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Analytica Chimica Acta 586 (2007) 163–170
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Formation of boldenone and boldenone-analogues by maggots of *Lucilia sericata*

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Received 19 June 2006; received in revised form 23 October 2006; accepted 3 November 2006
Available online 10 November 2006

Abstract

Current evidence suggests that neo formation of the anabolic steroid boldenone (androsta-1,4-diene-17-ol-3-one) occurs in calves' faecal material, making it difficult to distinguish between illegally administered boldenone and its potential endogenous presence. This strengthens the urgent need to elucidate the pathway leading to boldenone formation. In our laboratory, the invertebrate *Neomysis integer* (*Crustacea*, *Mysidacea*) was used since 2004 as an alternative model for the partial replacement of vertebrate animals in metabolisation studies with illegal growth promotors and veterinary drugs, e.g. boldenone. The present study evaluates the metabolic capacity of other invertebrates, the brine shrimp *Artemia franciscana* and maggots of the greenbottle fly *Lucilia sericata*. The first results indicate that maggots of *L. sericata* are able to convert phytosterols and –stanols, nowadays in substantial amounts added to animal feed, into androsta-1,4-diene-3,17-dione (ADD), the precursor of boldenone, at a yield of 0.10–0.14% ($p < 0.001$, significance compared to endogenous excretion of maggots) but not to boldenone itself. Furthermore, β-testosterone, an endogenous hormone, was transformed into androst-4-ene-3,17-dione (AED), ADD and β-boldenone at a significant ($p < 0.001$, significance compared to endogenous excretion of maggots) yield of circa 13%, 0.80% and 2.2%, respectively. In future studies these results are of value to further evaluate the use of maggots of *L. sericata* as an invertebrate model in metabolisation studies.

Keywords: Boldenone; Anabolic steroids; Phytosterols; Metabolisation; *Lucilia sericata*

1. Introduction

In recent years different European Union Member States have focused a lot of research on the presence and metabolisation of boldenone in a number of biological samples [1]. The discussion on the origin of boldenone remains a hot topic in those laboratories that determine residues of illegally used anabolic steroids. According to the review by De Brabander et al., an increased number of boldenone findings, especially in bovine faeces and urine samples, should be linked to endogenous formation of boldenone besides of illegal treatment of animals and improved analytical capabilities [1].

The metabolic pathway leading to the formation of boldenone in various animal species remains unknown. In order to elucidate this pathway, animal trials are asked for. Nevertheless, the use of vertebrate animal experiments for scientific purposes still has a serious economic, ethical and ecological impact [2,3]. Because of this ongoing debate, scientists are looking for alternative methods to answer their questions.

In our laboratory, experience has been build up using the mysid shrimp *Neomysis integer* (*Crustacea, Mysidacea*) as an invertebrate model partly replacing vertebrate animals in metabolic studies. An indication of the similarity between the metabolism of bovine species and the invertebrate *N. integer* could be given based on the metabolisation of several exogenous and endogenous anabolic steroids [2,4,5]. Results were promising, even leading to the consideration of introducing *N. integer* as suitable standard test organism for ecotoxicological studies [6,7]. Nevertheless, results were not always reproducible.

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doi:10.1016/j.aca.2006.11.009

According to literature, Verslycke et al. were the first to detect the anabolic steroid β-boldenone in invertebrates after exposure of *N. integer* to β-testosterone [4]. However, this conversion could not be repeated.

The weakness of the reproducibility of metabolisation experiments with *N. integer* and on the other hand, the strength of invertebrate models encouraged the consideration to perform *in vivo* studies with other invertebrate species. In this paper, nauplii of the brine shrimp *Artemia franciscana* (*Crustacea*, *Anostraca*) and maggots of the greenbottle fly *Lucilia sericata* (*Arthropoda*, *Diptera*) were used to set up metabolisation experiments.

The small crustacean *A. franciscana*, better known as brine shrimp, is a typical inhabitant of hypersaline environments that have low species diversity and simple trophic structures [8]. Brine shrimps' eggs are encapsulated in cysts giving them the characteristic to withstand extreme environmental conditions. Cysts can be reactivated by exposing them to more favorable conditions, allowing embryonic development to be completed. Within 24–48 h, depending on temperature, hatching of nauplii occurs. It takes about 3 weeks from hatching, over successive developmental stages or instars, to reach the adult stage [9].

The usefulness of this invertebrate species for ecotoxicological studies results from the fact that they are cheap, commercially available and easy to rear under laboratory circumstances [10].

