Journal of Steroid Biochemistry & Molecular Biology 119 (2010) 161–170

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Endogenous boldenone-formation in cattle: Alternative invertebrate organisms to elucidate the enzymatic pathway and the potential role of edible fungi on cattle's feed

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ARTICLE INFO

Article history:
Received 2 November 2009
Received in revised form 21 February 2010
Accepted 22 February 2010

Keywords:
Steroids
Testosterone
Phytosterols
LC–MS–MS
Model organism
Animal feed

ABSTRACT

Although β-boldenone (bBol) used to be a marker of illegal steroid administration in calves, its endogenous formation has recently been demonstrated in these vertebrates. However, research on the pathway leading to bBol remains scarce. This study shows the usefulness of *in vivo* invertebrate models as alternatives to vertebrate animal experiments, using *Neomysis integer* and *Lucilia sericata*. In accordance with vertebrates, androstenedione (AED) was the main metabolite of β-testosterone (bT) produced by these invertebrates, and bBol was also frequently detected. Moreover, *in vitro* experiments using feed-borne fungi and microsomes were useful to perform the pathway from bT to bBol. Even the conversion of phytosterols into steroids was shown *in vitro*. Both *in vivo* and *in vitro*, the conversion of bT into bBol could be demonstrated in this study. Metabolism of phytosterols by feed-borne fungi may be of particular importance to explain the endogenous bBol-formation by cattle. To the best of our knowledge, it is the first time the latter pathway is described in literature.

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1. Introduction

Because of their muscle-building and growth-enhancing properties [1–3], steroid hormones are still illegally administered to food-producing animals. Nevertheless, the possible risks to human health from residues of growth-promoting hormones in food products of animal origin resulted in the ban of hormonal substances in animal production [4]. This European Union legislation applies equally to novel steroids, not existing in nature, and synthetically produced versions of naturally occurring steroid hormones, i.e. exogenous and synthetic endogenous hormones, respectively.

As an anabolic steroid with low androgenic activity, boldenone (Bol) has frequently been subject of discussion in residue analysis. β-Boldenone (bBol), also called 1-dehydrotestosterone, only differs from the major circulating androgen β-testosterone (bT) by one double bond at the 1-position (Fig. 1). Important steroids closely related to bBol and bT are the bBol epimer α-boldenone (aBol), androstadienedione (boldione, ADD) and androstenedione (AED) (Fig. 1) [5]. Especially because of the structure-similarity of Bol and testosterone (T), Bol is known to be administered to cattle to promote growth [2]. However, this analyte has been shown to be a metabolite of other steroid hormones, such as T [6]. The incidence of Bol in biological samples has long been considered of exogenous origin. An increase in the amount of Bol-positive samples in the last decades has suggested the origin of Bol in cattle to be endogenous [7–10].

For some time now it has been possible to prove the abuse of exogenous steroid hormones in livestock using sophisticated analytical GC–MS^n or LC–MS^n techniques [3,9–19]. Unfortunately, these techniques do not allow to distinguish between synthetically manufactured endogenous steroids and the chemically identical endogenous hormones which occur naturally in the animal's body. Recently many laboratories have been focussing on the development of new analytical strategies to distinguish between synthetically endogenous and naturally endogenous Bol to prove or disprove the illegal use of this steroid [20–23].

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doi:10.1016/j.jsbmb.2010.02.020

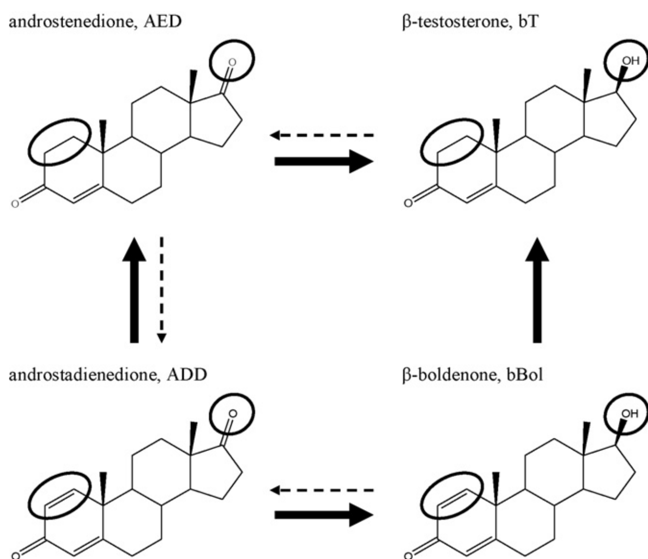

Fig. 1. Mutual reductive (bold arrows) and oxidative (dotted arrows) conversions of androstenedione, β-testosterone, androstadienedione and β-boldenone, as has been demonstrated in the exposure medium of *Neomysis integer* (adapted from Poelmans et al. [30]).

The origin of endogenous Bol and related substances in cattles' urine and faeces has often been linked to the conversion of phytosterols [8,20,21,24–26]. Animal feed is a natural source of phytosterols, such as β-sitosterol, especially since vegetable fat has been used commonly as an additive to animal feed in response to the crises caused by bovine spongiform encephalopathy, better known as mad cow disease, and polychlorobiphenyls (PCBs) food contamination [8,27]. Phytosterols only differ from steroid hormones by their side chain [28,29], and their conversion to steroids has been frequently reported [30–32].

