Ecdysteroids as natural doping substances in the blood of insectivorous bats

Sándor Hornok^{1*}, Róbert Berkecz², Endre Sós³, Attila D. Sándor^{1,4}, Tímea Körmöcz²,
Norbert Solymosi⁵, Jenő Kontschán⁶ and Attila Hunyadi^{7,8*}

¹Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest, Hungary

²Institute of Pharmaceutical Analysis, University of Szeged, Szeged, Hungary

³Budapest Zoo and Botanical Garden, Budapest, Hungary

⁴Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania

⁵Centre for Bioinformatics, University of Veterinary Medicine, Budapest, Hungary

⁶Plant Protection Institute, Centre for Agricultural Research, ELKH, Budapest, Hungary

⁷Institute of Pharmacognosy, Interdisciplinary Excellence Centre, University of Szeged, Szeged, Hungary

⁸Interdisciplinary Centre for Natural Products, University of Szeged, Szeged, Hungary.

* Correspondence should be addressed to S.H. (email: Hornok.Sandor@univet.hu) and A.H. (email: Hunyadi.A@pharmacognosy.hu)

Abstract

Bats have deserved much scientific attention due to their biological-ecological properties and increasingly recognized epidemiological significance. Ecdysteroids are insect-molting hormones that (under experimental conditions) have stimulating and anabolic effects in mammals, including humans. Therefore, these biologically active compounds are currently under consideration by the World Anti-Doping Agency (WADA) to become doping-controlled substances. Previously we demonstrated that low to high concentrations of ecdysteroids appear in the blood of insectivorous passerine birds. Since passerine birds and echolocating bats share several adaptive mechanisms in connection with flying, and insectivorism is also among their common traits, we hypothesized that ecdysteroids might also be present in the blood of insectivorous bats. To test this hypothesis, blood samples of eight insectivorous bat species were collected and analyzed for the presence of ecdysteroids with highly sensitive targeted ultra-high-performance liquid chromatography coupled to high-resolution quadrupole-orbitrap mass spectrometry method (UHPLC-HRMS). The results supported our hypothesis, because nine ecdysteroids were detected in bat blood. The spectrum of these ecdysteroids was similar in those bat species which have their most preferred food items from the same insect order, supporting insects as the most likely source of these hormones. It was also shown that the spectrum of blood-borne ecdysteroids was broader in the autumn than in the summer, and higher concentrations of 20-hydroxyecdysone were measured in samples of large size bat species in comparison with small size ones. Based on the known physiologic effects of ecdysteroids, we postulate that these results might have implications on the metabolic rate and parasite burdens of insectivorous bats.

Bats (Mammalia: Chiroptera) have attracted enormous scientific attention on account of their biological-ecological properties and epidemiological significance. Importantly, they are the only mammals that actively fly. This adaptation to flying entailed unique anatomical and physiological mechanisms, including a high metabolic rate¹. Insects, on which the great majority of bat species feed, contain molting hormones, so-called ecdysteroids. Recently it has been demonstrated that low to high concentrations of ecdysteroids appear in the blood of insectivorous passerine birds and these might possibly contribute to their "higher-than-avian-average" metabolic rate and physical performance^{2,3}. Because passerine birds and insectivorous bats share several adaptive mechanisms in connection with flying, and dietary guild is also among their common traits, we hypothesized that ecdysteroids may also be present in the blood of insectivorous bats. The aim of this study was to investigate this possibility.

In the blood of eight insectivorous bat species, sampled over a one-year period, nine ecdysteroids were detected (Figure 1; Supplementary Table 1). Ecdysteroids were the most diverse in *Eptesicus serotinus*, followed by *Nyctalus noctula* and *Pipistrellus nathusii* (Figure 1). Considering the whole study period, the spectra of ecdysteroids were identical or overlapping between bat species which have their most preferred food items from the same insect order (Technical Appendix). In particular, all compounds detected in *Hypsugo savii* were also present in *Miniopterus schreibersii* (preferentially feeding on Lepidopterans), all compounds shown to be present in *Myotis myotis* were also demonstrated in *E. serotinus* (preferentially feeding on beetles), and *My. nattereri* shared all its compounds with *N. noctula* and *P. nathusii* (Figure 1). In case of the latter two species the spectrum of ecdysteroids was the same, in accordance with their most preferred food items, Dipterans, but despite of the highly significant difference between their mean body weight (Supplementary Table 2: 28.1±3.63 g, n=192 vs 6.76±1.48 g, n=44, respectively) (t=62.015, df=170.38, P<0.0001). The mean number of compounds per sampling occasion was low in January, April and compared to the summer (median=4, IQR=1.25, n=20 occasions) it was significantly (t=2.34, df=26, P=0.028) higher during the autumn (median=6, IQR=2.5, n=7 occasions) (Supplementary Table 1).