In the past the insect *L. sericata*, or greenbottle fly, has been known especially for its parasitic properties. Larvae, or greenbottle maggots, are facultative ectoparasites. However, these maggots are now widely used in medicine as a fast and effective means of treating necrotic chronic wounds, both in humans and animals [11,12]. In addition, they serve as reliable substrates for qualitative analysis of drugs in human tissues, in order to provide data concerning postmortem interval for badly decomposed bodies [13]. Their value for toxicological analyses remains limited since maggots metabolise and eliminate ingested substances during their development [14,15]. Nevertheless, this fact strengthens their potential use in metabolisation experiments.

Our objective was to obtain initial data on the metabolic capacity of nauplii of *A. franciscana* and maggots of *L. sericata* to determine the endogenous origin of boldenone or its formation after exposure to the steroid hormone β-testosterone, the phytosterol β-sitosterol and the phytostanol stigmastanol.

17β-Boldenone (β-Bol), also called 1-dehydrotestosterone, is a steroid with androgenic activity. It only differs from the main steroid hormone 17β-testosterone (β-T) by a double bond at the 1-position. Other important steroids closely related to β-Bol and β-T are their precursors androstadienedione (ADD or boldione) and androstenedione (AED), respectively. The chemical

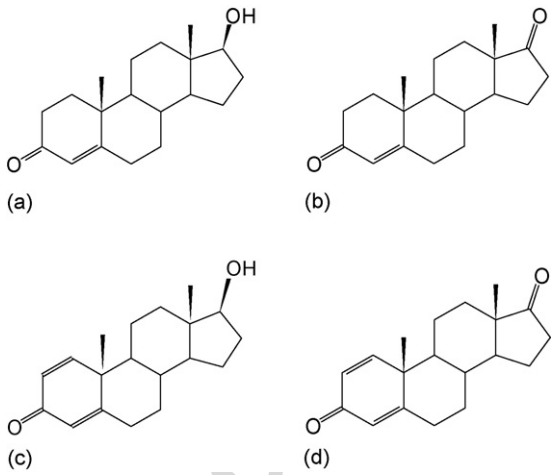

Fig. 1. Chemical structure of the most important steroids involved in this study: (a) testosterone (T), (b) androstenedione (AED), (c) boldenone (Bol) and (d) androstadienedione (ADD).

structures of these compounds are given in Fig. 1. β-Bol, esters of β-Bol and ADD are for sale as anabolic preparations. β-Bol improves the growth and feed conversion of cattle and might therefore be abused to achieve more efficient meat production. Transformation of β-T to β-Bol has previously been reported in several invertebrate and vertebrate species [2]. It has also been demonstrated that insects and prawns are able to synthesise steroid hormones out of phytosterols, with cholesterol as an intermediary product [16,17]. Phytosterols or plantsterols and cholesterol are 4-desmethyl sterols that share identical ring structures. The various sterols only differ in their side chain configuration. Hydrogenation in the 5α-position results in their saturated forms, the phytostanols. The most common representatives are β-sitosterol and stigmastanol, a phytosterol and –stanol, respectively, illustrated in Fig. 2 [16,18–21].

2. Experimental

2.1. Reagents and chemicals

β-Testosterone (androst-4-ene-17β-ol-3-one, β-T), methyltestosterone (androst-4-ene-17α-methyl-17β-ol-3-one, MT), androstadienedione (androsta-1,4-diene-3,17-dione, ADD), stigmastanol ((3β,5α)-stigmastan-3-ol) and β-sitosterol (3β-stigmast-5-en-3-ol) (contains 20% campesterol, 21% stigmasterol and 59% sitosterol) were obtained from Sigma–Aldrich (St. Louis, USA). Androstenedione (androst-4-ene-3,17-dione, AED) and β-boldenone (androsta-1,4-diene-17β-ol-3-one, β-Bol) were purchased from Steraloids (Newport, USA).

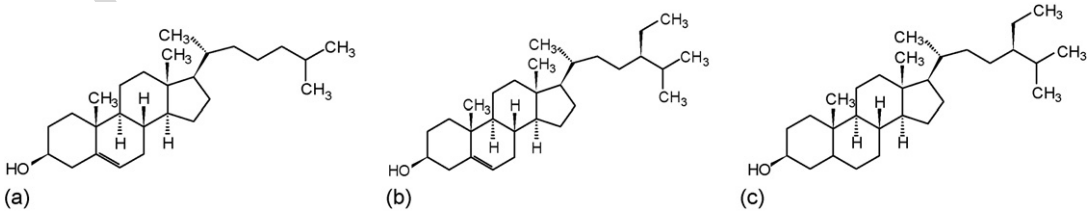

Fig. 2. Chemical structure of: (a) cholesterol, (b) β-sitosterol and (c) stigmastanol.