However, the pathway leading to endogenously formed Bol in cattle has not been studied extensively. Vertebrate animal trials are time consuming, have serious cost implications, and are often associated with ethical constraints [33,34]. For these reasons *in vivo* invertebrate models have been proposed to simulate vertebrate pathways. In common with Bol, ADD, AED and T have also been identified in several invertebrate species [32,33,35]. In some invertebrates these steroid hormones even seem to exert functions analogous to these of their vertebrate equivalents [36]. Moreover, the metabolic reactions involved in Bol-formation, have been shown in invertebrate as well as in vertebrate animals [33,34]. Over the last decade, our laboratory has built up a solid expertise in the use of the mysid shrimp *Neomysis integer* (Mysidaceae; Crustacea) as invertebrate model partly replacing vertebrate animals in metabolism studies. Based on this experience, experiments using *N. integer* are reported in this publication [8,31–33,36]. In addition, larvae of the greenbottle fly *Lucilia sericata* (Arthropoda; Diptera) are considered of potential use in metabolism experiments because of their value as reliable substrates for qualitative analysis of drugs in human tissues, in order to provide post-mortem data for badly decomposed bodies [37]. In addition to *in vivo* invertebrate models, *in vitro* biotransformation studies using microsomal preparations also appear a specifically useful tool to characterise metabolites of particular analytes [38–42].

The aim of this study was to provide evidence for the natural endogenous formation of Bol by cattle based on both alternative *in vivo* and *in vitro* experiments. Firstly, the feasibility of using invertebrates as models for vertebrate metabolism studies was evaluated by examining the metabolic pathway of testosterone *in vivo* in two invertebrate species: the mysid shrimp *N. integer* (Mysidaceae; Crustacea) and larvae of the greenbottle fly *L. sericata* (Arthropoda; Diptera). Secondly, the enzymatic activity of feed-borne fungal species, and more specifically microsomes of *Pleurotus sapidus* (Basidiomycota; Agaricales), to convert testosterone into steroid metabolites was evaluated *in vitro*.

2. Materials and methods

2.1. Reagents and chemicals

β-Testosterone (androst-4-ene-17β-ol-3-one, bT, purity ≥98%), methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one, MeT, purity ≥97%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥98%), stigmastanol (24α-ethyl-5α-cholestan-3β-ol, purity ≥95%), β-sitosterol (5-stigmasten-3β-ol, purity ≥70%, residual campesterol and β-sitostanol) and α-pinene (purity ≥98%) were obtained from Sigma–Aldrich Corp. (St-Louis, MO, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥96%), β-boldenone (androsta-1,4-diene-17β-ol-3-one, bBol, purity ≥98%), α-testosterone (androst-4-ene-17α-ol-3-one, aT, purity ≥98%), dihydrotestosterone (5α-androstan-17β-ol-3-one, DHT, purity ≥98%) and different testosterone metabolites (4-androstene-[2α-, 6α-, 7α-, 11α-, 15α-, 16α-, 11β-],17β-diol-3-one, purity ≥98%) were purchased from Steraloids (Newport, USA). Formic acid (HCOOH), ethyl acetate and ethanol were of analytical grade, while methanol (MeOH) and water were of HPLC grade quality. All solvents and reagents were purchased from VWR International (Merck, Darmstadt, Germany). Standard stock solutions were prepared in absolute ethanol at a concentration of 200 ng μL^{-1}. Working standard solutions were prepared by appropriate dilution of the stock solutions in ethanol. All standard solutions were stored at 4 °C following the quality assurance instructions of Belac accreditation (EN17025).

2.2. Sampling

N. integer populations were initially collected from the brackish lake Galgenweel (Antwerp, Belgium). After a 24 h acclimation period to the maintenance temperature of 15 ± 1 °C, the organisms were transferred to 200-L glass aquaria filled with artificial seawater (Instant Ocean®, Aquarium Systems, France) diluted with deionised carbon-filtered tap water to a final salinity of 5‰. Cultures were fed daily with 24–48 h old *Artemia* nauplii *ad libitum* [36].

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

Fungal species were isolated from grinded corn and inoculated on Sabouraud Dextrose agar in Petri dishes as performed by The Department of Pathology, Bacteriology and Poultry Diseases (Faculty of Veterinary Medicine, Ghent University, Belgium). Subsequently the plates were incubated at 26 °C for 4–7 days until harvesting.

Precultures of *P. sapidus* (8266 DSM) were prepared by The Technical Biochemistry Workgroup (Faculty of Biochemical and Chemical Engineering, Dortmund University, Germany) as described by Zorn et al. [43] and made available for the purpose of this article. As an up-regulation of several enzymes was shown when α-pinene was present in the growing medium, both α-pinene-induced (by addition of 1 mM α-pinene daily) and non-induced cultures were grown.

2.3. Experimental setup

2.3.1. N. integer

Juvenile *N. integer* (Mysidaceae; Crustacea) were individually exposed to an excess of 3.5 μM of β-testosterone for 6 h in 2 mL of

medium (water with a salinity of 5‰, artificial sea water (Instant Ocean®) diluted with deionised carbon-filtered tap water). During exposure, test organisms were placed in 5-mL glass tubes in a temperature-controlled chamber (15 °C, Liebher®, Laborimpex, Brussels) [44–46].

2.3.2. *L. sericata*

Maggots of *L. sericata* (Arthropoda; Diptera) were individually placed into 5-mL glass tubes containing 2 mL of medium (water with a salinity of 5‰, artificial sea water (Instant Ocean®) diluted with deionised carbon-filtered tap water) at room temperature. Test species were exposed to an excess of 3.5 μM of β-testosterone or 2.4 μM of a phytosterol (β-sitosterol or stigmastanol) for 4 h [32].

2.3.3. Unidentified fungal species

All media employed in fungal experiments were autoclaved prior to use and standard sterile techniques were applied throughout the procedure in accordance with the specific laboratory guidelines. Samples of fungal mycelium were transferred into 15-mL plastic screw-cap bottles. Subsequently samples were homogenised in HPLC-grade water and the mycelium was separated from the extracellular enzyme containing supernatant by centrifugation. Phosphate buffered saline (PBS), pH 7, was prepared (8 g NaCl, 0.134 g KH_2PO_4, 1.12 g K_2HPO_4) and 0.5 mL PBS buffer was added to 0.5 mL of supernatant and incubated with an excess of 3.5 μM of an analyte at 37 °C for 1 h.

