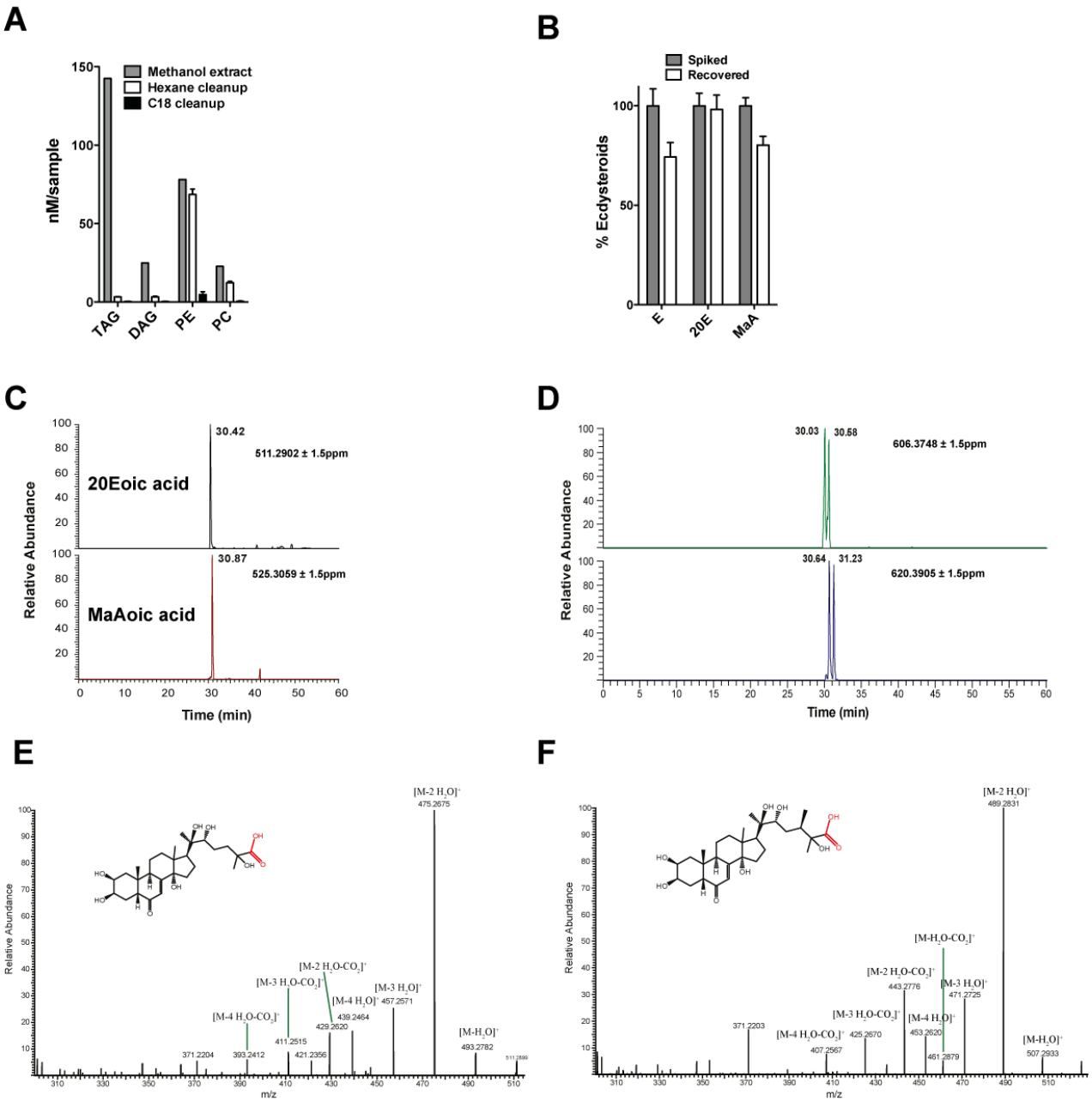
