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Effect of Nandrolone Decanoate on the Development of Three Species of *Chrysomya* (Diptera: Calliphoridae), Flies of Forensic Importance in Brazil

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ABSTRACT Necrophagous insects are valuable tools for postmortem interval (PMI) estimation or for determining the cause of death. Due to the increase in deaths related to drug abuse, it is crucial to know how these substances affect the development of flies that feed on corpses, to avoid errors in the PMI estimates. This study evaluated the effect of nandrolone decanoate, an anabolic androgenic steroid, on the development of immatures of *Chrysomya megacephala* (F.), *Chrysomya putoria* (Wiedemann), and *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) when added to an artificial rearing diet. Four experimental groups were delineated: three of them were given diets containing 4.5, 22.5, or 45 mg/kg nandrolone decanoate; and a drug-free control group. Weights were recorded at 12-h intervals from larval eclosion to pupation. No statistically significant differences were observed in mean larval weights, emergence interval, or emergence rates for all groups. However, differences in the three species were observed during the larval development. Initially, *C. putoria* reared in the highest concentration of decanoate showed greater weight gain. However, at older ages, immatures reached lower mean weights than the control group. For *C. albiceps*, the highest concentration of decanoate contributed to an effective lack of weight gain during almost the entire course of larval development. Therefore, the influence of any drug on development should always be considered.

KEY WORDS blow flies, hormone, postmortem interval, entomotoxicology

After death, animal tissues are attractive to a variety of invertebrates, especially sarcosaprophagous insects (Nuorteva 1977). The decomposition process may be influenced by many factors, among them the important activity of dipterans belonging to the families Calliphoridae, Sarcophagidae, and Muscidae as decomposers and scavengers (Souza and Linhares 1997, Byrd and Castner 2001). These flies are attracted from long distances, due to the presence of highly specialized odor-sensing sensilla, to visit a carcass or breed in this substrate. Certain species colonize a corpse for a limited time interval or during a specific stage of decomposition, so this produces a faunal succession (Catts and Goff 1992). For this reason, entomology within the forensic context has been defined as the application of the study of insects and other arthropods, aiming to collect useful information for a legal investigation.

Forensic entomology can be used to determine the postmortem interval (PMI), circumstances, or cause of death; investigate whether there was displacement of the corpse; link suspects to a criminal act; or analysis of any toxic substances, if an autopsy cannot definitively determine the cause of death (Nuorteva 1977, Smith 1986, Catts 1990). Some studies have demonstrated that the presence of drugs or toxins in decomposing tissues can affect the developmental rate of insects, when such tissues are used as food, thus potentially altering the estimate of the PMI (Goff et al. 1989, Goff et al. 1991, Bourel et al. 1999, Bourel et al. 2001, Carvalho et al. 2001, Oliveira et al. 2009). Therefore, failure to take this parameter into consideration leads to errors in the PMI estimate (Introna et al. 1998, Greenberg and Kunich 2002).

Drugs such as anabolic androgenic steroids (AASs) are used indiscriminately by athletes, bodybuilders, teenagers, and any person interested in obtaining rapid increase in muscle mass and strength, and to reduce recovery time between intense exercises (Lise et al. 1999). Questionnaires administered to strength-training apprentices in some Brazilian states have reported that the user rate is between 6.5 and 20%, according to the age range and economic and social conditions (Conceição et al. 1999, Silva and Moreau 2003, Frizon et al. 2005, Silva et al. 2007, Iriart et al. 2009).

Nandrolone decanoate is an injectable steroid belonging to the class of testosterone esters and is commercially known as Deca-Durabolin (Organon from Brazil) (Oga 1996). It has a low androgen level, and hence it promotes low water retention. For this reason, it is widely used by women and men in the pre-

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competition period, and by women outside of the competition season. The use of AAS is based on empirical experience, which has determined that for nandrolone decanoate, a sequence of use may include dosages ranging from 200 to 25 mg. The most evident collateral effects that may occur from the use of high dosages are baldness, prostate hypertrophy, acne, hypertension, increase in the cholesterol content intensifying the risk of heart attack and stroke, limiting of growth in young people (due to premature closure of bone epiphyses), virilization in women, gynecomastia, impotence, infertility, insomnia, liver toxicity, problems with tendons and ligaments (due to the increase in strength and size of muscle fibers), and retention of body fluids (Guimarães-Neto 2005). These effects also can be observed in the increased mortality rate of AAS users, which is ≈4.6 times higher than in nonusers of these substances (Cunha et al. 2004).

