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ORIGINAL ARTICLE

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Toxicological data and growth characteristics of single post-feeding larvae and puparia of *Calliphora vicina* (Diptera: Calliphoridae) obtained from a controlled nordiazepam study

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Abstract Larvae of the *Calliphora vicina* (Diptera: Calliphoridae) were reared on artificial food spiked with different concentrations of nordiazepam. The dynamics of the accumulation and conversion of nordiazepam to its metabolite oxazepam in post-feeding larvae and empty puparia were studied. Analysis was performed using a previously developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. This method enabled the detection and quantitation of nordiazepam and oxazepam in single larvae and puparia. Both drugs could be detected in post-feeding larvae and empty puparia. In addition, the influence of nordiazepam on the development and growth of post-feeding larvae was studied. However, no major differences were observed for these parameters between the larvae fed on food containing nordiazepam and the control group. To our knowledge, this is the first report describing the presence of nordiazepam and its metabolite, oxazepam, in single *Calliphora vicina* larvae and puparia.

Keywords Forensic entomology · Entomotoxicology · LC-MS/MS · Benzodiazepines · *Calliphora vicina*

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Introduction

Benzodiazepines are amongst the most widely prescribed psychoactive drugs for the symptomatic treatment of anxiety and sleep disorders. Unfortunately, misuse of these compounds is often reported. Nordiazepam, a long-acting benzodiazepine drug, has sedative and tranquilizing effects. In addition, it is the main metabolite of diazepam, which is the most commonly prescribed benzodiazepine [1].

Insects are a useful source of samples for toxicological analysis when the tissues or body fluids normally used for this purpose are not available. Since the first identification in 1980 of a drug (phenobarbital) in fly larvae found on a skeletonised corpse [2], entomotoxicology i.e. the study of drugs in insects, has become an established approach [3, 4, 5, 6]. Whereas previously published methods have required pools of insects in order to achieve detectable levels of toxic substances [7, 8, 9], we have developed a LC-MS/MS method with sufficient sensitivity to allow the quantitation of benzodiazepines in single larvae or empty puparia [10].

The objectives of the present study were twofold: firstly, to determine the dynamics of the accumulation of nordiazepam and its metabolite oxazepam in single larvae and puparia and, secondly, to study the effect of nordiazepam on the length and weight of post-feeding larvae and its influence on the patterns of larval development.

Material and methods

Flies and larvae from a stock colony of *Calliphora vicina* were maintained in an environmental chamber at 18–24°C and 60–70% humidity with cyclical artificial lighting simulating 16 h daylight and 8 h darkness.

Larvae were reared on different treatment regimes, i.e. beef heart spiked with a range of concentrations of nordiazepam (NOR 0: 0 µg/g, NOR 1: 0.5 µg/g, NOR 2: 1 µg/g, NOR 3: 2 µg/g).

For each treatment regime, 30 larvae per day (harvested from day 4 till day 8 inclusive) were collected for physical measurements. These were initially weighed and then boiled and conserved in a 75:25 v/v mixture of ethanol and acetic acid prior to length

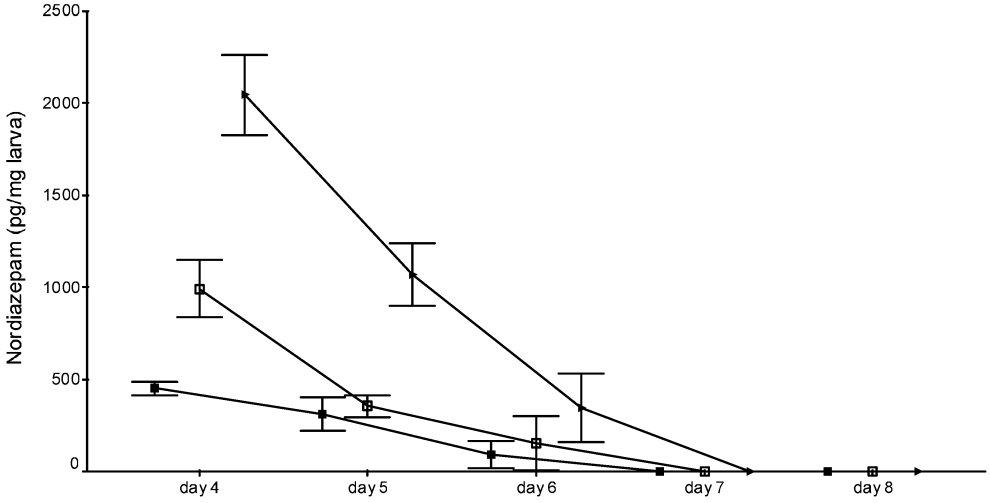

Fig. 1 Concentrations (pg/mg larva) of nordiazepam in single larvae, measured on 5 consecutive days. Data represent the mean ±1 standard deviation for 6 larvae for each diet condition (*closed squares* NOR 1, *open squares* NOR 2, *triangles* NOR 3)