Solvents and reagents were of analytical grade and purchased from VWR (Merck, Darmstadt, Germany).

For each component, a primary standard stock solution was prepared in ethanol (EtOH) at a concentration of 200 ng μL^{-1}. Working solutions for spiking of the exposure medium were made by appropriate dilution of these stock solutions in EtOH. All standard solutions were stored at 4 °C following the quality assurance instructions of Belac accreditation (EN17025).

2.2. Animal collection and maintenance

Cysts of *Artemia franciscana* were purchased from INVE (Dendermonde, Belgium). Hatching was performed by dissolving 5 g of *Artemia* cysts in 900 mL of artificial sea water (Instant Ocean®, Aquarium Systems, Sarrebourg, France) with a salinity of 30 g L^{-1} in a 1 L cylinder–conical vessel. Vigorous aeration and continuous illumination was maintained for 24–48 h at 25 °C.

Maggots of *Lucilia sericata* were a generous gift from PRO fishing (Oetingen, Belgium).

2.3. Animal experiments

For experiments with *A. franciscana*, 5 mL of nauplii in artificial sea water were diluted in 1 mL of medium (water with a salinity of 5 g L^{-1} diluted from artificial sea water with deionised carbon-filtered tap water). From this dilution 100 μL was added to 1900 μL of medium in a 5-mL glass tube. Maggots of *L. sericata* were individually placed into 5-mL glass tubes containing 2 mL of medium (water with a salinity of 5 g L^{-1} diluted from artificial sea water with deionised carbon-filtered tap water).

Test species were exposed to 2 μg of the different analytes (β-testosterone, β-sitosterol, stigmastanol) in 2 mL of medium. A 4 h exposure period was applied at room temperature (21 ± 3 °C). These test conditions were based on similar metabolisation experiments with the mysid shrimp *Neomysis integer* [22].

Modifications on above mentioned exposure conditions were evaluated in order to optimise metabolisation conditions for *A. franciscana*. The influence of the medium composition (tap water/water with a salinity of 5 g L^{-1}), the temperature (15 °C/room temperature of 21 ± 3 °C) and the exposure period (1 h/4 h/6 h) was investigated. All the exposure experiments with this test species were performed in three replicas. For *L. sericata*, exposures were performed in 10 replicas.

Different controls were incorporated in the experiments. These are described in more detail in Section 2.6.

2.4. Sample extraction and clean-up

After exposure and before extraction, methyltestosterone was added to the medium at a concentration of 12 ng as internal standard. Metabolites were extracted twice from the medium using 2 mL ethyl acetate and the organic phase was withdrawn after centrifugation (5 min, 14,000 × *g*, 4 °C). The two ethyl acetate fractions were combined and vacuum evaporated to dryness (Speedvac Plus SC210A, Savant Instruments Inc., Farmingdale, USA).

The extract was reconstituted in 30 μL of methanol (MeOH) and 90 μL of 0.02 M aqueous formic acid (HCOOH). Fifty microliters were injected on column [22].

Table 1
Quality assurance data (limit of detection (LOD), recovery ± standard deviation (S.D.) and calibration coefficient (R^2)) for the analysis of AED, ADD, β-Bol and β-T spiked in medium at a range of 2.5E−01 to 2.5E+01 ng mL^{-1}

	AED	ADD	β-Bol	β-T
LOD (ng mL^{-1})	2.5E−01	1.0E−01	1.0E+00	1.0E−01
Recovery ± S.D. (%)	108 ± 18.7	106 ± 20.4	104 ± 17.5	114 ± 21.9
R^2	0.93	0.92	0.99	0.97

2.5. LC–MS^2 analysis

The HPLC apparatus comprised of a HP 1100 series pump, an AS3000 autosampler (TSP, San Jose, USA) and vacuum degasser (Agilent, Palo Alto, USA). Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm, Waters, Milford, USA). For separation of the different compounds, a linear gradient was used starting with a mixture of 60% 0.02 M aqueous HCOOH and 40% MeOH. The methanol percentage increased from 40% to 80% in 25 min. The flow rate was set at 0.3 mL min^{-1}. Between each sample the column was allowed to equilibrate at initial conditions (8 min). Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyser (Thermo Electron, San José, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface and Xcalibur 2.0 software (Batavia, USA). The compounds were detected in positive ion mode MS full scan and MS^2 scan.