Approximately 97% of testosterone, including its synthetic forms, when present in the bloodstream, binds to plasma proteins to be transported to the tissues or degraded. The free testosterone is rapidly converted by the liver and then excreted. In the cells, this hormone is broken down into metabolites. However, some effects observed in the organism can occur even when the steroid is in its unmetabolized form (Guyton and Hall 2002).

In this study, we evaluated the larval developmental rate and the viability of *Chrysomya megacephala* (F.), *Chrysomya albiceps* (Wiedemann), and *Chrysomya putoria* (Wiedemann) (Diptera: Calliphoridae) reared on artificial diets containing nandrolone decanoate. These blowfly species, originally from the Old World, were introduced into southern Brazil in the 1970s (Guimarães et al. 1978), and they are important from the forensic entomology standpoint (Souza and Linhares 1997; Marchiori et al. 2000; Carvalho et al. 2000, 2004; Carvalho and Linhares 2001; Moretti et al. 2008).

Materials and Methods

Adults of *C. megacephala*, *C. albiceps*, and *C. putoria* were collected in the urban area of Campinas (22° 54′21″ S, 47° 03′39″ W), São Paulo, Brazil. Decaying beef liver, chicken gizzard, fish, or a combination were used as bait. The specimens collected were taken to the laboratory, where they were maintained in screened plastic cages in a temperature-controlled room at 27 ± 1°C, 60% ± 10% RH, and a photoperiod of 12:12 (L:D) h. The larvae used in the experiments were obtained from colonies established from these collected flies.

For each *Chrysomya* species, the following procedure was performed: the newly hatched larvae were counted and separated with a thin brush into groups of 150 individuals each, which were kept in nylon-covered plastic vials containing an artificial diet prepared according to Estrada et al. (2009). This artificial rearing diet was composed of powered milk, yeast, casein, antifungal compound, agar, and animal tissues (in this case, chicken heart and beef rumen in a 2:1 proportion). The vials were maintained in a growth chamber (Fanem model 387), with the same temperature, relative humidity, and photoperiod as for the adults. The drug was added during the preparation of the artificial diet. Nandrolone decanoate is available in an oil solution, marketed as Deca-Durabolin 50 mg (Organon from Brazil). Considering that the therapeutic dosage (TD) of this AAS for adults (≈60 kg) is equal to 0.83 mg/kg, four experimental groups were used, three of them containing the following nandrolone decanoate concentrations: 4.5 mg/kg (≈5× TD), 22.5 mg/kg (≈25× TD), and 45 mg/kg (≈50× TD); and a control group without the drug. Each treatment was replicated six times; three replicates were used for weight recording and determination of the larval instars, and the other replicates were maintained to determine developmental and emergence rates.

For each treatment, individual weights of 10 randomly chosen larvae were recorded at 12-h intervals using an analytical balance (Scientech AS 210), until pupation. The larval samples used for weight recording were cleaned with distilled water and dried with filter paper to remove diet residues. For instar determination, the number of stigmatic clefts in the posterior spiracle was determined with a stereomicroscope (Stemi SV 11, Carl Zeiss, Jena, Germany). Because of their light weight at the beginning of development, larvae were weighed in groups of ten. In this case, the experiments were done in three replicates. After 24 h, the weight of each larva was recorded individually, as described above. For each measurement, the immatures were taken from different vials and discarded after this procedure. When larvae reached the post-feeding stage, they were transferred to another vial containing sawdust for pupation. The emerged adults and the puparia were counted to determine the rates previously mentioned.