2.3.4. *P. sapidus*

As described by Zorn et al. [43], mycelia of the edible basidiomycete *P. sapidus* were collected by centrifugation and the culture supernatants were discarded. Mycelia were washed once with distilled water and frozen in liquid nitrogen. Frozen mycelia were grinded under liquid nitrogen, resuspended in phosphate buffer (pH 6.0) and centrifuged. The microsomal fraction was pelleted by high speed centrifugation, resuspended in phosphate buffer (pH 6.0) containing 10% (v/v) glycerol, and diluted in Hepes buffer (200 mM, pH 5.5) to reach a final protein-concentration of 0.2 mg mL^{-1} as determined by means of the Popov assay. In the case of freezing samples before analyses, storage occurred at −20 °C. To perform metabolism experiments, a 1 mL aliquot of the microsomal fraction was incubated with 20 μM of an analyte for 22 h at 24 °C while shaking at 150 rpm.

2.4. Extraction

N. integer and maggots of *L. sericata* were isolated from their exposure medium prior to extraction of analytes from these media. Analytes were extracted from exposure media of fungal species and *P. sapidus* without removal of any residual organic material. Methyltestosterone (MeT) at a concentration of 19.8 nM was added to the medium as internal standard before extraction, except for samples of *P. sapidus* to which a 10-fold of this concentration was added. Extracts were analysed for the following phase I metabolites: 2α, 6α, 7α, 11α, 15α, 16α, 11β-hydroxytestosterone (OHT), α-testosterone (aT), androstenedione (AED), androstadienedione (ADD), β-boldenone (bBol) and dihydrotestosterone (DHT). Extraction and clean-up are based on the method described by De Wasch et al. [33] for *N. integer*. In short, metabolites were extracted from the medium using 4 mL ethyl acetate and after centrifugation (5 min, 14,000 × *g*, 4 °C) the organic phase was withdrawn. Ethyl acetate fractions were combined and vacuum evaporated to dryness (Speedvac Plus SC210A, Savant Instruments, Inc., Farmingdale, USA).

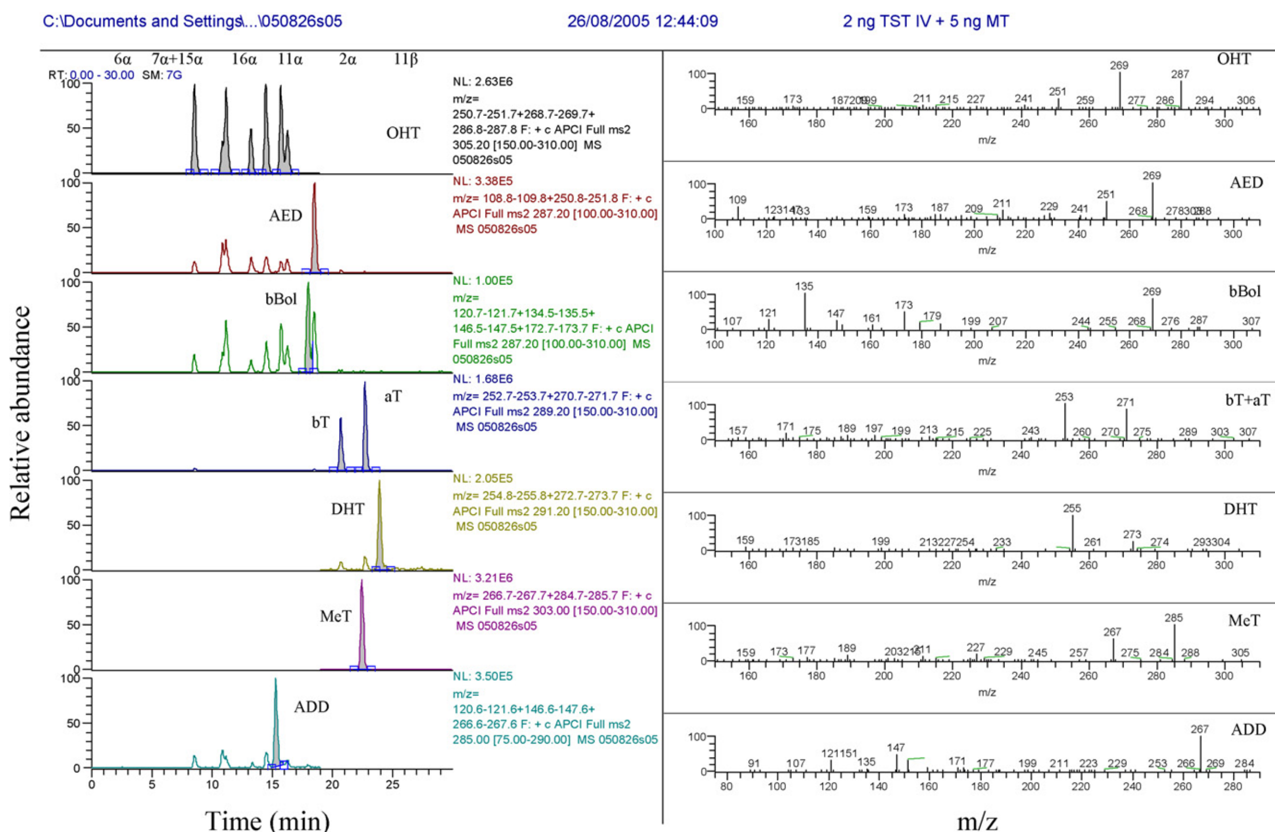

Fig. 2. Chromatograms and spectra of a standard solution of steroids. A concentration of 2 ng was brought on column. Abbreviations: OHT, hydroxytestosterone; AED, androstenedione; bBol, β-boldenone; bT, β-testosterone; aT, α-testosterone; DHT, dihydrotestosterone; MeT, methyltestosterone; ADD, androstadienedione.