2.6. Quality assurance

Prior to sample analysis, standard mixtures of the targeted metabolites were injected in order to check the operation conditions of the chromatographic devices. Different metabolites were identified based on their relative retention time and on the ion ratio of their product ions based on the performance criteria for analytical residue methods defined in Commission Decision 2002/657/EC [23]. Quantification occurred by fitting metabolites' area ratio in a calibration curve. For all targeted metabolites extracts of standard solutions spiked in medium at a concentration of 0.25–25 ng mL^{-1} were injected once to four times. For every single metabolite a calibration curve was constructed. The limit of detection for each component was set at the lowest detectable calibration point. Analyte recoveries were determined by adding known concentrations of the standard solutions to blank medium. The quality assurance data for AED, ADD, β-Bol and β-T are summarised in Table 1.

Extracts of the medium as such were run to account for the presence of any of the targeted metabolites in the exposure medium. Extracts of medium in which the analytes, β-testosterone, β-sitosterol and stigmastanol, were added individually were run to account for microbial transformation or impurity of the standard solutions in the absence of test

species. The influence of the dilution solvent, EtOH, on the metabolisation pattern of the test species was accounted for by integrating a solvent control. Extracts of medium in which the test species were not exposed to an analyte were analysed to determine the endogenous excretion of metabolites by the test species. All tests were performed in 10 replicas.

2.7. Statistics

All statistical analyses were assessed with the software package Statistica™ (Statsoft, Tulsa, USA). An outlier analysis was performed and data were checked for normality and homogeneity of variance, respectively, using Kolmogorov–Smirnov and Levene's tests, both with an α-error of 0.05. If these assumptions were met, the effect of the treatment was tested for significance by one-way analysis of variance (Dunnett's test). If the assumptions were not met, data were logarithmically transformed or the non-parametric Mann–Whitney *U*-test was used. All box plots were created with Statistica™ and show mean (small square), standard error (box) and the standard deviation (whisker).

3. Results

3.1. Artemia franciscana

In a preliminary experiment *A. franciscana* was exposed to a 1 mg L^{-1} concentration of β-T to assess its metabolic capacity. Several exposure conditions (temperature, exposure period, exposure medium) were evaluated. However, under none of the tested conditions metabolites could be detected in the medium.

3.2. Lucilia sericata

In this study extracts of the exposure medium of the maggots were specifically examined for the presence of AED, ADD and β-Bol. For exposure to β-sitosterol and stigmastanol, the presence of β-T as a metabolite was considered as well. The identification of the metabolites was based on what has been described previously for the mysid *N. integer* [2,4,7]. From our experience with *N. integer* [22], it was observed that excreted concentrations of metabolites in the medium were higher than those retained in the organism. Furthermore, in a preliminary experiment with *L. sericata* in which maggots were exposed to β-T, AED, ADD as well as β-Bol were detected in the medium while only AED was detected in the organism and in minor concentrations. Based on these facts and the fact that the method for extraction of the metabolites from maggots is not satisfactory yet, extraction of the metabolites of interest was, within the scope of this article, only performed on the medium, not on the organisms.

After exposure of larvae of *L. sericata* to β-T, each of the targeted metabolites AED ($p < 0.001$), ADD ($p < 0.001$) and β-Bol ($p < 0.001$) was detected in the medium. Mean concentrations of 1.3E+02 ± 4.3E+01 ng mL^{-1}, 8.0E+00 ± 3.8E+00 ng mL^{-1} and 2.2E+01 ± 4.8E+00 ng mL^{-1} were calculated for AED,