Two-way analyses of variance (ANOVAs) were performed to compare the effect of the treatments and fly species on the developmental rate, and to compare the effect of the treatments and fly species for each time period. For each test, the weight (milligrams) was the response variable. A series of one-way ANOVAs also were used to test the effect of the treatments on the mean weight of each species at each age. Duncan's multiple comparisons test was used to compare the means. All analyses were done with a 5% global level of significance. The PROC GLM procedure of the statistical package SAS (SAS Institute 2006) was used for the analyses.

Results

There was no relationship between drug concentration and total mean larval weight for *C. megacephala* ($F = 0.06$, $P = 0.9797$) (Fig. 1). A few peaks were observed at different ages for the mean larval weight (Table 1). At 24 h ($F = 38.62$, $P < 0.0001$), the immatures from the control group showed a lower weight gain than the groups exposed to the drugs. At 84 h ($F = 3.42$, $P = 0.0274$), the 25× TD showed a

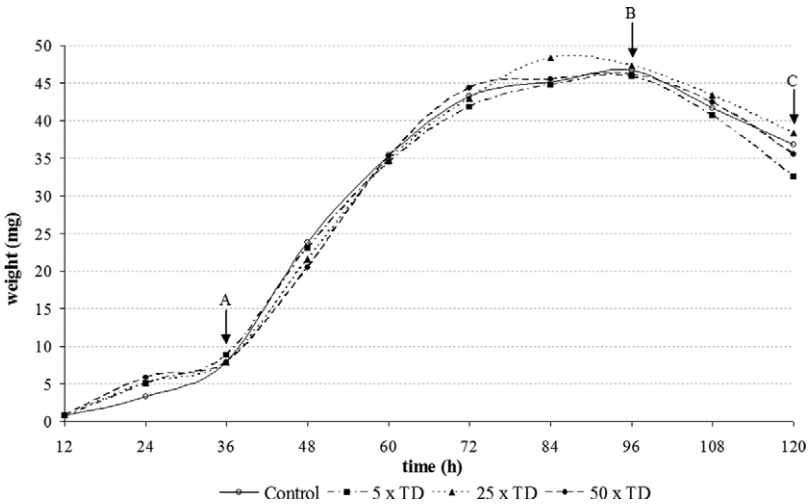

Fig. 1. Developmental curves (hours) of *C. megacephala* larvae reared in different concentrations of nandrolone decanoate, during the period from hatching until pupation. A, 2nd instar end; B, postfeeding stage; C, pupae.

higher weight gain compared with the other groups. The pupal stage was reached at the same time in all groups. The same was observed for the periods of instar change. The emergence intervals, emergence rates, and larval viabilities were similar for all groups (Table 2).

Although the different concentrations of nandrolone decanoate had no effect on the rate of weight gain in *C. albiceps* immatures ($F = 1.81$, $P = 0.1436$), the Duncan multiple comparisons test showed that the mean weight at 25× TD was significantly lower than that of the control group. The total larval developmental times were not significantly different among the groups. The control and drug-added groups underwent the instar change at the same time (Fig. 2). The larval mean weights were significantly different at most ages, except at 24 h ($F = 0.76$, $P = 0.5460$), 36 h ($F = 2.63$, $P = 0.0647$), 144 h ($F = 1.12$, $P = 0.3550$), 156 h ($F = 1.40$, $P = 0.2595$), and 180 h ($F = 1.38$, $P = 0.2659$) (Table 3). The emergence interval was similar for all groups as well. The larval viability was higher for the control group than for the AAS groups. For the 5× TD group this index was lower. The emergence rate was slightly lower for 5× TD than those for the other groups, where this rate was similar (Table 4).

The total rate of weight gain for *C. putoria* was not affected by the presence or concentration of the drug ($F = 0.10$, $P = 0.9615$). The instar changes occurred at the same time for immatures of all groups (Fig. 3). However, some weight variation among the groups was observed along the curves of weight gain. ANOVA and Duncan's multiple comparison tests showed differences at 12 h ($F = 35.12$, $P < 0.0001$), 24 h ($F = 10.99$, $P < 0.0001$), 36 h ($F = 5.21$, $P = 0.0043$), 48 h ($F = 36.02$, $P < 0.0001$), 120 h ($F = 5.36$, $P = 0.0037$), and 132 h ($F = 4.09$, $P = 0.0135$) (Table 5). The larval viability was similar between the control group and the higher concentration group (50× TD) and was slightly higher in the groups treated with intermediate concentrations of AAS (5 and 25× TD, in an increasing pattern). The emergence rate was similar among the control, 5× TD, and 25× TD groups, and lower for the higher concentration group (50× TD) (Table 6).

