, exposed during development to different concentrations of methamphetamine and/or *p*-hydroxymethamphetamine. Experimental groups are control; 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (0.1 MA:0.015 *p*-OHMA); 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-OHMA (1.0 MA:0.15 *p*-OHMA); 10 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (10 MA:1.5 *p*-OHMA); 0.1 mg/kg methamphetamine (0.1 MA); 1.0 mg/kg methamphetamine (1.0 MA); 10 mg/kg methamphetamine (10 MA); 0.015 mg/kg *p*-OHMA (0.015 *p*-OHMA); 0.15 mg/kg *p*-OHMA (0.15 *p*-OHMA); 1.5 mg/kg *p*-OHMA (1.5 *p*-OHMA). Groups not connected by the same letter are significantly different.

3.5. Survivorship

Kruskal–Wallis analysis of survivorship showed no significant difference in the number of larvae surviving after four days ($X_9^2 = 3.54$, $p > 0.432$). However, at the day 7 interval there was a significant difference in survivorship between drug and control groups ($X_9^2 = 28.01$, $p = 0.017$). When compared to the control, larval numbers had decreased in all pure methamphetamine (0.1 MA, 1.0 MA and 10 MA), and ratio treatments (0.1 MA:0.015 *p*-OHMA, 1.0 MA:0.15 *p*-OHMA and 10 MA:1.5 *p*-OHMA) but not in the treatments feeding on substrates spiked with pure *p*-hydroxymethamphetamine (0.015 *p*-OHMA, 0.15 *p*-OHMA and 1.5 *p*-OHMA). Adult survivorship was also significantly different between treatments ($X_9^2 = 29.00$, $p < 0.001$). The number of adult flies remaining in each drugged treatment was significantly smaller than the number of samples surviving in the control.

3.6. Development rates

Changes in growth rate due to the presence of methamphetamine and *p*-hydroxymethamphetamine in the larval food source were determined by recording, to the nearest hour, when samples in each replicate commenced and completed pupariation, and began and completed adult emergence. *Calliphora stygia* larvae exposed to any concentration of methamphetamine or *p*-hydroxymethamphetamine proceeded to show accelerated growth, commencing pupariation significantly before control larvae ($F_{2,\ 10} = 144.03$, $p < 0.0001$) (Fig. 6). This discrepancy was most obvious in the 10 MA and 10 MA:1.5 *p*-OHMA treatments. The initial colour change of the prepupae from white to orange began 44 h earlier in these treatments than in the control.

Pupariation was also complete in all methamphetamine- and metabolite-treated pupae significantly before the control ($F_{2,\ 10} = 293.47$, $p < 0.0001$). Samples then remained as pupae for significantly longer in treatments containing drug compounds ($F_{2,\ 10} = 441.63$, $p < 0.0001$), and emerged later than control samples ($F_{2,\ 10} = 91.47$, $p < 0.0001$). The 10 MA:1.5 *p*-OHMA treatment showed the greatest incongruity from the control. Emergence in this treatment began 34 h following the first control samples, equating to a 78 h total difference in development rate.

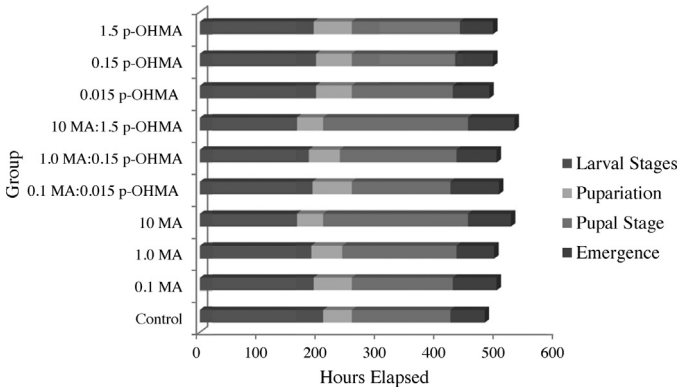

Fig. 6. Development rates of *Calliphora stygia* samples exposed to different concentrations of methamphetamine and/or *p*-hydroxymethamphetamine. Experimental groups are control; 0.1 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (0.1 MA:0.015 *p*-OHMA); 1.0 mg/kg methamphetamine:0.15 mg/kg *p*-OHMA (1.0 MA:0.15 *p*-OHMA); 10 mg/kg methamphetamine:0.015 mg/kg *p*-OHMA (10 MA:1.5 *p*-OHMA); 0.1 mg/kg methamphetamine (0.1 MA); 1.0 mg/kg methamphetamine (1.0 MA); 10 mg/kg methamphetamine (10 MA); 0.015 mg/kg *p*-OHMA (0.015 *p*-OHMA); 0.15 mg/kg *p*-OHMA (0.15 *p*-OHMA); 1.5 mg/kg *p*-OHMA (1.5 *p*-OHMA).