Table 1
Optimised working parameters of the LCQ Ion Trap Ion Source for ionisation of specific steroids.

Ionisation source	APCI
Detection mode	Positive
Capillary temperature (°C)	200
Source heater temperature (°C)	450
Sheath gas flow (au)	80
Auxillary gas flow (au)	3

2.5. Liquid chromatography–mass spectrometry

The HPLC apparatus comprised of a HP 1100 series pump, an AS3000 autosampler and a HP 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), which is illustrated in Fig. 2. 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 0.300 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 Jose, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface (Thermo Electron). In Table 1 the optimised working parameters for ionisation of the steroids under investigation are presented. The compounds were detected in positive ion mode MS full scan and MS–MS scan. For each specified steroid, Table 2 shows precursor and product ions as well as the optimal MS–MS conditions. The residue was reconstituted in 30 μL MeOH and 90 μL 0.02 M aqueous HCOOH, except for residues originating from *P. sapidus* which were reconstituted in 300 μL MeOH and 900 μL 0.02 M HCOOH. A volume of 50 μL was injected on the HPLC-system. This method was originally developed by Verslycke et al. [36] and modified as described by De Wasch et al. [33]. Data processing was performed using Xcalibur® 2.0 software (Thermo Electron).

2.6. Quality assurance

Prior to sample analysis, standard mixtures of the targeted metabolites were injected to check the operation conditions of the chromatographic devices (Fig. 2). Different metabolites were identified based on their relative retention time, relative to the internal standard methyltestosterone, and on the ion ratio of their product ions as described in the performance criteria for analytical residue methods defined in Commission Decision 2002/657/EC [47]. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3, was 0.1 ng on column for OHT, AED, bT, aT, MeT and 0.2 ng on column for ADD, bBol and DHT. All specified product ions were used for peak integration for quantification purposes (Table 2). Quantification occurred by fitting the metabolites' area ratio in a calibration curve established either in standard solution or in spiked matrix.

To account for the presence of any of the targeted metabolites in the exposure medium, extracts as such were run (Fig. 3). Extracts of media in which the analyte of exposure was added were run to account for microbial transformation or impurity of the standard solution in the absence of the test species. The influence of the dilution solvent, ethanol, on the metabolism pattern of the test species was accounted for by integrating a solvent control. Extracts of media in which the test species were not exposed to an analyte were analysed to determine the endogenous excretion of metabolites by the test species.

3. Results

3.1. Method development

For the metabolism experiments using *N. integer* and several fungal species, calibration curves were set up by injecting standard solutions of all targeted metabolites several times at relevant concentrations. For experiments using *L. sericata* and microsomal preparations of *P.* sapidus, extracts of standard solutions spiked in the appropriate exposure medium, were injected several times to set up the calibration curves. In all cases, linearity was achieved in the range of 0.3–25.0 ng mL^{-1} for all analytes. The limit of quantification, determined by a signal-to-noise ratio of at least 6, was set at the lowest concentration of the calibration curve (0.3 ng mL^{-1}).

Table 2
Precursor and product ions of several steroids and their optimal APCI(+) MS–MS conditions expressed by the relative retention time (t_r, min) and collision energy (eV).

Analyte	Relative retention time, t_r (min)	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy (eV)
Hydroxytestosterone	0.38–0.49–0.59–0.65–0.70–0.72	305.2	251 269 287	27
Androstadienedione	0.68	285.2	121 147 267	25
β-Boldenone	0.80	287.2	121 135 147 173	27
Androstenedione	0.82	287.2	109 251	27
β-Testosterone	0.92	289.2	253 271	28
Methyltestosterone*	1.00	303.0	267 285	27
α-Testosterone	1.01	289.2	253 271	28
Dihydrotestosterone	1.06	291.2	255 273	30

* Internal standard.