Table 1. Weights (mean ± SD, mg) during the developmental time (hours) of *C. megacephala* immatures exposed to different concentrations of nandrolone decanoate

Age	Control	5× TD	25× TD	50× TD
12	0.79 ± 0.06a,b	0.77 ± 0.07b	0.90 ± 0.10a	0.87 ± 0.02a,b
24	3.34 ± 0.50c	5.19 ± 0.53b	5.09 ± 0.73b	5.96 ± 0.45a
36	7.94 ± 1.17a	8.86 ± 1.44a	7.90 ± 1.30a	7.95 ± 0.90a
48	23.82 ± 3.45a	23.06 ± 2.82a,b	21.54 ± 2.21a,b	20.45 ± 4.29b
60	35.44 ± 3.74a	34.53 ± 3.77a	34.70 ± 5.28a	35.28 ± 3.77a
72	43.26 ± 6.23a	41.80 ± 4.34a	42.94 ± 7.04a	44.31 ± 2.89a
84	45.09 ± 3.81b	44.75 ± 1.81b	48.37 ± 2.59a	45.56 ± 2.72b
96	46.71 ± 4.29a	45.84 ± 2.96a	47.26 ± 2.98a	46.11 ± 3.58a
108	41.71 ± 3.93a	40.59 ± 2.61a	43.39 ± 2.02a	42.44 ± 3.47a
120	36.79 ± 2.54a,b	35.52 ± 2.52b	38.36 ± 3.13a	35.57 ± 1.95b

For each row, means followed by at least one letter in common differ, according to Duncan's multiple comparisons test.

Discussion

Few studies have examined the effects of hormones on the developmental rate of flies. Among these, Musvasva et al. (2001) investigated the influence of hydrocortisone, a steroid commonly used as an anti-

Table 2. Larval viability, emergence, and emergence interval (hours) rates of *C. megacephala* exposed to different concentrations of nandrolone decanoate ($n = 150$)

Group	Larval viability	Emergence rate	Emergence interval
Control	133 (89)[a]	126 (84)	102 ± 22.45
5× TD	138 (92)	128 (85)	102 ± 22.45
25× TD	137 (91)	127 (85)	102 ± 22.45
50× TD	135 (90)	128 (85)	102 ± 22.45

[a] Data are mean ± SD. Values in parentheses are percentages.

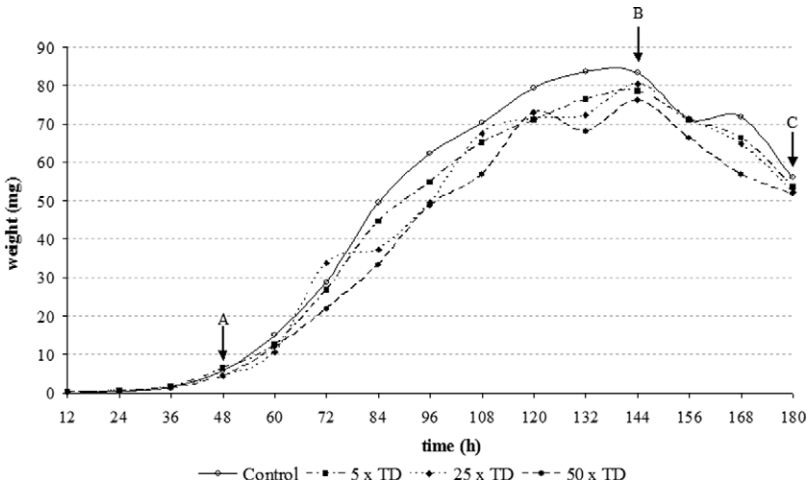

Fig. 2. Curves of development (hours) of *C. albiceps* larvae reared in different concentrations of nandrolone decanoate, during the period from hatching until pupation. A, 2nd instar end; B, postfeeding stage; C, pupae.