3.7. HPLC analysis

Standards for methamphetamine hydrochloride and *p*-hydroxymethamphetamine hydrochloride were established for HPLC under isocratic conditions, with the former showing a retention time of 27.3 min and the latter 14.2 min. Analysis of samples of each life stage by HPLC with UV detection qualitatively confirmed the absence of methamphetamine and *p*-hydroxymethamphetamine in the feeding substrate of the control (Fig. 7).

Methamphetamine could not be detected in homogenised larval samples of treatments 0.1 MA, 1.0 MA or 10 MA. However, HPLC chromatograms qualitatively confirmed its presence in pupal and adult samples (Fig. 7). *p*-Hydroxymethamphetamine was detected in larval, pupal and adult preparations of treatments 0.015 *p*-OHMA, 0.15 *p*-OHMA and 1.5 *p*-OHMA. In ratio treatments 0.1 MA:0.015 *p*-OHMA, 1.0 MA: 0.15 *p*-OHMA and 10 MA:1.5 *p*-OHMA, only *p*-hydroxymethamphetamine could be detected.

4. Discussion

In this study, methamphetamine-spiked kangaroo meat was utilised to simulate the postmortem environment of a methamphetamine overdose. Kangaroo mince was selected in favour of a live laboratory animal as the major and minor metabolites of methamphetamine and absorption and excretion rates are known to vary between vertebrate species [20]. The application of drug or metabolite directly to the meat ensured that larvae were exposed to known concentrations and types of drug