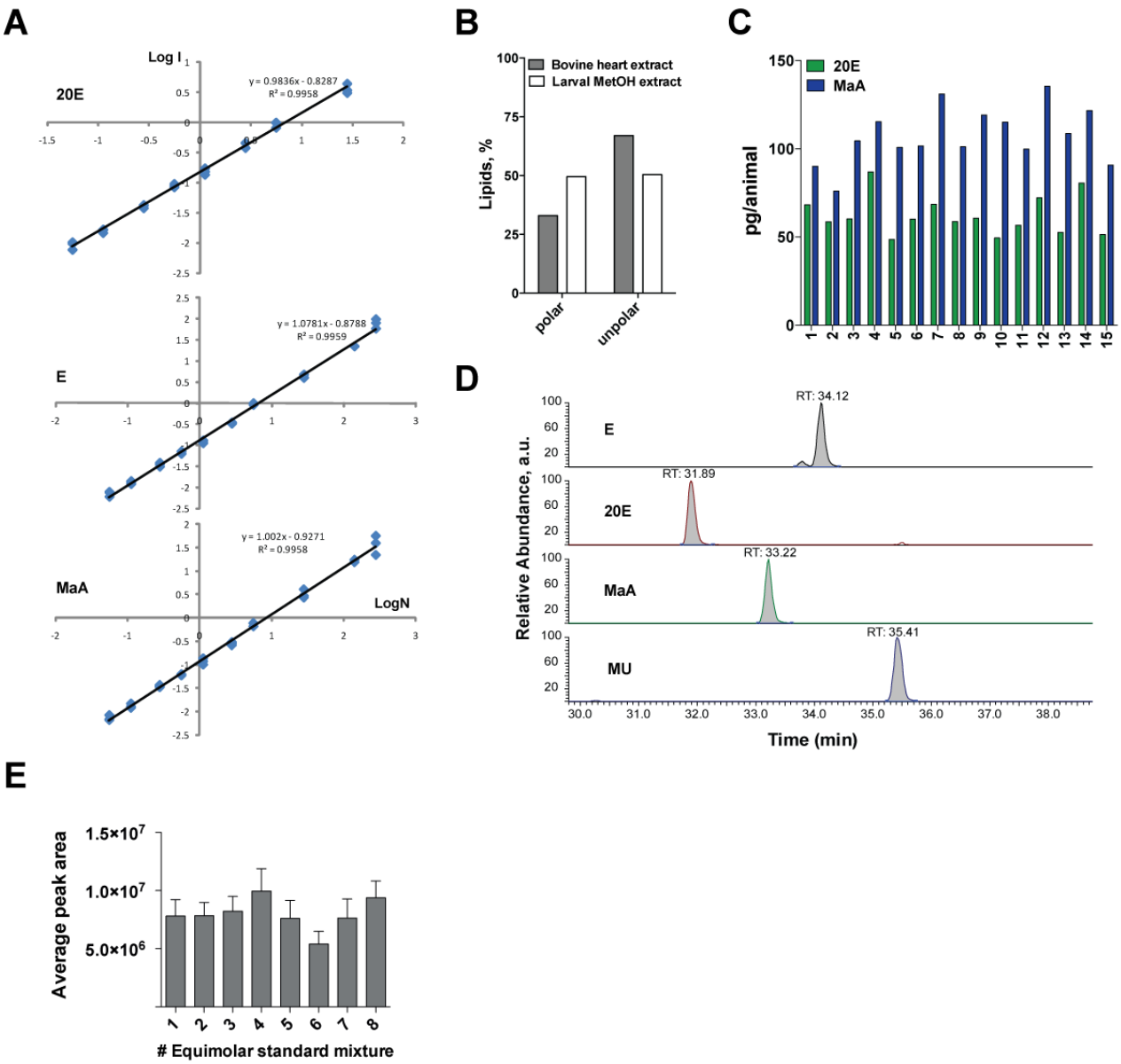
Supplementary Figure 1.