The most prevalent ecdysteroid (detected in all evaluated bat individuals) was 20-hydroxyecdysone (20E). Among the three large size bat species (*N. noctula*, *E. serotinus*, and *My. myotis*), the mean 20E concentration was significantly higher in *E. serotinus*

(232.82±287.23 pmol/ml, n=8) compared to *N. noctula* (38.49±27.92 pmol/ml, n=11) (W=77, P=0.012). In addition, the mean concentration of 20E was significantly higher in bat species predominantly feeding on beetles (order Coleoptera) (204.96±260.09, n=10) than in those typically feeding on flies (order Diptera) (49.74±59.58, n=15) (W=129, P=0.009) (Figure 1).

Thus, in the blood of all eight insectivorous bat species studied here the presence of a broad range of naturally acquired ecdysteroids was demonstrated, to the best of our knowledge for the first time in a worldwide context. Hitherto the general view persisted that experimentally administered ecdysteroids are rapidly cleared from the blood of mammals, e.g., mice⁴. However, the continuous uptake of insects can apparently counterbalance this rapid clearance in bats.

Regarding the physiologic importance of these findings, various ecdysteroids were shown to have significant anabolic effect in mammals, sometimes exceeding that of the well-known doping agents. For instance, 20E enhances physical performance, acting as a "natural dope",⁵ and it is currently under consideration by the World Anti-Doping Agency to become a doping-controlled substance. Based on such data, it can be hypothesized that bats may take advantage of these biologically active compounds in their blood, particularly in association with their high metabolic rate necessitated by flying.

The predominant source of ecdysteroids detected in bats should have been insects, considering that all eight bat species investigated here are insectivorous. While none of the evaluated bat species are known to feed on fruits, they may still have access to plant-derived ecdysteroids via their insect prey items which feed on plants. This should explain the surprising occurrence of phytoecdysteroids in bat blood as shown here (i.e., those which are not known to be naturally produced by insects, such as polypodine B, ajugasterone C, calonysterone, dacryhainansterone and shidasterone).

Based on our results, identical spectra of detected ecdysteroids in *N. noctula* and *P. nathusii* was in accordance with their common most preferred food items (Figure 1), but not with their significantly different body weight. Assuming that the spectrum of blood-borne ecdysteroids is interrelated with the taxonomic diversity of insect food items, these results confirm previous observations, according to which dietary diversity was not related to the body mass of bats⁶. At the same time, variations in the spectra of ecdysteroids according to sampling months might reflect changes in the daily or seasonal availability/uptake of insects from various taxonomic orders.

Based on the dataset of this study, within the category of large size bats, higher 20E concentrations were measured in *E. serotinus* than in *N. noctula*. *Eptesicus serotinus* (unlike *N. noctula*) belongs to the category of "ground-gleaning" bats⁷, i.e., obtains a significant portion of its food items from surface objects such as the ground and its plant covering. From such surfaces *E. serotinus* predominantly feeds on beetles, which might significantly contribute to its high 20E blood concentrations. It was observed that in case of beetles the adult diapause is induced by the commencing short-day photoperiod in the autumn, concomitantly with an ecdysteroid peak value nearly as high as those found during metamorphosis⁸. In a broader context, this may also explain why the blood of bat species predominantly feeding on beetles contained significantly higher concentrations of 20E compared to those species which typically feed on flies.

The oral uptake of excess amounts of ecdysteroids by blood-sucking ectoparasites is known to affect them in several ways. Experimentally, these hormones have antifeedant effect and induce early salivary gland degeneration in ticks⁹ and were shown to naturally affect ticks taking a blood meal from birds with ecdysteroids in their blood³. In support of the possibility that a similar phenomenon might exist among insectivorous bats, these were shown to have significantly lower ectoparasite loads than sympatric fruit-eating bats¹⁰.