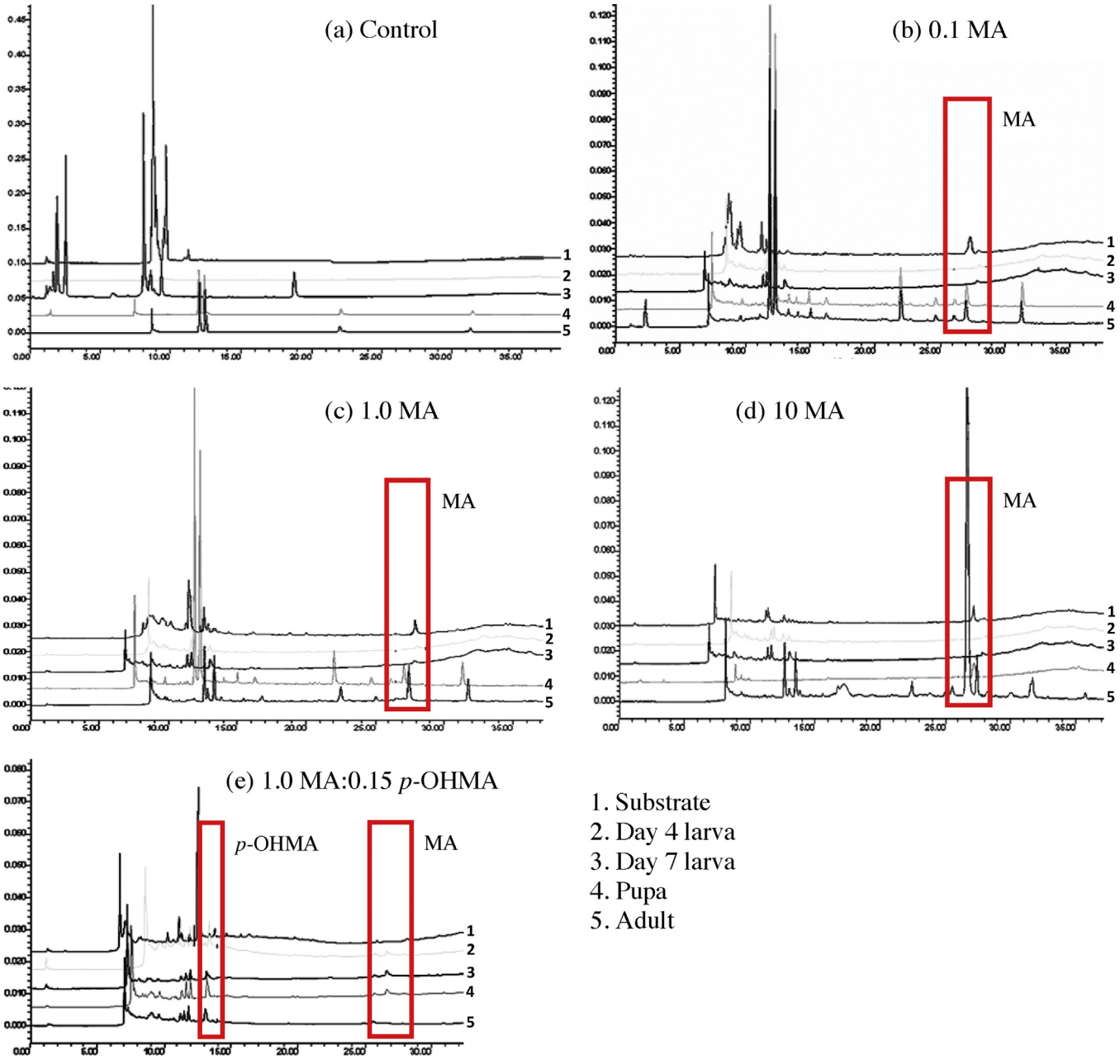

Fig. 7. Selected examples of HPLC traces qualitatively showing the presence or absence of methamphetamine (MA) and *p*-hydroxymethamphetamine (*p*-OHMA): (a) control; (b) 0.1 MA; (c) 1.0 MA; (d) 10 MA; and (e) 1.0 MA:0.15 *p*-OHMA.

compound, a recognised issue in entomotoxicological research [31]. Sustained mixing ensured a homogenous distribution of methamphetamine and/or *p*-hydroxymethamphetamine.

These compounds were found to significantly alter the size of all life stages at the concentrations investigated. After four days of growth, larvae in all treatments, with the exception of the highest *p*-hydroxymethamphetamine (1.5 *p*-OHMA) and the intermediate ratio treatments (1.0 MA:0.15 *p*-OHMA), were longer, wider and heavier than the control samples. After seven days, samples exposed to any methamphetamine treatment had developed into significantly longer, wider and heavier larvae than the control maggots.

These findings contrast with those of Goff et al. [12] in their studies of the flesh fly *Sarcophaga ruficornis*. When reared on methamphetamine-dosed rabbit tissues, individuals exposed to the highest concentrations of the drug were significantly shorter in length than control samples. This may have resulted from the different metabolism of methamphetamine in flesh flies and blowflies and highlights the inadvisability of extrapolating outcomes from entomotoxicological studies between species.

In forensic practice, the lengths of the largest larvae sampled from a body are used to estimate the age of the oldest specimens, and hence, give an estimate of the minimum PMI [32]. The results of this study strongly suggest that, in a maggot-infested corpse containing methamphetamine, the length of a larva of *C. stygia* might not necessarily be a valid indication of its age. If this enhanced growth is not considered, estimates of minimum PMI based on the larval stages of *C. stygia* could be erroneous.

Furthermore, the presence of methamphetamine and *p*-hydroxymethamphetamine in the feeding substrate significantly affected the growth rates of *C. stygia* larvae at the concentrations investigated. The onset of pupariation, and its duration, was significantly altered in drug-exposed treatments. *Calliphora stygia* larvae exposed to these compounds as immatures reached the pupal stage up to 44 h prior to, and remained as pupae up to 34 h longer than controls, a total divergence of 78 h. These findings again contrast with those of Goff et al. [12], who found the duration of the pupal period of *S. ruficornis* to be significantly shorter than the control for all samples exposed to methamphetamine during the larval stage. Additionally, the previous study on *C. stygia* by George et al. [27] found that its development was unaffected by pure morphine at sub-lethal, lethal, and twice-lethal doses. These contradictory findings are most likely due to the different type of drug used in each study, and the ease with which they are absorbed by the tissues of an organism.

While morphine is a potent analgesic [33], and represses the central nervous system [34], methamphetamine is a psychostimulant [35]. In humans, it immediately induces an increase in metabolism and the release of 'pleasure' neurotransmitters [18]. Although both types of drug are water soluble, morphine is poorly solubilised in lipids [33], whereas both methamphetamine and *p*-hydroxymethamphetamine are highly lipid soluble [36]. Methamphetamine compounds may therefore be better suited to the internal environment of blowfly larvae, which have a high fat content [37]. It is possible that these compounds are able to cross the lipid bi-layer of larval cells and accelerate metabolism. An increase in metabolic rate could manifest as an accelerated rate of development, or enlarged larvae, pupae and adults, which were all observed in the current study.

