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Short Communication

Effects of cocaine and heroin, and their combination, on the development rate of *Calliphora vomitoria* (Diptera: Calliphoridae)

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ABSTRACT

Insects present on or near decomposing bodies are collected by forensic entomologists and used to estimate the post-mortem interval. Drugs metabolized by a person before death may affect the rate of development of insects feeding on the corpse. This study aimed to determine the effects of cocaine and heroin main metabolites on the development rate of the *Calliphora vomitoria* (Diptera: Calliphoridae) and their implications on minimum post-mortem interval determination. Groups of 250 eggs each were placed into four separate pots of 150 g of minced pork meat being either un-spiked, or spiked with benzoylecgonine, morphine, or a combination of both. Larval length (mm) and weight (mg) measurements were taken twice daily and the rate of development of the insects' life cycle was monitored until eclosion. Results show that cocaine-fed larvae developed less in length and weight than the control group. Heroin-fed larvae showed a more fluctuating pattern, being smaller and lighter than the control group for most of their larval cycle, but overtaking them in both parameters towards pupation. Combination-fed larvae seemed to favour the effects of cocaine. The three conditions also had a significant impact on the length of the insects' life cycle. Cocaine and drug combination treatments increased the length of the second and third instar stages, but led to the shortening of pupation and accelerated eclosion. Conversely, heroin treatment led to lengthier pupation. Interestingly, the effects of the drug combination seemed to mirror more precisely those of cocaine.

These findings indicate that both cocaine and heroin, singularly and in combination, have sizable effects on blowflies' development rates, potentially biasing post-mortem interval estimations.

1. Introduction

Blowflies (Diptera: Calliphoridae) are the most widely analysed forensic entomologist species because they have a well-researched and easily recognizable life cycle and they are usually the first colonizers of a corpse [1,2]. Research has shown that drugs present in carrion can affect blowflies, lengthening or shortening their development rate. This has important ramifications for forensic entomologists when estimating the post-mortem interval (PMI), the time that has elapsed between death and discovery of the body [3–7]. Cocaine has been shown to accelerate the development rate in *Calliphora vicina, Chrysomya albiceps,* and *Chrysomya putoria* [4,5]. This body of research is limited and there is no information on cocaine's effect on *C. vomitoria.* Moreover, the effects of heroin on blowfly development rate vary from species to species. Goff et al. [6] found that heroin accelerated *Boettcherisca peregrina* larval development and slowed pupal development. Conversely, George et al. [7] found that heroin had no significant effects on *Calliphora stygia* development rate, while Ishak et al. found that not only heroin led to longer and heavier larvae, but also shortened the overall life cycle of *Lucilia cuprina* [8]. Furthermore, although the effects of single drugs on insects' growth rate have been researched, similar investigations have not yet been performed using a combination of drugs. This is considered an important area of research due to the increasing number of yearly fatalities related to combined drug toxicity, particularly of cocaine and heroin [9,10].

From the scant published literature, we see a clear need for more entomotoxicological research gathering data on the effects of different drugs on a variety of forensically relevant species. This study wants to expand our understanding of the effects of cocaine and heroin main metabolites on *Calliphora vomitoria.* Furthermore, to the best of the authors' knowledge, this study is the first of its kind focusing on the effects of a combination of drugs on the development of a forensically relevant

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species.

2. Methodology

The research has been carried out complying with the ARRIVE guidelines and following the U.K. Animals (Scientific Procedures) Act 1986, after receiving approval from the institution's ethics committee.

C. vomitoria was chosen due to its prevalence in the United Kingdom, the lack of existing research, and its forensic relevance. Insects were reared at an average temperature of 24.3 °C and average humidity of 37%.

Four transparent tanks (25.5 cm × 60 cm × 30.5 cm) were set up to house the larvae in the different experimental conditions. Each tank had a 2.5 cm-deep layer of vermiculite to allow pre-pupal larvae borrowing. Inside each tank, a short-sided 450 mL plastic cup containing meat was used for insects' rearing.