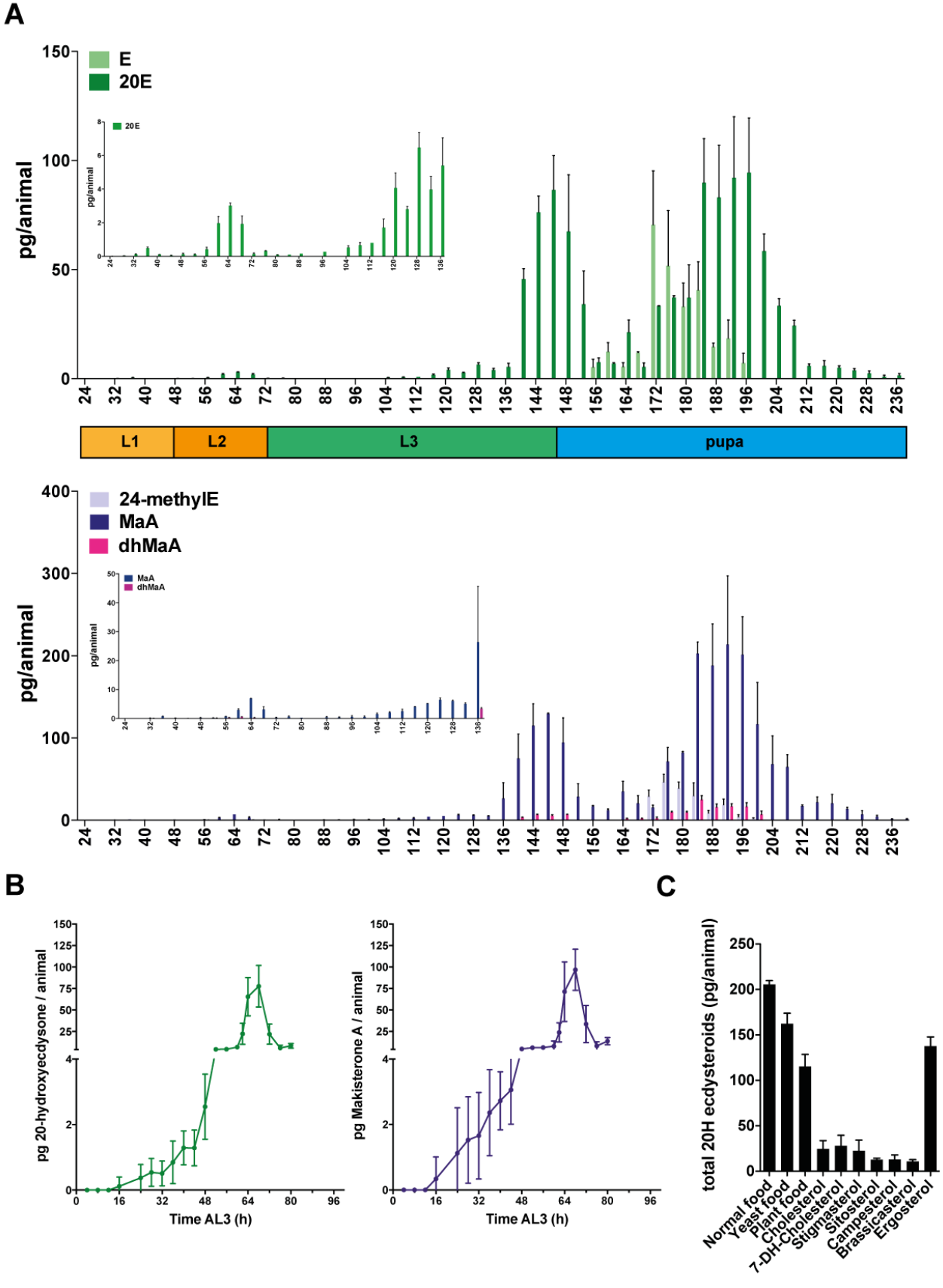
Supplementary Figure 1 Development of the analytical method and identification of ecdysteroids catabolites. **A:** Partitioning of the cold (4°C) methanol extract against n-hexane and clean-up on a C18 cartridge removes the bulk of both apolar and polar lipids. The amount of lipids in nmols in the extract of five 3rd instar larvae is shown at the *y*-axes. **B:** The absolute recovery of endogenous ecdysteroids was, on average, better than 80% as compared to the unprocessed samples. Adding the internal standard of muristerone C further compensates possible losses. Apart from ecdysteroids, in pupae extracts we also detected two of their catabolites 20E- and MaA-oic acids by *t*-SIM in the native form (C) and as their Girard derivatives (D) and confirmed structural assignments by *t*-MS2 in positive ion mode (**E** and **F**) by facile neutral loss of CO_2.