Compared with the findings of George et al. [27], a significant difference in survivorship was recorded between drug treatments and controls. There was a significant decrease in the number of larvae in all methamphetamine-spiked treatments after 7 days. Similarly, overall survival, calculated once all flies had emerged, showed that there were significantly fewer flies in treatments exposed to methamphetamine as immatures. This suggests that methamphetamine compounds have a toxic effect on *C. stygia* larvae at any concentration, and may influence their ability to undergo metamorphosis.

Methamphetamine and *p*-hydroxymethamphetamine were detected both in meat and *C. stygia* samples. HPLC analysis confirmed that methamphetamine and/or *p*-hydroxymethamphetamine were absent from the control group, but present in the larval food source of the remaining treatments. Consequently, larvae would have ingested the drug compounds. However, when larvae sampled at the first and second comparison intervals were analysed with HPLC–UV, methamphetamine could not be detected. These findings differed from those of Wilson et al. [38] in their studies of *Calliphora vicina.* When reared on skeletal muscle from a suicidal overdose of co-proxamol and amitriptyline, amitriptyline and its active human metabolite, nortriptyline, were both detected in *C. vicina* larvae.

This negative detection may have been due to the way in which samples were prepared for analysis. Larvae were ground with a mortar and pestle and combined with distilled water to solubilise any cellular material released. This was a relatively crude method of homogenization and the complete lysis of all cells, and the release of stored methamphetamine, was not guaranteed. Studies by Kinnear et al. [39] have shown that the concentration of lipids in *C. stygia* larvae increases sharply between days 3 and 6 of development, before decreasing after day 7. As larvae were sampled on days 4 and day 7, within this period of increased lipid production, increased fat content, combined with poor homogenization techniques, could be responsible for the absence of methamphetamine in larval preparations analysed by HPLC.

It is also possible that drug compounds were not detected in larval samples due to the lower sensitivity of HPLC–UV, when compared to chemiluminescence or fluorescence detection [40]. Takayama et al., in their studies on methamphetamine deposits in hair, were unable to detect methamphetamine in small hair samples when UV detection was employed. However, minute amounts of the drug in single hairs were able to be isolated and detected by chemiluminescence techniques [41]. The use of alternate detection methods might yield more conclusive results in future studies.

Although the results of the present study are notable, it is recommended that further investigations at different temperatures, and alternative concentrations of methamphetamine, be carried out to form a comprehensive bank of data against which forensic cases can be compared. As corpses are rarely found in environments with stable temperatures, a greater understanding of the effects of methamphetamine at different temperatures could assist with interpreting forensic cases. Similarly, the postmortem concentrations of drugs in a corpse may vary according to tissue type and location [42,43] and also may differ from the concentrations at the time of death due to postmortem redistribution by passive releases from the drug reservoirs of the gastrointestinal tract, lungs and myocardium, or at later stages, from the autolysis of cells and putrefaction processes [44,45]. Basic lipophilic drugs, such as methamphetamine, appear to be particularly susceptible to postmortem redistribution processes [44]. It is also known that larval growth can be influenced by tissue type and age in animal models [46–48]. Therefore, further investigation of the effects of methamphetamine on blowfly larvae must be carried out at different concentrations and in different substrates for its influence to be conclusively understood. Of course, once a comprehensive set of data is available for *C. stygia*, the influence of this drug on other fly species of forensic importance would also need to be investigated.

5. Conclusions

These findings hold significance for forensic science with particular regard to minimum PMI calculations using flies. The

altered growth exhibited in this study suggests that any estimate of minimum PMI based on the normal rates of *C. stygia* development at 23 °C could be overestimated by up to 44 h when based on the larval stage, and by up to 78 h when based on pupal samples. Indeed, because of the resultant developmental acceleration, caution should be exercised whenever *C. stygia* is used to estimate the minimum PMI of methamphetamine-dosed corpses. The developmental changes of this species of blowfly are also yet unknown at temperatures, drug concentrations and in substrates other than those used here. Further research is therefore essential to more comprehensively understand the effects of methamphetamine on blowfly development. Until then, *C. stygia* cannot be assumed to be a reliable model for aging corpses containing this drug.

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