Four tanks were prepared and labelled as:

- Control group
- Cocaine group
- Heroin group
- Combination group (cocaine + heroin)

Four 150 g portions of 5% fat pork mince were weighed and added to each labelled pot. The main metabolite of cocaine and heroin, benzoylecgonine tetrahydrate C-11 (Sigma-Aldrich, lot n: 015M4057V) and morphine (Macfarlan Smith, lot n: 08-00468) respectively, were used to spike the meat. For the cocaine-spiked meat, a concentration of 17 mg/kg was reached by adding 2.5 mg of benzoylecgonine to 150 g of minced pork meat. For the heroin-spiked meat, a concentration of 34 mg/kg was reached by adding 5 mg of morphine to 150 g of minced pork meat. For the combination group, the same drug concentrations were used in combination. The chosen drug concentrations were optimised from the literature [3,4,6,11].

Each drug was weighed out and diluted in 2 mL of water. The diluted drugs were vortexed for 30 s before being poured into 150 g of minced pork meat and homogenized by a food processor for 2 min, followed by hand mixing for 1 min.

Third instar larvae were obtained from a bait shop and placed into a tank containing 150 g of minced pork meat. Once the adult flies emerged and opened their wings, 10% of the fly population was removed. These specimens were frozen and identified using taxonomic identification keys [12]. Adult flies were provided with water and a protein-based food source of milk powder and cane sugar. The eggs of this insect generation were collected and an estimated 250 eggs were placed in each of the four tanks. A total of 170 insects were sampled from each of the control and test groups throughout their larval and pupal stages, comprising 68% of the total population in each group (n = 250).

The larvae length and weight were measured twice daily, from 24 h after hatching until adult emergence. For each measurement session, five larvae from each tank were randomly selected, boiled in water for 30 s, and measured. Samples were observed under a high-power microscope to determine the instar stages. Finally, pupae were collected and placed into emergence containers, to observe the insects' rate of emergence. As this was developed as a pilot study, repeated measurements of the same individuals were not performed.

Statistical analyses were performed on RStudio (version 4.0.3). The data was analysed through MANOVA, as this was found to be the most reliable statistical analysis tool to test the potential effect of the different experimental conditions on insects' length and weight. Testing for normality, a p-value of 0.01 was chosen. This was informed by literature detailing MANOVA to be a robust test even when normality assumptions are breached [13]. Where the normality assumption was not met, a Multivariate Kruskal-Wallis was performed, after testing for homogeneity of variances through Bartlett's test. A significance level of.

$p < 0.05$ was chosen to identify statistically significant differences. Effect size was evaluated through eta squared values calculated independently for both length and weight [14].

3. Results

The average lengths and weights from the morning and afternoon daily measurements were pooled into a daily average for each variable and compared (Figs. 1 and 2). The first set of measurements, referred to as Day 1, were taken 24 h after the eggs were placed into each of the experimental groups.

On Day 1, a statistically significant difference in length was found between the four experimental conditions. The analytes explained 31% of the variation in length of the individuals ($eta^2 = 0.31$; $p = 0.0125$). On average, cocaine treated insects were 0.53 mm longer ($p = 0.036$), heroin treated ones were 0.75 mm longer ($p = 0.0039$) and combination treated ones were 0.92 mm longer ($p = 0.0006$) than the control. A statistically significant difference in weight was also found between the four experimental conditions. The analytes explained 30.5% of the variation in length of the individuals ($eta^2 = 0.305$; $p = 0.0074$). Insects treated with cocaine and heroin separately did not show a statistically significant difference in weight against the control. However, the combination group was lighter in weight (average weight transformation coefficient $-2.3e^{-4}$) than the control ($p = 0.0007$). On Day 2, 3 and 4, no statistically significant differences were found in either length or weight between the four experimental conditions. On Day 5, a statistically significant difference in length was found between the four experimental conditions. The analytes explained 47.24% of the variation in length of the individuals ($eta^2 = 0.4724$; $p = 0.0001$). On average, cocaine treated insects were 2.43 mm shorter ($p < 0.0001$), heroin treated ones were 1.04 mm shorter ($p = 0.0244$) and combination treated ones were 1.09 mm shorter ($p = 0.\ 0159$) than the control. The analytes also explained 37% of the variation in weight of the individuals ($eta^2 = 0.37$; $p = 0.0002$). Insects treated with heroin and a combination of drugs did not show a statistically significant difference in weight against the control. However, the cocaine group was lighter in weight (average weight transformation coefficient −0.039) than the control ($p < 0.0001$).