inflammatory drug, on the development of the flesh fly *Sarcophaga* (*Curranea*) *tibialis* Macquart (Diptera: Sarcophagidae). Even though the time of larval development was accelerated and the pupation time was delayed, a relationship between the concentration of this drug and the developmental time of immatures of this sarcophagid fly could not be established. The rate of mortality, although apparently high, was not statistically significant. Moreover, Musvasva et al. (2001) found no morphological changes in pupae and adults, similarly to the current study.

Ferrari et al. (2008) assessed the action of testosterone for veterinary use on the development of the blowfly *C. albiceps.* Differently from what was observed for nandrolone decanoate, the maggots exposed to testosterone gained more weight, despite a lack of difference in their developmental time compared with the drug-free group.

Da Silva and Villet (2006) evaluated the action of progesterone, a steroid obtained from two contraceptives for human use, on the developmental rate of *Chrysomya chloropyga* (Wiedemann). They observed a trend toward higher mortality rates in third-instar larvae but considered it a consequence of decay of the breeding substrate. A slightly accelerated development observed in second-instar larvae feeding on the progesterone was not significant.

The lack of a significant effect of AASs added to the artificial diet offered to *Chrysomya* species could be due to a lack or deficiency of assimilation of these compounds by the maggots. That is because vertebrate hormones, like their synthetic versions, are apolar and therefore show low solubility in water, whereas insect hormones, also derived from the precursor steroid molecule, are hydrophilic (Klowden 2002). This difference in solubility could have interfered with the absorption of nandrolone decanoate and its metabolites, because flies may lack effective apolar carriers to allow the compound to be metabolized.

C. albiceps is more frequently found in decaying carcasses (Souza and Linhares 1997; Carvalho and

Table 3. Weights (mean ± SD, mg) during the developmental time (hours) of *C. albiceps* immatures exposed to different concentrations of nandrolone decanoate

Age	Control	5× TD	25× TD	50× TD
12	0.11 ± 0.01d	0.22 ± 0.02c	0.29 ± 0.01b	0.34 ± 0.01a
24	0.43 ± 0.03a	0.45 ± 0.15a	0.48 ± 0.14a	0.36 ± 0.02a
36	1.46 ± 0.24a,b	1.45 ± 0.33a,b	1.52 ± 0.42a	1.17 ± 0.18b
48	5.72 ± 0.41a	6.13 ± 0.51a	4.17 ± 0.78b	4.17 ± 0.69b
60	14.94 ± 300a	12.43 ± 1.50b	10.38 ± 1.61c	11.84 ± 1.81c
72	28.80 ± 2.58b	26.80 ± 2.91b	33.70 ± 3.60a	21.87 ± 3.25c
84	49.58 ± 5.29a	44.44 ± 3.56b	37.22 ± 4.46c	33.23 ± 4.91c
96	62.25 ± 5.11a	54.69 ± 6.44b	49.52 ± 4.70c	48.76 ± 4.84c
108	70.29 ± 6.47a	65.05 ± 5.77a	67.54 ± 6.22a	56.75 ± 7.24b
120	79.28 ± 6.85a	71.11 ± 6.31b	71.14 ± 6.56b	72.71 ± 4.25b
132	83.38 ± 6.07a	76.23 ± 5.00b	72.23 ± 6.97b,c	68.10 ± 9.18c
144	83.67 ± 7.48a	78.46 ± 9.92a	80.26 ± 11.33a	75.93 ± 9.83a
156	71.16 ± 6.22a	70.92 ± 7.10a	71.37 ± 6.97a	66.20 ± 6.22a
168	71.79 ± 9.24a	66.36 ± 9.32a	64.78 ± 9.51a,b	56.66 ± 8.07b
180	56.06 ± 4.97a	53.53 ± 4.70a	52.33 ± 4.55a	51.67 ± 6.43a

For each row, means followed by at least one letter in common differ, according to Duncan's multiple comparisons test.