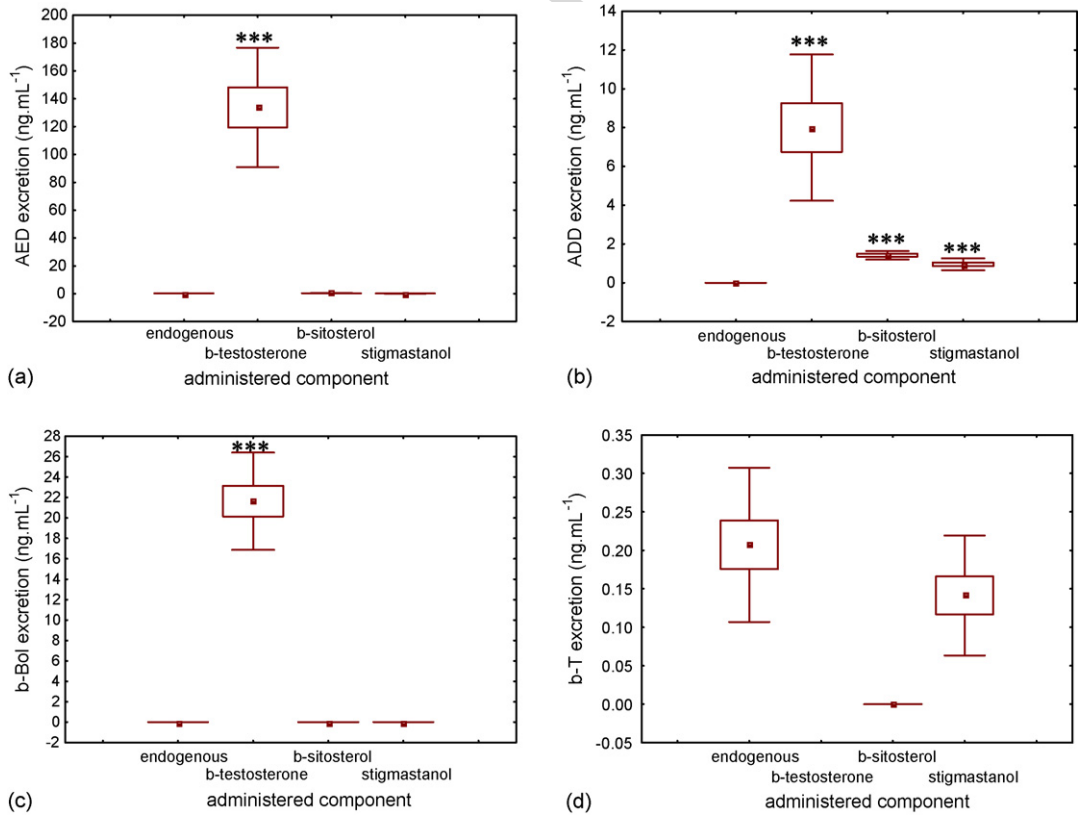

Fig. 3. Metabolite excretion in the medium by maggots of *L. sericata* after exposure for 4 h to β-testosterone, β-sitosterol and stigmastanol compared to the endogenous excretion (***$p < 0.001$, significance compared to endogenous excretion): (a) androstenedione (AED), (b) androstadienedione (ADD), (c) β-boldenone (β-Bol) and (d) β-testosterone (β-T).

Table 2
Metabolite excretion in the medium by maggots of *L. sericata*, expressed as mean concentration ± standard deviation, relative to the wet body weight (ng g ww^{-1})

Administered (2.1E+04 ± 2.4E+03) (ng g ww^{-1})	Excretion concentration (ng g ww^{-1})			
	AED	ADD	β-Bol	β-T
Endogenous	5.8E+00 ± 2.2E+00	ND[b]	ND[b]	4.6E+00 ± 2.0E+00
β-Testosterone	2.8E+03 ± 1.2E+03***	1.7E+02 ± 9.6E+01***	4.5E+02 ± 1.2E+02***	NC[a]
β-Sitosterol	0.0E+00	3.1E+01 ± 8.2E+00***	ND[b]	0.0E+00
Stigmastanol	0.0E+00	1.9E+01 ± 5.8E+00***	ND[b]	3.2E+00 ± 1.8E+00

Concentrations were corrected for impurities in the standard solutions. Significant differences of metabolite excretion to endogenous excretion are indicated by *** $p < 0.001$.
[a] Not considered a metabolite.
[b] Not detected.

ADD and β-Bol, respectively (Fig. 3a–c). These concentrations were significantly higher than what was excreted by unexposed organisms. Indeed, endogenous metabolite excretion by maggots of *L. sericata* did not contain any ADD or β-Bol. AED and β-T, however, were detected in the medium of unexposed organisms at concentrations of 2.6E−01 ± 1.0E−01 ng mL^{-1} for AED and 2.1E−01 ± 1.0E−01 ng mL^{-1} for β-T, indicating their endogenous origin (Fig. 3a–d). In Table 2 an overview is