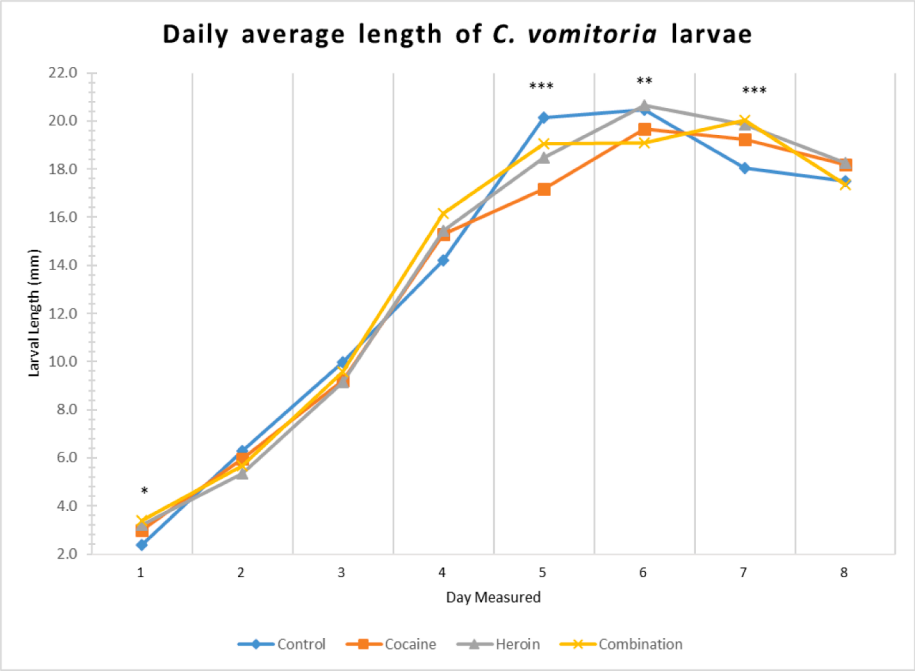
On Day 6, a statistically significant difference in length was found between the four experimental conditions. The analytes explained 30.1% of the variation in length of the individuals ($eta^2 = 0.301$; $p = 0.0066$). Insects treated with cocaine and heroin separately did not show a statistically significant difference against the control. However, the combination group was on average 1.35 mm shorter than the control ($p = 0.0043$). The analytes also explained 38.8% of the variation in weight of the individuals ($p = 0.0005$). Insects treated with heroin alone did not show a statistically significant difference against the control. However, the cocaine and combination groups were lighter (average weight transformation coefficient −0.011 and −0.016 respectively) than the control ($p = 0.0045$ and $p = 0.0002$ respectively).

On Day 7, a statistically significant difference in length was found between the four experimental conditions. The analytes explained 47.96% of the variation in length of the individuals ($eta^2 = 0.4796$; $p < 0.0001$). On average, cocaine treated insects were 1.21 mm longer ($p = 0.0035$), heroin treated ones were 1.82 mm longer ($p < 0.0001$) and combination treated ones were 1.99 mm longer ($p < 0.0001$) than the control. No statistically significant difference was found, in weight, among the four conditions.