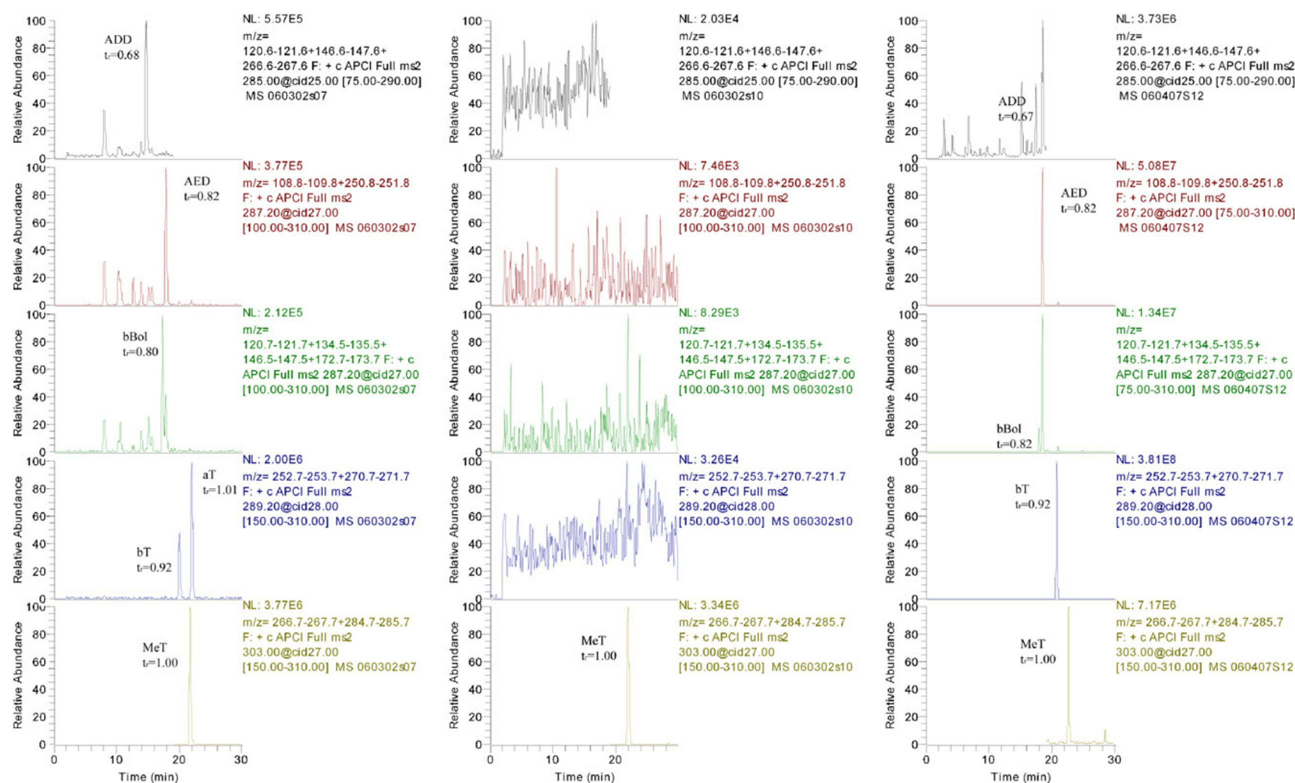

Fig. 3. Chromatograms of (left) a standard solution, (center) a blank exposure medium, and (right) a sample. All were spiked with the internal standard at a concentration of 5 ng on column. A standard solution of steroids was brought on column at a concentration of 2 ng. The sample was an extract of β-testosterone-metabolism by *L. sericata*. Abbreviations: ADD, androstadienedione; AED, androstenedione; bBol, β-boldenone; bT, β-testosterone; MeT, methyltestosterone.

3.2. *N. integer*

In vivo testosterone-metabolism by *N. integer* collected from the Scheldt estuary revealed that, according to mammalian experiments, AED was the main metabolite (Table 3). Testosterone was also converted into several hydroxy-metabolites and DHT (Table 3). Even bBol was detected as metabolite, albeit in low concentrations up to 23 ± 11 ng g^{-1} ww (Table 3). Nevertheless, the variability in excreted concentrations was high between organisms, for AED ranging from 151 ± 74 ng g^{-1} ww to 11511 ± 3321 ng g^{-1} ww. This batch dependency was confirmed by experiments performed in the laboratory. Although organisms were acclimatised under controlled environmental conditions (Section 2.3), hormone levels between different replica of this species upon testosterone exposure remained highly variable, marked by AED-concentrations from 479 ± 218 ng g^{-1} ww up to 15000 ± 10500 ng g^{-1} ww (Table 4).

3.3. *L. sericata*

Upon bT-metabolism, maggots of the greenbottle *L. sericata* mainly excreted AED in the medium, at mean concentrations of 2831.3 ± 1206.0 ng g^{-1} ww (Table 5). ADD and bBol were also detected in the medium, at concentrations of 170.5 ± 96.3 ng g^{-1} ww and 452.2 ± 120.1 ng g^{-1} ww, respectively (Table 5). This indicates the metabolic capability of *L. sericata* maggots (Fig. 3).

3.4. Fungal species

Several unidentified fungal species, grown on corn, converted bT into several hydroxy-metabolites at minor concentrations, and aT and AED at a mean concentration of 72.0 ± 24.9 ng g ww^{-1} and 64.1 ± 25.4 ng g ww^{-1}, respectively (Table 6).

Table 3
Metabolite excretion by *Neomysis integer*, sampled at different locations in the Scheldt estuary, after exposure to β-testosterone (mean ± S.D., ng g^{-1} ww) (adapted from Verslycke et al. [53]). Abbreviations: OHT, hydroxytestosterone; aT, α-testosterone; bT, β-testosterone; AED, androstenedione; ADD, androstadienedione; bBol, β-boldenone; DHT, dihydrotestosterone.

	OHT	aT	bT	AED	ADD	bBol	DHT
Sampling point 1	50 ± 14	na	[a]	2258 ± 719	na	6 ± 6	693 ± 585
Sampling point 2	98 ± 25	na	[a]	11511 ± 3321	na	23 ± 11	–
Sampling point 3	13 ± 10	na	[a]	368 ± 252	na	22 ± 21	–
Sampling point 4	120 ± 35	na	[a]	2515 ± 1358	na	7 ± 7	663 ± 576
Sampling point 5	63 ± 20	na	[a]	924 ± 288	na	10 ± 4	–
Sampling point 6	29 ± 9	na	[a]	151 ± 74	na	–	–
Sampling point 7	139 ± 27	na	[a]	2524 ± 1512	na	7 ± 9	18 ± 17
Sampling point 8	81 ± 17	na	[a]	1180 ± 153	na	22 ± 9	–
Sampling point 9	4 ± 4	na	[a]	33 ± 28	na	*y*[b]	–

na: no data available.
[a] Analyte added in excess to exposure medium, value not quantified.
[b] *y*: metabolite was only detected in one replicate sample.

Table 4
Overview of metabolite excretion by *N. integer* after exposure to β-testosterone (mean ± S.D., ng g^{-1} ww). Abbreviations: OHT, hydroxytestosterone; aT, α-testosterone; bT, β-testosterone; AED, androstenedione; ADD, androstadienedione; bBol, β-boldenone; DHT, dihydrotestosterone.

	OHT	aT	bT	AED	ADD	bBol	DHT
bT (n = 10)[b] [41]	45 ± 14	na	[a]	2005 ± 1447	na	25 ± 12	358 ± 453
bT (n = 10)[b] [42]	39 ± 14	na	[a]	1137 ± 933	na	–	–
bT (n = 10)[b] [42]	51 ± 23	na	[a]	1027 ± 298	na	–	–
bT (n = 10)[b]	143 ± 54	na	[a]	479 ± 218	–	–	–
bT (n = 7)[b] [43]	61 ± 25	na	[a]	12,109 ± 6774	5171 ± 2329	1113 ± 810	na

na: no data available.
[a] Analyte added in excess to exposure medium, value not quantified.
[b] n: number of replicates analysed.