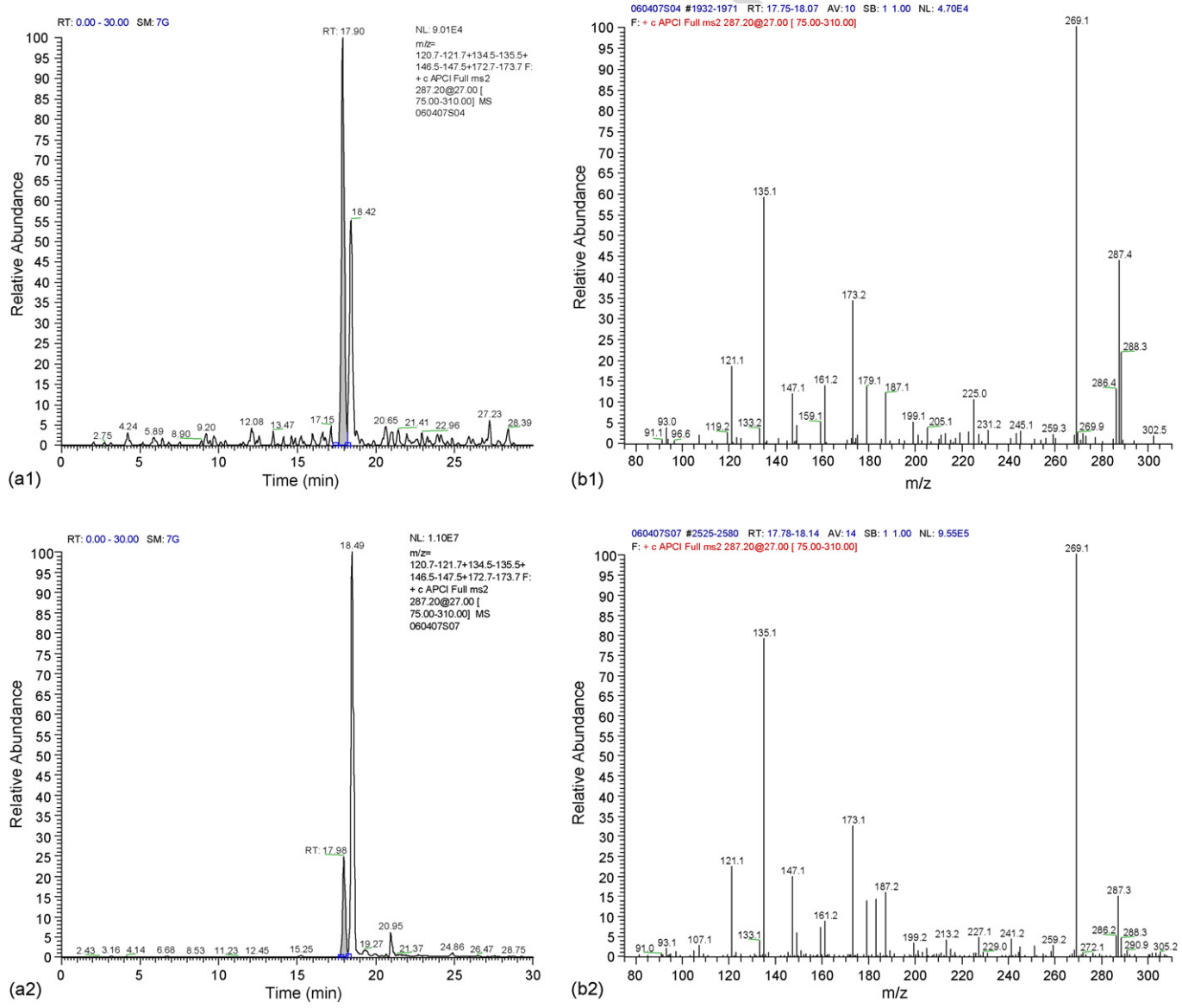

Fig. 4. LC–MS^2: (a) chromatograms and (b) spectra of β-boldenone (β-Bol) in medium (1) spiked at a concentration of 5 ng mL^{-1} and (2) excreted after exposure of *L. sericata* to β-testosterone (β-T).

given of the mean concentrations of this metabolite excretion by maggots, relative to their wet body weight.

In Fig. 4 a chromatogram and MS^2 spectrum of β-Bol, as detected in extracts of the medium in which an individual maggot of *L. sericata* had been exposed to β-T for 4 h, is compared to that of β-Bol spiked in medium. From the chromatograms it is clear that based on the retention time a peak of interest, highlighted in grey, shows up after 17.9 min. The peak at 18.4 min corresponds to AED. Confirmation for the identification of the peak at 17.9 min, corresponding to β-Bol, was achieved from comparison of the MS^2 product ions with *m/z* 121, 135, 147 and 173 of the precursor ion with *m/z* 287 of sample and spiked sample.

Exposure to β-sitosterol of maggots of *L. sericata* resulted in AED-concentrations in the medium with a mean of $3.8E{-}01 \pm 1.0E{-}01$ ng mL^{-1}, not significantly different from the concentrations in the medium of unexposed maggots, being $2.6E{-}01 \pm 1.0E{-}01$ ng mL^{-1}. Significantly higher concentrations of ADD, $1.4E{+}00 \pm 2.3E{-}01$ ng mL^{-1} ($p < 0.001$), were detected in the medium compared to unexposed organisms in which no endogenous excretion of ADD could be observed. In Fig. 3a and b these results are graphically represented. Mean concentrations of metabolite excretion expressed relative to the wet body weight of the maggots are summarised in Table 2. The excretion of β-T was negligible after correction for impurities in the standard solution of β-sitosterol as described in Section 2.6. Because the β-sitosterol standard consisted of a mixture of three phytosterols (campesterol, stigmasterol and sitosterol) it was not clear which phytosterol was responsible for the formation of AED and ADD by *L. sericata*.