Finally, on Day 8 no statistically significant differences were found in length or weight among the four experimental conditions.

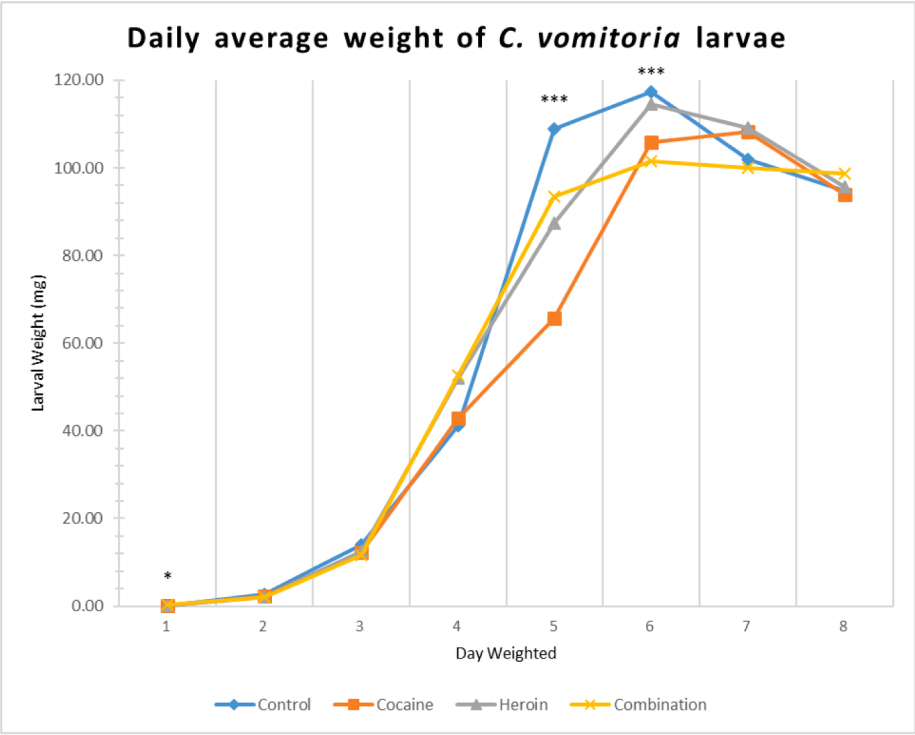
Day 9 measurements were not statistically analysed, as larvae from the control group were few and needed to be brought to pupation.

Each milestone in the life cycle of both control and test groups was documented and compared (Fig. 3). The control and all three test groups reached the second instar at the same point, between the first 24–28 h after the eggs were placed into the respective containers. The control group was the first to reach the third-instar larval stage, at the 48-hour mark. The cocaine and heroin groups had third instar larvae at the 52-



(*) Indicates statistical significance between the control and one or more of the test groups.

Fig. 1. Average length of total daily larvae samples.



(*) Indicates statistical significance between the control and one or more of the test groups.

Fig. 2. Average weight of total daily larvae samples.

hour mark, and the combination group was the last, at the 72-hour mark. The control and heroin groups began to pupate first, with over 50% pupated at the 192-hour mark, followed by the cocaine group. The combination group was last to reach full pupation, approximately 24 h after the other groups. The first groups to reach eclosion were the heroin and combination groups, with adult flies emerged between the 364-hour and 384-hour marks. The control group had one emerged adult fly by hour 384, and the fly was much less developed than the other emerged groups. The wings on the control fly were heavily wrinkled and the fly's body was a dull grey shade. The heroin group was the last to emerge, beginning eclosion only at the 388-hour mark.