Supplementary Figure 2.



Supplementary Figure 2 Quantification of endogenous ecdysteroids. **A:** calibration curves for 20E, E and MaA obtained by the method of MRM on a triple quadrupole mass spectrometer. For calibration ecdysteroid standards were mixed into one million fold excess of polar lipid extract from bovine brain used as a surrogate matrix: relative content of polar and apolar lipids. (**B**) in the commercially available ecdysteroid-free bovine heart extract was similar to cold methanol extract from larvae. MRM method was sufficiently sensitive (**C**) to quantify major ecdysteroids 20E and MaA in individual animals at white pupae stage. The method of *t*-SIM on a Q Exactive mass spectrometer was applied to quantify ecdysteroids whose standards were not available. The *t*-SIM analysis of an equimolar mixture of ecdysteroids (**D**) revealed that areas of their XIC peaks differed by less than 15% from the average value (**E**).

Supplementary Figure 3.



Supplementary Figure 3. (**A**) Alternative display of the time course profile in Figure 2; ecdysteroids content is presented in pg/animal with no further normalizations. **(B)** Time course of 20E and MaA quantified in *phm-Gal4/+* larvae synchronized at the 2nd to 3rd instar larval molt (time after L2-L3 transition; AL3) till the early pupal stage. Step increases in ecdysteroid levels preceded the main peak at the pupariation. **(C)** The total content of 20-hydroxylated ecdysteroids in white pupae reared on complex (normal, yeast and plant) foods and in lipid depleted food spiked with equal amounts of indicated sterols.

Supplementary Table S1

Target *m/z* of putative ecdysteroids and their characteristic fragments used for ecdysteroidome screening by *t*-SIM and *t*-MS2

$[M+H]^+$	$[M-2H_2O+H]^+$	GirT[1]	GirT-TMA[2]	Core structure[3]	Modification
349.2373	313.2173	444.3218	385.2484	E	hydrocarbon chain cleavage
365.2323	329.2123	460.3168	401.2434	E	hydrocarbon chain cleavage
449.3262	413.3062	544.4107	485.3373	E	dehydroxylation
463.3054	427.2854	558.3899	499.3165	E	oxidation
463.3418	427.3218	558.4263	499.3529	methE	dehydroxylation
465.3211	429.3011	560.4056	501.3322	E	none
477.3211	441.3011	572.4056	513.3322	methE	oxidation
477.3575	441.3375	572.4420	513.3686	ethyl E	dehydroxylation
479.3003	443.2803	574.3848	515.3114	20E	oxidation
479.3367	443.3167	574.4212	515.3478	E	methylation
479.3367	443.3167	574.4212	515.3478	MaA	dehydroxylation
481.3160	445.2960	576.4005	517.3271	20E	none
491.3367	455.3167	586.4212	527.3478	ethyl E	oxidation
493.3160	457.2960	588.4005	529.3271	MaA	oxidation
493.3524	457.3324	588.4369	529.3635	MaC	dehydroxylation
495.2952	459.2752	590.3797	531.3063	E	carboxylation
495.3316	459.3116	590.4161	531.3427	20E	methylation
495.3316	459.3116	590.4161	531.3427	MaA	none
497.3108	461.2908	592.3953	533.3219	MU	none
497.3108	461.2908	592.3953	533.3219	20E	hydroxylation
497.3108	461.2908	592.3953	533.3219	20 H	hydroxylation
507.3316	471.3116	602.4161	543.3427	MaC	oxidation
507.3316	471.3116	602.4161	543.3427	E	acetylation
509.3108	473.2908	604.3953	545.3219	meth E	carboxylation
509.3472	473.3272	604.4317	545.3583	20E	ethylation
509.3472	473.3272	604.4317	545.3583	MaA	methylation
509.3473	473.3273	604.4318	545.3584	MaC	none
511.2902	475.2702	606.3747	547.3013	20E	carboxylation
511.3265	475.3065	606.4110	547.3376	MaA	hydroxylation
521.3473	485.3273	616.4318	557.3584	meth E	acetylation
523.3265	487.3065	618.4110	559.3376	ethyl E	carboxylation
523.3265	487.3065	618.4110	559.3376	20E	acetylation
523.3265	487.3065	618.4110	559.3376	E	glucosylation
523.3629	487.3429	618.4474	559.3740	MaC	methylation
523.3629	487.3429	618.4474	559.3740	MaA	ethylation
525.3058	489.2858	620.3903	561.3169	MaA	carboxylation