Maggots of *L. sericata* were able to convert stigmastanol into ADD and β-T after a 4 h exposure period. Excreted concentrations of ADD in the medium of exposed organisms, with a mean of $9.6E{-}01 \pm 3.1E{-}01$ ng mL^{-1}, were significantly higher ($p < 0.001$) than those of unexposed organisms, being absent or below the limit of detection (Fig. 3b). Concentrations of β-T, $1.4E{-}01 \pm 7.8E{-}02$ ng mL^{-1}, were not significantly different from what was excreted by unexposed test species, $2.1E{-}01 \pm 1.0E{-}01$ ng mL^{-1}, as shown in Fig. 3d. An overview of this metabolisation expressed as mean concentrations of excreted metabolites relative to the wet body weight of the maggots is given in Table 2. Since AED was only encountered at concentrations equalling the endogenous excretion of *L. sericata*, it was not considered a metabolite of stigmastanol.

Table 3
Metabolite excretion by maggots of *L. sericata*, expressed as mean conversion percentages ± standard deviation of the exogenous administered compounds β-testosterone, β-sitosterol and stigmastanol

Administered component	Conversion percentages (%)			
	AED	ADD	β-Bol	β-T
β-Testosterone	13 ± 4.2	0.80 ± 0.38	2.2 ± 0.48	NC[a]
β-Sitosterol	0.0	0.14 ± 0.022	ND[b]	0.0
Stigmastanol	0.0	0.10 ± 0.031	ND[b]	0.0

Percentages were corrected for endogenous excretion and for impurities in the standard solutions.
[a] Not considered a metabolite.
[b] Not detected.

4. Discussion

The aim of the present study was to gain information on the metabolic capacity of two invertebrates, the brine shrimp *A. franciscana* and maggots of the greenbottle fly *L. sericata*. Furthermore, results from this study provide information on their use as a model for the partial replacement of vertebrate animals in metabolisation studies. Finally, the potential importance of these results in unravelling the pathway leading to boldenone formation and thus elucidating its origin is discussed.

Because of the simplicity of invertebrate animal experiments in comparison to vertebrate animal experiments, a lot of valuable time and money can be saved and in the end, less vertebrate animals have to be sacrificed. In this context, we performed *in vivo* metabolisation studies with *A. franciscana* nauplii. The knowledge already available on testosterone metabolisation in invertebrates encouraged the use of β-T as steroid substrate for exposure to provide initial data on the metabolic capacity [2,4,22]. The present studies indicate that *A. franciscana* were not able to metabolise β-T into detectable concentrations of the targeted metabolites. Although different exposure conditions were assessed, AED, ADD and β-Bol could not be detected in the medium as possible metabolisation products of β-T. This might be explained by the fact that the currently used biomass–water ratio was too low to become detectable concentrations of the metabolites. Future studies are required to assess the metabolic capacity of *A. franciscana* at different biomass–water ratios. Based on the present data, this test species was not considered a valuable invertebrate model for metabolisation experiments. However, there is a need for more research to confirm or reject the present findings.

Exposure of another invertebrate species, maggots of *L. sericata*, to an excess of β-T significantly increased the metabolite concentrations of AED, ADD and β-Bol compared to unexposed species. In Table 3 the conversion percentages of β-T into the targeted metabolites are presented. AED, the direct precursor of β-T, was the main metabolite excreted in the medium. This oxidative reaction comprises the oxidation of the enol- to the keto-form, a conversion that has also been described for *N. integer* [22]. ADD and β-Bol were detected in minor concentrations in the medium, though still significantly different from the endogenous excretion. This observation requires further confirmation to assure the reproducibility of the conversion of β-T into β-Bol, nevertheless these results clearly indicate the metabolic capacity of *L. sericata* maggots and consequently their value for metabolisation experiments. Furthermore, the metabolic pathway of β-T as observed for *L. sericata* in our experiments resembles what has been described earlier for *N. integer* [22]. This suggests the potential value of maggots of *L. sericata* as invertebrate model in animal trials. However, more studies are required to elucidate the similarity to vertebrate animals' metabolism.