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Figure Legends:

Figure 1. Diversity of ecdysteroids in bat species according to their most preferred food items. The topology is based on a cytochrome *b* phylogenetic tree. The color codes of ecdysteroids are: red – 20-hydroxyecdysone, orange – polypodine B, yellow – poststerone, light green – 14-deoxy-20-hydroxyecdysone, dark green – ecdysone, light blue – ajugasterone C, dark blue – calonysterone, black – dacryhainansterone, purple – shidasterone. The pictograms of food items stand for beetles (order Coleoptera), moths (order Lepidoptera), flies (mosquitoes also included: order Diptera) and caddisflies (order Trichoptera).

Supplementary Figure 1. Chemical structures of the tested ecdysteroids. 20E – 20-hydroxyecdysone, pB – polypodine B, pS – poststerone, 14d – 14-deoxy-20-hydroxyecdysone, 2d – 2-deoxy-20-hydroxyecdysone, E – ecdysone, AjC – ajugasterone C, Cal – calonysterone, Dac – dacryhainansterone, Shd – shidasterone.

Supplementary Figure 1.

Technical Appendix: Methods

Origin of samples

In this study, blood samples of 36 bats were evaluated, representing eight insectivorous species from two families (Supplementary Table 1; Figure 1). The first group of blood samples (n=14) were collected from bats mist-netted at either a cave entrance (*My. myotis*, n=2) in Chavdarts (Bulgaria), or near their roosts in buildings or mines at Canarua Fetii, Romania (*N. noctula*: n=5; *P. nathusii*: n=2; *My. nattereri*: n=1 and *Mi. schreibersii*: n=4). Sampling dates included early September in 2017, the end of July and the beginning of August in 2018. All soft and hard ticks were removed from these bats and stored in 96% ethanol until identification according to standard keys¹.

The second group of blood samples originated from 22 bats, which were transported to the Animal Rescue Centre of the Budapest Zoo, after found weak or in a moribund state in Budapest between mid-August, 2017 and early September, 2018 (i.e., in 2017: August, September, October, November, December; in 2018: January, April, August, September). In this category, all winter samples were obtained from the common noctule bat (*N. noctula*) which is known to have flying and foraging activity during winter months in Central Europe², therefore its non-hibernating individuals could be included in our study from both December and January. All rescued bats were sampled as part of their veterinary care within one day of their arrival, therefore their blood ecdysteroids were regarded to originate from previous (thus natural) feeding. No ticks were found on these bats. Since no body mass data were available for bats in the second group, the mean weight of the species was calculated from a larger sample size encompassing the period 2015-2020 and including bats captured at various locations in Romania (Supplementary Table 2: A. D. Sándor, unpublished data).

Chemicals and standards

All chemicals (water, methanol, acetonitrile, formic acid) for sample preparation and analysis with LC-MS grade were purchased from VWR (Radnor, PA, USA). Poststerone (20E)-oxime was used as internal standard (IS), and it was synthesized from poststerone as published before³.

The samples were tested for the presence of ten natural ecdysteroids, these compounds were obtained from our previous phytochemical work^{4,5,6}. Abbreviations of these

compounds' trivial names are as follows: 20E – 20-hydroxyecdysone, pB – polypodine B, pS – poststerone, 14d – 14-deoxy-20-hydroxyecdysone, 2d – 2-deoxy-20-hydroxyecdysone, E – ecdysone, AjC – ajugasterone C, Cal – calonysterone, Dac – dacryhainansterone, Shd – shidasterone; chemical structures of these compounds are presented in Supplementary Figure 1.

Sample preparation and calibration

The 150 µL of diluted blood sample (15 µL whole blood mixed with 135 µL saline solution) was spiked with 7 µL of IS solution (100 ng/mL in MeOH), and then 1 mL MeOH was added. After vortexing, the sample was shaken for 15 min at room temperature. Next, the sample was centrifuged at 4°C for 15 min at 21,000 g (Universal 320 R, Hettich, Tuttlingen, Germany) and 900 µL of the upper phase was dried under nitrogen at ambient temperature. The dried extract was dissolved in 65 µL of water/methanol (1/1, v/v) and centrifuged at 21,000 g at room temperature for 10 min, and the upper phase was collected for analysis.

External calibration was used for quantitative analysis of 10 ecdysteroids with the following concentrations of standards: 0, 0.033, 0.333, 1.667, 3.333, 16.667, 33.333, 333.333 and 3333.333 pmol/mL in water/methanol (1/1, v/v).