Table 4. Larval viability, emergence, and emergence interval (hours) rates of *C. albiceps* exposed to different concentrations of nandrolone decanoate (n = 150) (x ± SD)

Groups	Larval viability	Emergence rate	Emergence interval
Control	139 (93)[a]	133 (89)	132 ± 18.97
5× TD	116 (77)	115 (77)	132 ± 18.97
25× TD	132 (88)	128 (85)	132 ± 18.97
50× TD	133 (89)	126 (84)	132 ± 18.97

[a] Data are mean ± SD. Values in parentheses are percentages.

Linhares 2001; Carvalho et al. 2001, 2004; Moretti et al. 2008), and thus it is one of the most forensically important necrophagous insects in Brazil. If developing larvae present in carrion are taken for PMI estimates and their weight is used as a parameter, the PMI could be underestimated because of the smaller weight observed in larvae exposed to AASs, especially the highest AAS concentration compared with the weight gain of the other groups. Larvae of *C. putoria* exposed to AASs responded similarly, and at specific ages they showed significant differences in weight gain from the controls, as shown by ANOVA and Duncan's multiple comparisons tests. At 48 h, the weight recorded for the control group was significantly lower than that recorded for the individuals exposed to all decanoate nandrolone concentrations. This difference can indicate a developmental time ≈6 h faster in immatures exposed to the drug, which could produce an error in the PMI estimate.

Although the results showed a significant effect of nandrolone decanoate, at specific ages, on the fly larvae, it is important to mention that, because the experimental design used artificial media, direct extrapolation of these results to real human cases must be done carefully. This is because after nandrolone decanoate is metabolized by the organism, the molecule can be found in tissues, unaltered or in the form of metabolites such as 5-α-dihydronandrolone, 19-norandrosterone, 19-norepiandrosterone, 19-nortiocolanolone, compounds that are absent in the artificial media used to rear the immatures.

Cyclopentanoperhydrophenanthrene, the precursor molecule of insect steroid hormones such as ecdysteroids (e.g., ecdysone is basically related to molting but also is associated with events in other developmental stages), is obtained exclusively from feeding (Klowden 2002). Therefore, another hypothesis to explain why significant effects of nandrolone decanoate on the development rates of these flies were not observed is that the AAS metabolized during the feeding period of the immatures was broken down, and the recovered cyclopentanoperhydrophenanthrene nucleus may have been incorporated into the metabolism of the fly's own hormones and therefore did not affect its feeding physiology and metabolism.

In addition, maggots are able to accumulate and also to eliminate several compounds that can be obtained from feeding (Nuorteva and Nuorteva 1982, Sohal and Lamb 1977, 1979). This ability may determine whether their development, either during larval or pupal development, will be affected. In this case, the steroid may have been eliminated more rapidly than it accumulated. Because of this, the small variations observed in the rates of development, survivorship, and emergence were not significant. However, this assumption is merely speculative because the experimental model was not designed to test this hypothsis. Furthermore, this ability depends on individual physiology and on the developmental stages of each species.

Some blowfly larvae, such as *C. albiceps*, commonly behave as predators when food is insufficient or when the abundance of predators and prey in the decomposing substrate is very high. This behavior may be influenced by many factors, such as prey vulnerability due to the reduction of larval agility, palatability, and action of pheromones (Faria et al. 2004). Therefore,