Table 5
Metabolite excretion by maggots of *L. sericata* after exposure to β-testosterone, β-sitosterol or stigmastanol (mean ± S.D., ng g^{-1} ww). Abbreviations: OHT, hydroxytestosterone; aT, α-testosterone; bT, β-testosterone; AED, androstenedione; ADD, androstadienedione; bBol, β-boldenone DHT, dihydrotestosterone.

	OHT	aT	bT	AED	ADD	bBol	DHT
Endogenous	na	na	4.6 ± 2.0 (10/10)[b]	5.8 ± 2.2 (10/10)	– (0/10)	– (0/10)	na
bT	na	na	[a]	2831.3 ± 1206.0 (9/10)	170.5 ± 96.3 (9/10)	452.2 ± 120.1 (10/10)	na
β-Sitosterol	na	na	– (0/10)	– (0/10)	31.7 ± 8.3 (8/10)	– (0/10)	na
Stigmastanol	na	na	3.2 ± 1.8 (10/10)	– (0/10)	19.4 ± 5.8 (10/10)	– (0/10)	na

na: no data available.
[a] Analyte added in excess to exposure medium, value not quantified.
[b] (x/n) with x: number of replicates in which the metabolite was detected and n: total number of replicates analysed.

Table 6
Metabolite excretion by fungal species after incubation with β-testosterone, androstenedione, androstadienedione, β-sitosterol or stigmastanol (mean ± S.D., ng g^{-1} ww). Abbreviations: OHT, hydroxyl-testosterone; aT, α-testosterone; bT, β-testosterone; AED, androstenedione; ADD, androstadienedione; bBol, β-boldenone; DHT, dihydrotestosterone.

	OHT	aT	bT	AED	ADD	bBol	DHT
Endogenous	– (0/5)[b]	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)
bT	9.9 ± 14.4 (13/30)	72.0 ± 24.9 (5/5)	[a]	64.1 ± 25.4 (4/5)	– (0/5)	– (0/5)	– (0/5)
AED	3.0 ± 0.7 (3/30)	– (0/5)	691.6 ± 330.0 (5/5)	[a]	247.5 ± 163.1 (5/5)	– (0/5)	– (0/5)
ADD	– (0/30)	– (0/5)	288.6 ± 289.4 (5/5)	678.7 ± 259.6 (5/5)	[a]	87.9 ± 20.1 (5/5)	– (0/5)
β-sitosterol	– (0/30)	– (0/5)	– (0/5)	136.6 ± 67.6 (5/5)	– (0/5)	– (0/5)	– (0/5)
Stigmastanol	– (0/30)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)

[a] Analyte added in excess in incubation medium, value not quantified.
[b] (x/n) with x: number of replicates in which the metabolite was detected and n: total number of replicates analysed.

Upon exposure to β-sitosterol, AED was mainly produced at a mean concentration of 136.6 ± 67.6 ng g ww^{-1} (Table 6). No ADD, OHT, aT, bT, bBol or DHT could be detected upon β-sitosterol-exposure of fungi in our study (Table 6). Nevertheless, exposure to AED resulted in the production of ADD at a concentration of 247.5 ± 163.1 ng g ww^{-1} (Table 6). Moreover, exposure to ADD led to the production of bBol at a concentration of 87.9 ± 20.1 ng g ww^{-1} (Table 6).

3.5. *P. sapidus*

Results of these experiments are presented in Table 7. Irrespective of the experimental circumstances (fresh or frozen, induced or non-induced), AED was the main metabolite of bT with concentrations ranging from 20.6 ± 3.1 to 583.1 ng mg^{-1} protein solution. The only hydroxy-metabolite in the medium, detected at a maximum concentration of 113.3 ng mg^{-1} protein solution, was 6α-OHT.

Metabolism of β-sitosterol or stigmastanol was evaluated as well for fresh as for frozen non-induced microsomal preparations isolated from *P. sapidus*. However, none of the investigated metabolites (AED, ADD, bBol, aT, bT, P, OHP) could be detected.

4. Discussion

Research by several laboratories in Europe on the prevalence of Bol in untreated cattle indicates the frequent detection of aBol in urine samples up to a concentration of 80.0 ng mL^{-1} (Table 8). Besides aBol, bBol has even been detected in urine samples of untreated animals but not as frequently and in concentrations up to 7.0 ng mL^{-1}. Both Bol isomers have also been reported to be present in the faeces of untreated cattle (Table 8). Although bBol-positive urine samples have been used to indicate illegal animal treatment until the late 1990s, Bol may now be considered as an endogenous analyte as well [7,8]. In this study, the pathway leading to endogenous Bol in cattle was simulated by *in vivo* experiments with invertebrate species as alternative models for vertebrates. Moreover, feed-borne fungal species were shown to convert the phytosterol β-sitosterol into AED, potentially contributing to an increase of steroid hormones in the animal feed hence increasing the chance of endogenous Bol-formation.

Several authors have suggested that a combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) is the most powerful analytical technique to study drug-metabolism [17,53]. In this study, identification and quantification of known metabolites was required. For this purpose, an ion-trap mass spectrometer with a high detection sensitivity, offering real MS^n capability, was used in atmospheric pressure chemical ionization (APCI) mode (Section 2.5).

Most publications dealing with *in vivo* trials on Bol-formation focused on the metabolism- and excretion-profiles upon oral or intramuscular steroid administration to vertebrate animals [10,17,22,41,54]. However, to downscale expensive and time-consuming vertebrate animal experiments, intensive research on the use of alternatives has been conducted during the last decade [33,34,55].

Table 7
Metabolite production by microsomes isolated from *Pleurotus sapidus* activated with β-testosterone (mean ± S.D., ng mg^{-1} protein solution). Abbreviations: OHT, hydroxytestosterone; aT, α-testosterone; bT, β-testosterone; AED, androstenedione; ADD, androstadienedione; bBol, β-boldenone; DHT, dihydrotestosterone.