For calibration, standard solution mixtures were prepared with concentrations of 0, 0.033, 0.333, 1.667, 3.333, 16.667, 33.333 and 333.333 pmol/mL of each of pB, pS, 14d, E, AjC, Cal, Dac, 2d, and Shd in water/methanol (1/1, v/v). The concentration of 20E was set to 10 times higher than other standards and the concentration of IS was 28 pmol/mL in each calibration mixture.

UHPLC-HRMS and HRMS/MS analysis

The UHPLC-HRMS and HRMS/MS measurements were performed on Waters Acquity I-Class UPLC™ (Waters, Milford, MA, USA) coupled to Thermo Scientific Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer.

The UHPLC separation was carried out on an Accucore C30 column (150×2.1 mm, 2.6 µm) with an equivalent guard column (10×2.1 mm, 2.6 µm) from Thermo Fisher Scientific (Waltham, MA, USA). The mobile phase A consisted of 0.1% formic acid aqueous solution, and mobile phase B was composed of acetonitrile with 0.1% v/v formic acid. Total run time was 29 min and the following gradient program was used: 0 min 10% B held for 1.5 min;

ramped to 35% B in 18.5 min; then ramped to 100% B in 0.1 min; held for 4.9 min; and finally, returned to initial conditions within 4 min. The flow rate was 0.4 mL/min during the analysis. Column temperature was maintained at 50 °C and the injection volume was 8 µL. Each sample was measured three times.

The mass spectrometer was operated in positive mode using single ion monitoring (SIM) for quantitative analysis of ecdysteroids and HRMS/MS parallel reaction monitoring (PRM) for their identification. The heated ESI source was used with the following conditions: capillary temperature 262.5 °C, S-Lens RF level 50, spray voltage 4.0 kV, sheath gas flow 50, spare gas flow 2.5, and auxiliary gas flow 12.50 in arbitrary units. For SIM mode the maximum injection time (IT) was 50 ms with a resolution of 35,000 (FWHM) and the automatic gain control (AGC) setting was defined as 1×10^6 charges. In PRM mode with resolution of 17,500 (FWHM), the AGC setting was defined as 1×10^6 charges, the maximum IT was set to 50 ms and 20 eV collision energy was used. The precursor ion window was set to 1 Da in both scan modes. The monitored m/z values of protonated compounds with related retention times are detailed in Supplementary Table 3.

The UHPLC system was controlled with MassLynx V4.1 SCN 901 (Waters, Milford, MA, USA). The control of mass spectrometer, data acquisition and processing were performed by Xcalibur 4.3 software (Thermo Fisher Scientific, Waltham, MA, USA).

In whole blood samples, the ecdysteroids were reliably confirmed by UHPLC-HRMS/MS analysis which were based on literature data and the fragmentation pattern of the reference standards¹¹. The mass tolerance for the processing of UHPLC-HRMS spectra was set to 5 ppm. Three replicate quantitative analyses of samples were performed by external calibration using an internal standard and the linear calibration curves of ecdysteroids were based on analyte/IS peak area ratios against concentrations.

Ethical approval

Permission for bat capture was provided by the Underground Heritage Commission (Romania) and the local protected area management authorities. Bat banding license numbers are 718/2017 (Bulgaria), 24/2017 and 111/2018 (Romania). Bats were handled according to the current law of animal welfare regulation (Law 206/2004), and the Research Bioethics Commission of USAMV CN approved the used methodology of bat handling and blood-collection protocol. Permission from the Institutional Animal Care and Use Committee

(IACUC) was not necessary, because all wild-caught bats were released immediately in the field (none taken to participating institutes).

Phylogenetic analysis

Circular phylogenetic tree was prepared to show the gross relationship of the eight bat species involved in this study (Figure 1) by including cytochrome *b* sequence data from GenBank⁷. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Neighbor-Joining method, p-distance model, by using MEGA version 7.0.