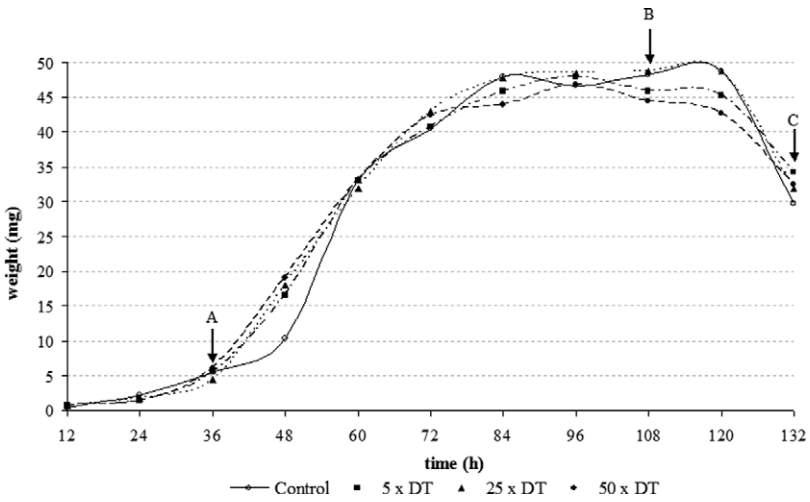

Fig. 3. Curves of development (hours) of *C. putoria* larvae reared in different concentrations of nandrolone decanoate, during the period from hatching until pupation. A, 2nd instar end; B, postfeeding stage; C, pupae.

Table 5. Weights (mean ± SD, mg) during the developmental time (hours) of *C. putoria* immatures exposed to different concentrations of nandrolone decanoate

Age	Control	5× TD	25× TD	50× TD
12	0.36 ± 0.08b	0.70 ± 0.03a	0.64 ± 0.04a	0.64 ± 0.02a
24	2.15 ± 0.38a	1.43 ± 0.31c	1.76 ± 0.33b	1.41 ± 0.30c
36	5.53 ± 1.08a	5.52 ± 0.95a	4.47 ± 0.53b	6.11 ± 1.11a
48	10.38 ± 1.44c	16.55 ± 1.66b	17.98 ± 2.08a,b	19.00 ± 2.72a
60	32.95 ± 3.48a	33.03 ± 3.44a	31.89 ± 2.75a	33.13 ± 4.10a,b
72	40.51 ± 7.20a	40.71 ± 4.76a	42.89 ± 4.61a	42.35 ± 3.93a
84	47.95 ± 2.47a	45.91 ± 6.61a	47.83 ± 3.12a	42.99 ± 5.29a
96	46.74 ± 5.07a	47.99 ± 3.47a	48.48 ± 2.37a	46.89 ± 2.64a
108	48.15 ± 3.84a	45.80 ± 6.41a	48.71 ± 2.57a	44.50 ± 3.48a
120	48.79 ± 4.14a	45.33 ± 3.93a,b	48.71 ± 3.01a	42.70 ± 4.78b
132	29.78 ± 2.40b	34.24 ± 4.70a	31.94 ± 1.81a,b	32.47 ± 1.85a,b

For each row, means followed by at least one letter in common differ, according to Duncan's multiple comparisons test.

another aspect that should be investigated is whether the presence of drugs such as the AAS tested in the current study may influence these parameters, making the species which had contact with this type of compound more vulnerable to predation, and therefore significantly changing the decomposition process.

Although the experimental design has some limitations due to the use of artificial media, this methodology is advantageous in rationalizing the use of animal models in research (with respect to animal ethics), and in reducing the cost of studies (Estrada et al. 2009). Although this study is one of the first to assess the effect of AAS on the development of forensically important dipterans, similar results may well occur in real human cases. It is necessary to consider carefully the complexity of extrapolating from data obtained from studies using artificial media or animal models, considering the existence of differences between the same tissues in different organisms.

Further experiments using AASs can be carried out with animal models, higher concentrations, or AASs in combination with other substances, to corroborate the data obtained in this study of a possible error induced by larval weight gain of *C. megacephala*, *C. albiceps*, and *C. putoria*, when this parameter is used to estimate PMI.

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Table 6. Larval viability, emergence rate, and emergence interval (hours) of *C. putoria* exposed to different concentrations of nandrolone decanoate (n = 150)

Group	Larval viability	Emergence rate	Emergence interval
Control	114 (76)[a]	106 (71)	126 ± 22.45
5× TD	120 (80)	115 (77)	126 ± 22.45
25× TD	130 (87)	126 (84)	126 ± 22.45
50× TD	118 (79)	90 (60)	126 ± 22.45

[a] Data are mean ± SD. Values in parentheses are percentages.

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