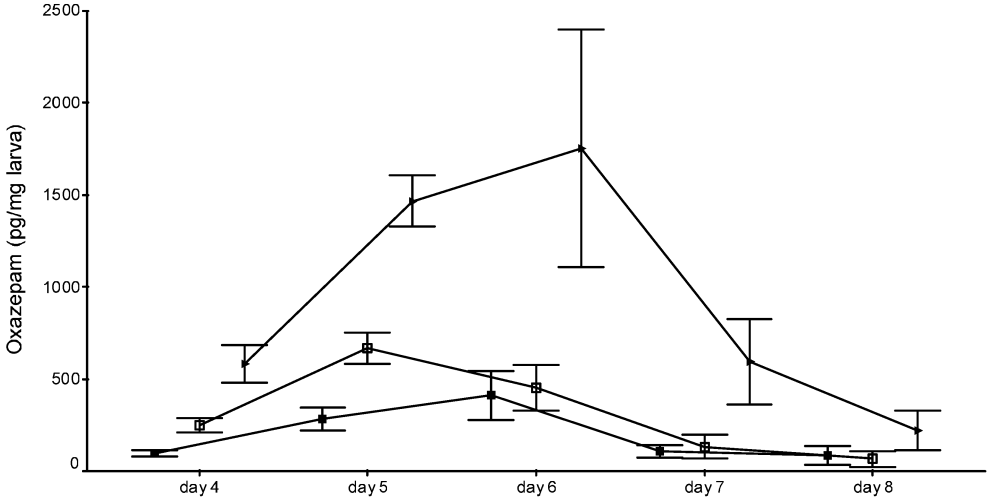

Fig. 2 Concentrations (pg/mg larva) of oxazepam in single larvae, measured on 5 consecutive days. Data represent the mean ±1 standard deviation for 6 larvae for each diet condition (*closed squares* NOR 1, *open squares* NOR 2, *triangles* NOR 3)

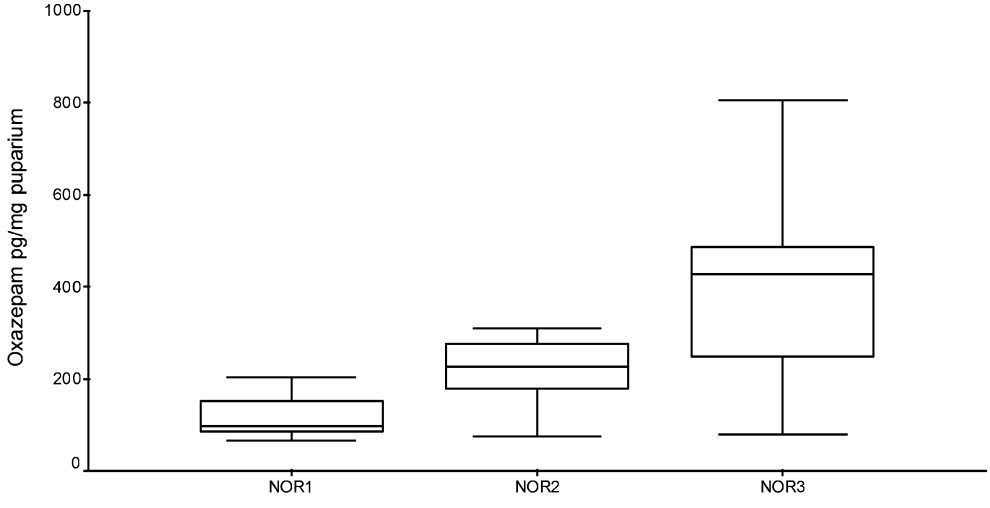

Fig. 3 Box-and-whisker plots showing concentrations of oxazepam (pg/mg puparium) in single puparia. Data represent results from 10 puparia for each diet condition. The *boxes* represent the interquartile ranges and contain 50% of the values. The *line* across each box indicates the median. The *error bars* indicate the highest and lowest values when outliers are excluded