Statistical analyses

Statistically analyzed data included the concentrations of 20E (which was available in the case of all bats) compared between bat groups distinguished according to the following characters: bat species (*N. noctula* vs *E. serotinus*), body size characteristic of bat species (small species: mean body weight below 13 g, large species: mean body weight above 24 g; Supplementary Table 2), the predominant food item (from orders Coleoptera, Diptera, Trichoptera or Lepidoptera) characteristic of each bat species^{8,9,10}. The mean concentrations of 20E were compared in male vs female bats, and between tick-infested and non-infested bats. In addition, the number of ecdysteroid compounds was analyzed according to astronomical seasons (summer sampling: July to early September; autumn sampling: October to early December). Where the assumption of normality was not satisfied, the Wilcoxon rank sum test¹¹ was used, otherwise the *t*-test. Normality was verified by the Shapiro-Wilk test¹². The P-values estimated by testing the groups on E20 were adjusted by the method of Benjamini and Hochberg¹³. The comparison of compound number between the summer and spring was performed using a negative binomial model¹⁴. All statistical analyzes were done in the R environment¹⁵. Differences were considered significant if $P < 0.05$.

Data availability

Important data are included in the manuscript and its references. Additional data are available in Supplementary Tables.

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Author contributions

S.H. initiated and designed the study, identified ticks, wrote most parts of the manuscript. R.B. and T.K. performed chemical analyses, E.S. and ADS provided expertise and important samples to the study, N.S. performed statistical analyses, J.K. performed phylogenetic analysis, A.H. supervised chemical analyses and wrote a significant part of the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary Table 1. Ecdysteroid concentrations according to bat species, sex, sample group and sampling month.																						
			Concentration (pmol/mL)																			
			20E		pB		pS		BKE11		α		AjC		Calo		DeO- β		Dakri		Shida	
Species	Sex	Habitat (month)	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%	pmol/mL	RSD%
PNAT	F	R (Aug)	245,53	7,68	ND		273,41	2,6	130,71	7,43	ND		9,18	0,66	55,80	6,4	ND		9,53	8,1	5,32	16,7
NNOC	M	R (no data)	49,90	3,84	ND		ND		12,05	10,71	ND		ND		8,05	23,0	ND		ND		ND	
ESER	F	R (Aug)	54,02	5,79	ND		ND		3,71	4,84	ND		ND		4,78	4,4	ND		ND		ND	
ESER	M	R (Oct)	93,71	3,62	ND		49,27	9,0	7,43	4,66	ND		6,40	2,06	13,20	4,5	ND		4,49	4,3	LOD	
ESER	M	R (Oct)	56,32	3,33	5,65	3,50	6,53	19,4	3,85	7,44	154,10	3,51	3,70	5,76	5,81	3,3	ND		4,90	2,0	ND	
no data	F	R (Oct)	33,99	4,40	ND		24,48	5,2	11,34	3,31	ND		6,59	10,88	6,86	4,2	ND		17,84	8,4	5,78	7,5
ESER	F	R (Nov)	247,68	5,55	25,28	7,11	15,61	5,3	5,21	5,61	LOD		LOD		7,36	1,9	ND		5,37	10,3	ND	
NNOC	F	R (Dec)	68,14	5,63	ND		14,24	8,8	9,60	6,25	ND		9,53	4,12	8,75	5,4	ND		37,85	4,0	4,38	14,7
NNOC	F	R (Dec)	54,23	6,81	ND		12,74	6,2	5,35	2,54	ND		4,75	9,10	8,98	8,2	ND		10,53	9,8	ND	
NNOC	F	R (Dec)	5,96	13,09	ND		9,79	5,0	LOD		ND		ND		ND		ND		ND		ND	
MMYO	F	N (Sept)	104,76	8,92	ND		12,51	28,8	7,12	18,01	ND		LOD		LOD		ND		5,98	22,1	LOD	
PNAT	M	N (Aug)	34,29	5,00	ND		11,44	23,2	6,64	2,76	ND		LOD		LOD		ND		9,60	6,7	ND	
MSCH	F	N (Aug)	45,21	2,64	ND		10,40	15,7	4,72	4,86	ND		LOD		LOD		ND		5,44	20,4	LOD	
MSCH	M	N (Aug)	48,30	0,92	ND		19,76	4,8	5,61	3,46	ND		LOD		LOD		ND		14,86	3,5	LOD	
NNOC	M	N (July)	49,94	5,84	ND		25,17	1,6	4,18	4,89	ND		LOD		72,80	3,8	ND		48,59	4,1	LOD	
NNOC	M	N (July)	22,77	11,02	ND		11,35	4,4	LOD		ND		ND		LOD		ND		LOD		ND	
PNAT	M	N (Aug)	8,31	1,27	ND		LOD		ND		ND		ND		LOD		ND		ND		ND	
VMUR	F	R (Apr)	8,67	11,64	ND		ND		ND		ND		ND		43,38	2,6	ND		LOD		ND	
NNOC	M	N (July)	16,66	16,86	ND		ND		LOD		ND		ND		ND		ND		ND		ND	
NNOC	M	R (Jan)	3,01	11,19	ND		ND		ND		ND		ND		ND		ND		ND		ND	
ESER	M	R (no data)	23,52	7,73	ND		ND		LOD		ND		ND		ND		ND		3,43	14,1	ND	
ESER	F	R (Aug)	296,29	3,07	44,93	1,58	ND		LOD		250,01	2,47	LOD		ND		ND		6,80	10,9	ND	
HSAB	M	R (Aug)	48,08	5,52	ND		LOD		5,20	2,79	ND		LOD		LOD		ND		9,84	13,6	ND	
ESER	M	R (Sept)	190,96	3,05	ND		LOD		10,65	18,31	LOD		21,28	9,12	12,60	18,1	ND		19,27	16,1	LOD	
no data	no data	R (no data)	124,65	5,73	LOD		LOD		LOD		ND		LOD		ND		ND		LOD		ND	
no data	no data	R (no data)	62,05	3,97	ND		LOD		6,88	9,97	ND		5,27	19,71	9,09	5,4	ND		14,86	16,6	LOD	
no data	no data	R (no data)	13,54	4,21	ND		ND		ND		ND		LOD		ND		ND		LOD		ND	
ESER	no data	R (no data)	900,08	0,16	12,78	3,50	ND		4,39	7,21	40,98	0,48	ND		ND		ND		5,27	33,8	ND	
NNOC	F	R (Nov)	47,24	3,39	ND		ND		LOD		ND		LOD		ND		ND		LOD		7,07	19,7
MMYO	F	N (Sept)	82,22	2,95	ND		LOD		8,62	4,63	ND		ND		18,84	5,2	ND		5,51	9,1	ND	
MNAT	F	N (Aug)	34,60	8,50	ND		LOD		6,72	17,87	ND		4,19	30,17	ND		ND		9,98	11,8	ND	
MSCH	M	N (Aug)	8,34	30,64	ND		LOD		5,37	5,10	ND		ND		ND		ND		3,52	5,8	ND	
MSCH	M	N (Aug)	8,90	32,97	ND		LOD		ND		ND		ND		ND		ND		4,07	3,9	ND	
NNOC	M	N (July)	15,24	7,83	ND		LOD		6,46	4,08	ND		ND		ND		ND		3,88	16,0	ND	
NNOC	M	N (July)	91,07	5,15	ND		LOD		14,70	6,64	ND		12,68	8,51	ND		ND		24,97	5,1	ND	
VMUR	M	R (Sept)	12,06	3,71	ND		ND		LOD		ND		LOD		ND		ND		5,33	7,2	ND	