	6α-OHT	aT	bT	AED	ADD	bBol	DHT
Frozen, non-induced							
Non-activated	1.3 ± 0.2 (2/2)[b]	– (0/2)	– (0/2)	41.6 ± 1.3 (2/2)	– (0/2)	– (0/2)	– (0/2)
bT-activated	12.5 ± 1.5 (2/2)	– (0/2)	[a]	65.7 ± 5.8 (2/2)	– (0/2)	– (0/2)	– (0/2)
Frozen, induced							
Non-activated	0.9 ± 0.1 (2/2)	– (0/2)	– (0/2)	22.5 ± 5.5 (2/2)	– (0/2)	– (0/2)	– (0/2)
bT-activated	9.0 ± 1.5 (2/2)	– (0/2)	[a]	89.3 ± 29.6 (2/2)	– (0/2)	– (0/2)	– (0/2)
Fresh, non-induced							
Non-activated	1.1 ± 0.2 (2/2)	– (0/2)	– (0/2)	20.6 ± 3.1 (2/2)	– (0/2)	– (0/2)	– (0/2)
bT-activated	21.1 ± 3.7 (2/2)	– (0/2)	[a]	159.1 ± 15.0 (2/2)	– (0/2)	– (0/2)	– (0/2)
Fresh, induced							
Non-activated	1.2 ± 0.0 (2/2)	– (0/2)	– (0/2)	– (0/2)	– (0/2)	– (0/2)	– (0/2)
bT-activated	113.3 (1/1)	– (0/1)	[a]	583.1 (1/1)	– (0/1)	– (0/1)	– (0/1)

[a] Analyte added in excess in incubation medium, value not quantified.
[b] (x/n) with x: number of replicates in which the metabolite was detected and n: total number of replicates analysed.

Experiments with the mysid *N. integer*, performed at our laboratory, suggest similarity between the enzymatic steroid metabolism pathway of these crustaceans and mammals [33,36]. The vertebrate type steroid β-testosterone was used to study metabolic pathways dependent on cytochrome P450 enzymes, the latter having a major role in the oxidative metabolism of drugs in mammals [55]. Moreover, it was demonstrated by Poelmans et al. [31] that exposure of *N. integer* to bT primarily leads to AED-production, subsequently followed by the formation of bBol, with ADD as intermediary product (Fig. 1). Field as well as laboratory experiments proved that AED was the main metabolite (Tables 3 and 4). These experiments, however, indicated both spatial (Table 3) and seasonal (Table 4) variability in excreted concentrations [56], forming a serious drawback in using *N. integer* as an invertebrate model organism to investigate hormonal pathways. Especially with regard to bBol, reproducibility of *in vivo* exposure experiments with *N. integer* has been reported by De Wasch et al. [33] to be poor. In contrast, the use of *N. integer*'s testosterone-metabolism has proven its use in eco-toxicological studies [44–46,57]. For the purpose of simulating vertebrate metabolic pathways however, another invertebrate *L. sericata*, was evaluated as model organism [32].

The study of drugs in insects, entomo-toxicology, has become an established approach in toxicological studies since 1980 [58]. In fly larvae, detection of controlled substances and drugs has been carried out by different research groups [58,59]. It should be noted, however, that in these experiments analyses were done on whole organism extracts whereas in our study metabolites were determined upon excretion in the medium, as initial experiments with *N. integer*, revealed that testosterone metabolites are preferably excreted in the medium rather than maintained in the organism's body [36]. Nevertheless, future experiments investigating extraction of metabolites from fly larval bodies might provide additional information on Bol-formation. bT-Metabolism by maggots of the greenbottle *L. sericata* mainly resulted in the excretion of AED in the medium. This reaction, comprising the common oxidation of the enol- to the keto-form, has been described as the most important conversion of bT for *N. integer* as well. The similarity of its metabolic pathways to these described for *N. integer*, suggests the potential value of *L. sericata* as invertebrate model in animal trials (Tables 3–5). Nevertheless, this observation requires further confirmation to assure the reproducibility of the conversion of bT into bBol and consequently the value of this organism for *in vivo* metabolism experiments. Until now, excreted steroid-concentrations upon bT-exposure were highly variable for fly larvae of different origin, e.g. Belgium, Great Britain, The Netherlands and Italy (data not shown). It should be noted that the conversion of bT into ADD and bBol was only observed in maggots originating from Italy, as shown in Table 5. As for *N. integer*, this variability in invertebrate metabolism of bT might be explained by the intestinal system of the invertebrates. Within the purpose of this article no distinction was made however between the enzymatic and the intestinal metabolism of invertebrates. In calves' faeces, the most likely origin of 1-dehydro compounds such as Bol is believed to be through conversion of specific precursors, such as phytosterols or other steroids, by gut bacteria [17,51]. However, the present results indicate the potential relevance of the presence

Table 8
Literature overview of α-boldenone and β-boldenone findings in urine (ng mL^{-1}) and faeces (ng g^{-1}) of untreated cattle. Abbreviations: aBol, α-boldenone; bBol, β-boldenone.