Based on experiments with *N. integer* performed in our laboratory, it has been suggested that boldenone can be formed

after exposure of this mysid to several steroid hormones and their precursors [22]. This theory is based on the biosynthesis pathway of β-T. *N. integer* produced β-T after exposure to its precursor AED. The latter can be formed from ADD by dehydrogenation in the 1,2-position. On the other hand, reduction of the keto-group of ADD to an enol-group would result in the formation of β-Bol. When exposing *N. integer* to ADD, the formation of both AED and β-Bol was observed. Moreover, dehydrogenation in position 1,2 of β-T might even directly lead to the formation of β-Bol (Fig. 1). Poelmans hypothesised that exposure to a high concentration of an anabolic steroid, leads to a shift in the equilibrium between β-T and AED towards AED production, subsequently followed by the formation of β-Bol, with ADD as intermediary product [22]. Our results, indicate the existence of a similar pathway in *L. sericata* for the formation of β-Bol out of β-T, when administered in excess. Testosterone metabolism activity or at least the presence of androgenic compounds as a prerequisite for boldenone formation has also been suggested by De Brabander et al. [1]. Their hypothesis was based on the fact that boldenone is mostly reported together with substantial amounts of testosterone and in male animals.

Another theory on the origin of boldenone focuses on the transformation of phytosterols and –stanols. Since the mid 1990s there has been considerable interest in and commercial marketing of phytosterol enriched products, which are suggested to lower human cholesterol levels [19]. Furthermore, the importance of phytosterols as a substitute for fat from animal origin in animal feed has increased due to the crisis from bovine spongiform encephalopathy, as in Belgium in the late 1990s. The popularity of these components has caused the medical and biochemical community to focus on phytosterols. Consequently, research activity on phytosterols has increased dramatically. Side chain cleavage of plantsterols with the formation of steroid hormones or their precursors has been described in literature by several authors [5,24,25]. If ADD and other androgens, such as AED and T, are generated after degradation of phytosterols and –stanols to steroidal precursors, other effects may need to be considered. This was the incentive for investigating boldenone formation out of phytosterols and –stanols. Exposure of *L. sericata* maggots to high concentrations of β-sitosterol and stigmastanol resulted in the excretion of ADD, the precursor of Bol, in the medium. However, direct formation of β-Bol out of β-sitosterol or stigmastanol could not be proven based on the present data.

In this study, initial data on the metabolic capacity of *L. sericata* were obtained after exposure of these organisms to unrealistically high concentrations of β-T, β-sitosterol and stigmastanol. The results are of value in future exposure experiments to evaluate the metabolisation of these components at environmentally and biologically relevant concentrations and in different matrices. In faeces of veal calves, for example, neo formation of the anabolic steroids ADD, α-Bol and β-Bol was hypothesised by microbial conversion of steroidal feed components, such as phytosterols, present in the faeces [26]. In literature the detection of Bol in faeces is described and discussed by several authors [1,27–29]. Apart from the screening of faeces for boldenone, current studies also investigated urine samples [26,29–31]. Consequently, metabolisation of phytosterols and anabolic steroids by maggots of *L. sericata* in a matrix such as faeces might provide novel insights into our understanding of boldenone formation. Furthermore, the interference of faecal contamination with boldenone formation in urine requires further investigation.

Finally, it should also be noted that the ability of maggots of *L. sericata* to metabolise β-testosterone and also phytosterols and phytostanols, makes it interesting to extract metabolites from the organisms. In this respect, the method for androgenic metabolite extraction from the organism as developed for *N. integer* should be optimised.

5. Conclusion

The use of maggots of *L. sericata* as an invertebrate model for metabolisation studies has shown to be promising. Compared to experiments with other invertebrate species, such as *N. integer* and *A. franciscana*, the yield as well as the reproducibility of the metabolic reactions have considerably been improved.

It was demonstrated that *L. sericata* is able to transform β-testosterone into AED, ADD and β-Bol with a substantial yield of 13%, 0.80% and 2.2%, respectively. Lower conversion percentages of 0.14% and 0.10% were reported for the transformation of β-sitosterol and stigmastanol into ADD.

To better understand the origin of boldenone, its precursors, such as ADD, and its metabolites, more research is required. The results presented here can hopefully further advance the current knowledge on the metabolic pathway and the origin of boldenone. More studies regarding the usefulness of *L. sericata* and related species in metabolisation experiments with phytosterols and anabolic steroids under more realistic circumstances are currently ongoing.

Acknowledgements

This research was supported by a research grant of the Ghent University Research Fund (BOF, B/06856/01). The authors gratefully acknowledge PRO fishing (Oetingen, Belgium) who supplied the maggots of *L. sericata*.

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