Supplementary Table 2. Body weights (shown in grams) of bat species involved in this study, calculated from 2046 bats captured at various locations in Romania in the period 2015-2020.

	N	Minimum	Maximum	Mean	Std. Deviation
<i>Nyctalus noctula</i>	192	18,79	39,60	28,0968	3,62792
<i>Pipistrellus nathusii</i>	44	4,10	9,80	6,7632	1,48102
<i>Hypsugo savii</i>	6	8,60	11,00	9,8667	,98319
<i>Vespertilio murinus</i>	143	8,40	20,60	12,4116	2,29530
<i>Eptesicus serotinus</i>	24	18,60	33,00	24,8792	3,76078
<i>Myotis myotis</i>	236	19,90	39,90	28,2642	3,45991
<i>Myotis nattereri</i>	105	3,50	10,20	7,1829	,94822
<i>Miniopterus schreibersii</i>	1296	6,80	25,00	12,8472	1,83764

Supplementary Table 3. The main UHPLC-HRMS parameters of the targeted ecdysteroids and internal standard (IS).

Compound	Theoretical mass of [M+H] ⁺ (m/z)	Retention time (min)
20E	481.3160	8.06
IS	378.2275	8.10
pB	497.3109	8.17
pS	363.2166	8.48
14d	465.3211	10.10
E	465.3211	10.70
Cal	477.2847	11.49
AjC	481.3160	11.51
2d	465.3211	11.61
Dac	463.3054	14.52
Shd	463.3054	17.13