Overall, the group with the shortest duration between the second and

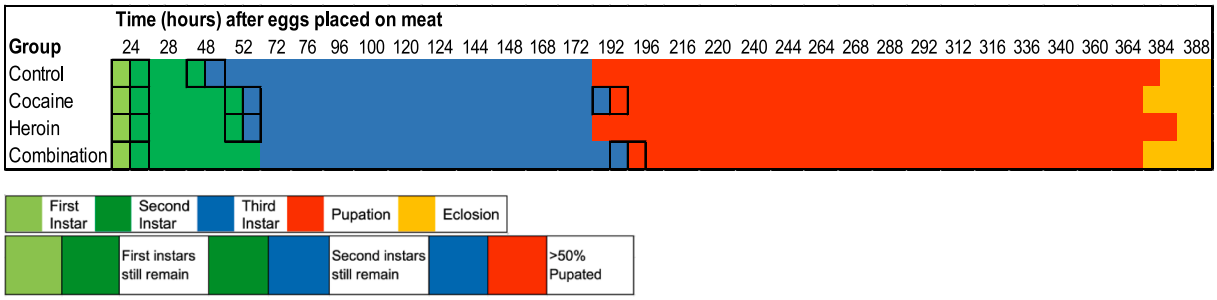


Fig. 3. Timeline comparison of C. vomitoria life stages with legend, showing when each test group reached specific life stages in comparison to the others.

third instar was the control group (20–28 h), followed by the cocaine (24–28 h), the heroin group (24–48 h), and lastly the combination (44–48 h). The combination group had the shortest duration between the third instar andpupation (120–124 h), followed by the cocaine and heroin groups (140 h) and the control (140–144 h). The pupation period from the start of pupation until eclosion was fastest in the combination group (168–188 h), followed by the cocaine group (172–188 h), the control group (192 h), and lastly the heroin group (196 h). In relation to the control group, the combination group's overall pupation period was approximately 24 h shorter, the cocaine group's pupation period was approximately 20 h shorter, and the heroin group's pupation period was approximately 4 h longer than the control.

4. Discussion

The use of entomological species for PMI estimations has been widely accepted in the field of forensic science. Amongst the host of factors that can affect forensically relevant insects' development, and hence the reliability of PMI estimation, the focus has recently shifted to the presence of drugs in carcasses. The field of forensic entomotoxicology has evolved since the 1990 s and it is now clear that more research is needed on the effect of drugs on forensically relevant species. Cocaine and heroin are of particular interest as they are one of the most common drug combinations [15]. Following further research, the gap between applied science and general research must always be considered, when dealing with PMI estimations. The misuse of technical terms may cause incorrect interpretation or misunderstanding regarding insect evidence [16]. A letter in the Entomological Society of America journal displays the controversial topic of the chasm between research and application in the field of forensic science. [16–19]. All forensic scientists should be aware that the results found in research are obtained in a controlled environment, avoiding false or unrealistic expectations in their investigations.

Throughout development, the cocaine-fed larvae developed less in length and weight than the control. These results are consistent with findings on *C. albiceps* [4]. However, the same study showed opposite effects on *C. putoria*. Being a pharmacological stimulant, it is generally believed that cocaine should cause accelerated feeding and enhance growth in blow flies [20]. However, this effect was not seen in the current study. The heroin group was longer and heavier than the control group on day one, but was smaller overall on days five and six, before gaining both length and weight on days six to eight. These results contrast the findings on *Boettcherisca peregrina* [6], where heroin-fed larvae were consistently larger in both length and weight for the entirety of the larval stages. Furthermore, these results also contrast the findings on *L. cuprina* [8]. The combination group developed slower than the control in both length and weight for most of the insects' development. Only towards the end of the larval period, the combination group became larger than the control in both weight and length. There is no direct research to compare the combination group results to, but it is worth noting that the combination group seems to have favoured the cocaine rather than heroin effects, which have previously been shown to increase the development rate towards the end of the larval stages and into pupation [4]. It is important to highlight the difficulty in comparing results on the effect of different drugs on different species. The lack of existing research regarding the effects of cocaine or heroin on *C. vomitoria* prevents a more accurate understanding of the current results. In the field of forensic entomotoxicology, there is now a general understanding that methodically developed databases on the interaction of specific drugs with distinct species are needed [21].