measurement. A further 30 were collected for toxicological analysis. These were killed by freezing (–20°C) and stored at this temperature prior to analysis. Empty puparia were collected after the emergence of the adult fly and stored at –20°C until analysis. For each concentration, a total of 6 larvae per day (harvested over the 5 consecutive days) and 10 puparia were prepared and analysed for nordiazepam and oxazepam according to the method described by Wood et al. [10]. The drugs were quantified in single larvae and puparia rather than pools. The data were analysed using single-factor analysis of variance (ANOVA) with the different concentrations in the diet as fixed factors. Pair-wise comparisons of the means of different concentrations were performed using a LSD-test (least

significant difference). Results were considered as statistically significant if $P<0.05$.

Results and discussion

Control larvae and puparia were negative for nordiazepam and oxazepam.

The concentration of nordiazepam in the diet affected the detected concentrations of nordiazepam and oxazepam in larvae and puparia (Figs. 1, 2, 3): a step-wise increase of larval drug concentrations was observed with increasing drug concentrations in the diet. Peak concentrations were significantly different for the three nordiazepam regimes on day 4 (F=83.663, P=0.0001) (Fig. 1). Levels declined through days 5 and 6 and no significant differences were seen on day 6. From day 7 on, nordiazepam was no longer detectable in the larvae. In addition, significantly different concentrations of the metabolite oxazepam were seen for all conditions on days 4 (F=85.487, P=0.0001) and 5 (F= 183.436, P=0.0001) (Fig. 2). Maximum larval concentrations were observed on day 6 for NOR 1 and NOR 3 and on day 5 for the NOR 2 group. Oxazepam was still detectable on day 8 larvae from the 3 treatment conditions.

Both nordiazepam and the metabolite oxazepam could be detected in individual empty puparia (Fig. 3). Due to the low concentrations no statistical analysis was performed for nordiazepam. For oxazepam a significant difference in concentrations was observed between the NOR 2 and NOR 3 groups (F=13.348, P=0.002) and NOR 1 and NOR 3 groups (F=13.348, P=0.0001). The difference between the NOR 1 and NOR 2 groups was close to being significant (P=0.06).

Since oxazepam is a known metabolite of nordiazepam and peak concentrations of oxazepam were seen 1 or 2 days later than peak concentrations of nordiazepam, it is likely that nordiazepam is metabolised in the larvae to oxazepam. The detection of both nordiazepam and the metabolite in empty puparia suggests that a metabolisation and possible bioaccumulation may occur rather than an excretion. Recently Carvalho et al. reported a bioaccumulation of diazepam in larvae of *Chrysomya albiceps* and *Chrysomya putoria* [11]. However, they only determined the presence of diazepam and not that of its metabolites.

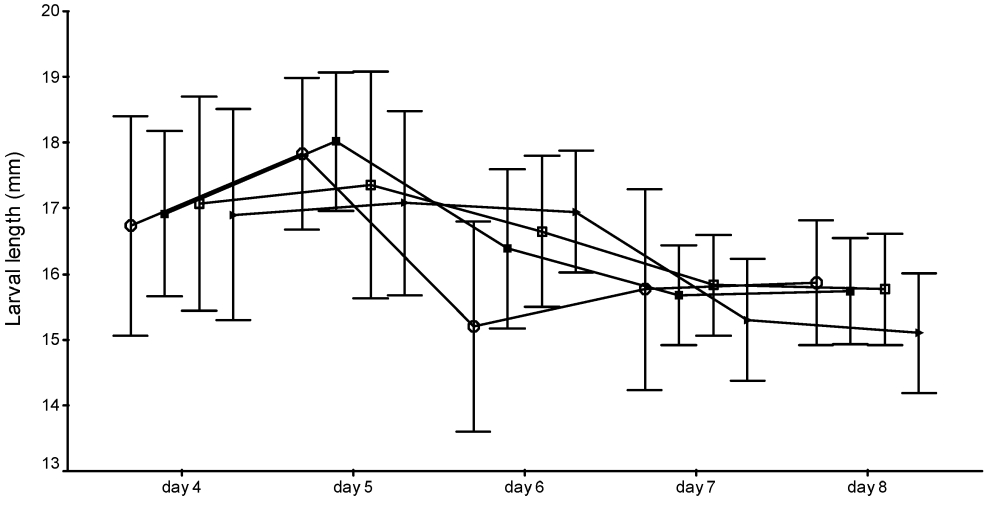

Fig. 4 Larval length (mm) measured on 5 consecutive days. Data represent the mean ±1 standard deviation for 30 larvae for each diet condition (*open circles* NOR 0, *closed squares* NOR 1, *open squares* NOR 2, *triangles* NOR 3)