	Urine (ng mL^{-1})		Faeces (ng g^{-1})	
	aBol	bBol	aBol	bBol
Arts et al. [7]	<0.1–2.6	<0.1	na	na
De Brabander et al. [8]	nd–>80.0	nd–≤7.0	1.0–10.0[a]	0.1–2.0[a]
Rossi et al. [48]	≤7.9	≤0.2[b]	na	na
Nielen et al. [9]	nd	nd	0.1–2.0[a]	0.1–2.0[a]
Sangiorgi et al. [10]	nd	nd	–	–
Arioli et al. [49]	na	na	nd–10.0	nd
Arioli et al. [49]			168.7–124664.0[a]	331.5[a]
Draisci et al. [50]	nd–0.9	nd	na	na
Pompa et al. [51]	nd	nd	nd–5.9	27.6–89.0
Pompa et al. [51]			nd–3090.0[a]	nd–482.0[a]
Nielen et al. [25]	nd–2.7	nd	100.0[a]	4.0[a]
Gallina et al. [52]	<1.0	nd	na	na

nd: not detected; na: no data available.
[a] Analyte was only detected in dried faeces, not in fresh rectal faeces.
[b] Analyte was only detected in urine after faecal contamination.

of maggots living on faecal material to the origin of Bol-positive faeces samples. Moreover, this mechanism could possibly explain the observed increase in Bol-detection in dried out faeces (Table 8), being more exposed to flies and consequently maggots than fresh faeces.

Even though the role of phytosterols in the formation of Bol and other steroids has often been suggested, it has only been described in a few studies [50,52,60,61]. In this study, the ability of edible fungi – potentially growing on animal feed – to convert phytosterols into steroids was hypothesised. Evidence is existing on the occurrence of fungal species in corn [62]. However, this has mainly been linked to contamination of corn, and consequently animal feed, with mycotoxins [63–65]. To the best of our knowledge, interference of feed-borne fungi in steroid-formation has so far not been considered. The hormonal activity of animal feed ingredients, was taken into account in one study analysing steroid hormone excretion in veal calves' urine and faeces [25]. Unfortunately, only the estrogenic activity of animal feed was evaluated during this study.

Exposure of several fungal species to β-sitosterol mainly resulted in AED detection (Table 6). This is consistent with a study performed by Lin et al. [30] in which AED was the main transformation product of phytosterols in corn flour (mainly containing β-sitosterol) produced by an isolated *Fusarium* strain. The production of ADD could not be proven during their study. Accordingly, no ADD could be detected upon β-sitosterol-exposure in our experiment. Nevertheless, exposure to AED was shown to result in ADD-production, and ADD-exposure led to the production of bBol by several fungi. These data support the assumption that isolated fungal species are able to transform phytosterols into bBol (Fig. 1), which strengthens the hypothesis that animal feed rich in phytosterols may be a potential source of bBol or its precursors. Important in this context is the fact that during this study fungal species were exposed to micrograms of phytosterols only. A more realistic approach would be exposure to grams of phytosterols. Indeed, cattle at an age of 8–12 months old can be fed approximately 15 kg corn-rich animal feed [66]. Depending on the specific type of animal feed and/or the addition of vegetable oils, this equals to about 20 g of β-sitosterol [67]. Further research should therefore focus on growing fungal strains under laboratory conditions on real animal feed samples to establish steroid hormone production on feed.

To further investigate the P450 enzymatic activity of fungal species, *in vitro* evaluation of their testosterone-metabolism was performed. This resulted in the detection of several hydroxytestosterone, aT and AED (Table 6), in accordance with literature on testosterone biotransformation by fungi [68,69]. Furthermore, T-conversion by specific microsomal preparations isolated from the edible fungus *P. sapidus* was evaluated *in vitro*. In accordance with the above-mentioned experiments, AED was the main metabolite of bT. The only hydroxy-metabolite of bT detected was 6α-OHT. According to Van Puymbroeck et al. [41], hydroxylation in position 6 of steroid hormones appears to be a common metabolic pathway by microsomal preparations. Additionally, these authors reported some interesting advantages for the use of microsomes as an *in vitro* tool for metabolism experiments. The easy preparation of microsomes combined with the relatively low costs and the possibility to preserve microsomal material for years are clear advantages. This is in contradiction with our results, since the AED-concentration of fresh induced bT-activated microsomes was about 6-fold the concentration of their frozen counterparts: i.e. 583.1 and $89.3 \pm 29.6\,ng\,mg^{-1}$ protein solution, respectively (Table 7). Possibly this could have been prevented by cryopreserving the microsomal preparations which appears to be a recognised way of conserving [55]. Since the applicability of fungal microsomes to predict the metabolic pathway of bT was demonstrated in this study, the metabolism of β-sitosterol or stigmastanol was also evaluated using fresh and frozen non-induced microsomal preparations isolated from *P. sapidus*. However, none of the investigated metabolites (AED, ADD, bBol, aT, bT, P, OHP) could be detected. Considering only the fresh microsomal preparations, induction with α-pinene resulted in a 3-fold increase of AED, 583.1 compared to $159.1 \pm 15.0\,ng\,mg^{-1}$ protein solution (Table 7). Consequently, these results clearly indicate the importance of a cofactor, e.g. α-pinene, to up-regulate enzyme activity. Although the enzymatic activity of fresh induced microsomes isolated from the edible fungus *P. sapidus* was demonstrated, these results are preliminary and further confirmation is needed.

5. Conclusion

While many laboratories are focussing on developing novel analytical methods to distinguish between exogenous and endogenous Bol in cattle, this study examined the potential pathways of endogenous Bol-formation.

Vertebrate enzymatic activity was simulated *in vivo* using the invertebrates *N. integer* and *L. sericata*. However, model organism variability was too high to consider these invertebrate species as alternative biotransformation models. Besides cattles' natural enzymatic metabolic pathway, this study also proposes a novel mechanism of endogenous Bol-formation. Feed-borne fungi, potentially living on animal feed, were shown capable to convert phytosterols into steroids. Consequently, increased amounts of steroid hormones, probable precursors of Bol, may unintentionally be offered to cattle through their feed.

To consider the prevalence and the amount of Bol residues in cattle's urine or faeces as proof of illegal animal treatment, a more integrated approach is required, taking into account, e.g. the phytosterol-concentration in animal feed, the environmental circumstances and the ecology of gut microbiota.

Acknowledgements

The authors wish to thank the Special Research Fund (BOF) for financial support. Furthermore, A. Van Bastelaere and V. Mortier are gratefully acknowledged for their practical assistance in the laboratory.

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