The development rates of *C. vomitoria* seemed to be affected by the presence of drugs in all three test groups, but the effect of each drug type depended on the specific life stage of the insects. The four groups showed similar timelines for the transition between the first and second instar. During the transition to the third instar, the cocaine and heroin groups developed approximately four hours later than the control, and the combination group developed 24–28 h later. Interestingly, the combination group then spent the shortest amount of time between the third instar and pupation, with the control, cocaine, and heroin groups following behind by 16–24 h. This may indicate that the presence of both cocaine and heroin may have caused a sudden increase in the insects' development rate. Furthermore, the combination group had a pupal stage 24 h shorter, the cocaine group 20 h shorter, and the heroin group 4 h longer than the control.

Heroin seemed to delay the overall development rate of the blowflies, in agreement with findings of similar research performed with *B. peregrina* and *Lucilia sericata* [6,11]. Interestingly, George et al. [7] reported no modification of growth rate in *C. stygia* fed on morphine up to a concentration of 20 μg/g, while the current study has shown a clear shift in *C. vomitoria* growth rate with a concentration of 30 μg/g. Cocaine appeared to delay the development during the transition between second and third instar, did not impact the transition between third instar and pupation, and then shortened the overall pupation period by 20 h. These findings are consistent with previous research that found cocaine to slow larval development slightly and speed up pupation in *B. peregrina*, *C. albiceps*, and *C. putoria* [4,22]. Overall, the effects of cocaine and heroin were relatable to the results of previous research and set a baseline for comparison with the combination group. The blowflies in the combination group showed the highest number of physical and developmental differences, compared to the control group. As this is the first study of its kind, no comparable literature is present on the effects of a mixture of drugs on forensically relevant blowflies. The results obtained highlight the need for more data gathering on the effects of drugs, singularly and in combination, on forensically relevant entomological species. When these findings are compared with other related studies, differences in methodology and larvae growth rate may be seen. Campobasso et al. [23] and Gosselin et al. [24] discuss the need for standardisation within entomotoxicological experiments focusing on the effects of drugs on insects. This effort will likely lead to increased applicability and presence of validated protocols and methods, which could be introduced for the toxicological analysis of entomological samples. Standardisation may also make it possible to compile a database for professionals to refer to when utilising entomology in death investigations. Furthermore, a standardised methodology for larvae

sampling, killing, storage and measurement would prevent unnecessary and detrimental variability [25].

5. Conclusion

The current study explored the effects of cocaine and heroin main metabolites, and their combination, on the development rate of *C. vomitoria*. It can be concluded that the metabolites of cocaine, heroin and their combination significanlty impacted on insects' development. All three experimental conditions altered the insects' morphology during development from first to third instars, producing shorter and lighter specimens. Furthermore, the three conditions also had a significant impact on the length of the insects' life cycle. The cocaine and drug combination treatments increased the length of the second and third instar stages, but led to the shortening of pupation and accelerated eclosion. Conversely, the heroin treatment led to lengthier pupation. Interestingly, the effects of the drug combination seem to mirror more precisely those of cocaine.

These results have sizable implications for forensic entomotoxicology and forensic casework. The modulation of insects' growth rates has the potential of biasing PMI estimates if the presence of drugs is not taken into consideration. In turn, wrong PMI estimates may divert investigations and lead to miscarriages of justice. This proof-of-concept study is the first of its kind focusing on the effects of a combination of drugs on blowflies' development. Our study has provided further insight on the effect of drugs on forensically relevant species, but a more robust data gathering is needed to confirm findings and lead to the development of viable species-specific databases.

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CRediT authorship contribution statement

T. Wood: Investigation, Formal analysis, Writing – original draft. **K. Pyper:** Formal analysis. **F. Casali:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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