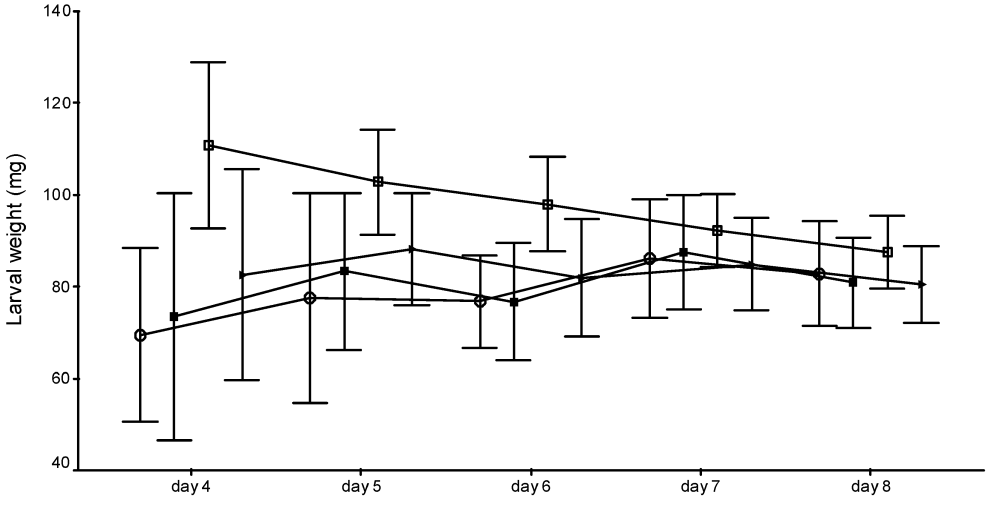

Fig. 5 Larval weight (mg) measured on 5 consecutive days. Data represent the mean ±1 standard deviation for 30 larvae for each diet condition (*open circles* NOR 0, *closed squares* NOR 1, *open squares* NOR 2, *triangles* NOR 3)

In general, the post-feeding larvae fed under the four different conditions developed at approximately the same rate. Each group exhibited wandering behaviour, which occurred on day 6 for NOR 0 group, in the night from day 6 on day 7 for the NOR 1 and NOR 3 groups and on day 8 for NOR 2 larvae. For the NOR 0, 1 and 3 groups pupariation started at day 8 and adult flies emerged on day 18, whereas pupariation for NOR 2 larvae was seen on day 9 and adult flies emerged on day 19. This is in contrast to earlier publications where the rate of development was significantly altered by the presence of drugs [11].

No consistent significant differences were seen in larval length (Fig. 4). This was in contrast to the larval weight, where significant differences were noticed between NOR 2 and the other groups, from day 4 (F=21.456, P=0.0001) till day 6 (F=22.569, P=0.0001) (Fig. 5). However, from day 7 onwards, this difference was no longer significant. As the larvae were all reared on the same foodstuff, no possible effect of this parameter has to be taken into account [12].

Conclusions

Larvae of the *Calliphora vicina* (Diptera: Calliphoridae) were reared either on artificial food containing three different concentrations of nordiazepam or on a control regimen. Due to the low detection limits of our LC/MS-MS method we were able to quantify nordiazepam and its metabolite oxazepam in single larvae and empty puparia under the three conditions. A day to day analysis of post-feeding larvae suggested that a metabolisation of nordiazepam and an accumulation of nordiazepam and oxazepam were taking place. A small effect was observed on larval development and larval weight for the larvae reared on food with a concentration of 1 µg/g of nordiazepam in comparison with the other groups. However, no effect was seen on the larval length parameter. The reason why this effect was observed only in these larvae and not in the other groups is not yet understood.

The purpose of this study was to detect nordiazepam and oxazepam even in low (therapeutic) doses. These are often encountered in cases of drug-facilitated assaults. However, further experiments with higher concentrations (overdoses) are needed to determine a possible effect of these higher concentrations on the development rate of larvae of the *Calliphora vicina* [13].

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