525.3421	489.3221	620.4266	561.3532	MaC	hydroxylation
525.3422	489.3222	620.4267	561.3533	MaA	carboxylation
535.3629	499.3429	630.4474	571.3740	ethyl E	acetylation
537.3422	501.3222	632.4267	573.3533	MaA	acetylation
537.3785	501.3585	632.4630	573.3896	MaC	ethylation
539.3214	503.3014	634.4059	575.3325	MaC	carboxylation
539.3215	503.3015	634.4060	575.3326	20E	glucosylation
545.2779	509.2579	640.3624	581.2890	E	sulfation
545.2874	509.2674	640.3719	581.2985	E	phosphorylation
551.3578	515.3378	646.4423	587.3689	ethyl E	glucosylation
551.3578	515.3378	646.4423	587.3689	MaC	acetylation
553.3371	517.3171	648.4216	589.3482	MaA	glucosylation
559.2935	523.2735	654.3780	595.3046	meth E	sulfation
559.3030	523.2830	654.3875	595.3141	meth E	phosphorylation
561.2728	525.2528	656.3573	597.2839	20E	sulfation
561.2823	525.2623	656.3668	597.2934	20E	phosphorylation
567.3527	531.3327	662.4372	603.3638	MaC	glucosylation
573.3092	537.2892	668.3937	609.3203	ethyl E	sulfation
573.3187	537.2987	668.4032	609.3298	ethyl E	phosphorylation
575.2884	539.2684	670.3729	611.2995	MaA	sulfation
575.2980	539.2780	670.3825	611.3091	MaA	phosphorylation
589.3041	553.2841	684.3886	625.3152	MaC	sulfation
589.3136	553.2936	684.3981	625.3247	MaC	phosphorylation
627.3739	591.3539	722.4584	663.3850	E	glucosylation
641.3895	605.3695	736.4740	677.4006	meth E	glucosylation
643.3688	607.3488	738.4533	679.3799	20E	glucosylation
655.4052	619.3852	750.4897	691.4163	ethyl E	glucosylation
657.3845	621.3645	752.4690	693.3956	MaA	glucosylation
671.3527	635.3327	766.4372	707.3638	MaC	glucosylation

1 $[M+H]^+$ ion of the dehydrated GirardT derivative (Lavrynenko et al, 2013)
2 characteristic fragment produced by TEA neutral loss from the dehydrated GirardT derivative
3 core ecdysteroid molecule subjected to the indicated modification

Supplementary Table S2

Standards of ecdysteroid conjugates used for validating ecdysteroidome screening by LC-MS/MS[1]

1	Silenoside A
2	20E-2-glucoside
3	20E-3-glucoside
4	20E-25-glucoside
5	20E-22-palmitat
6	E-22-palmitoleat
7	E-22-palmitat
8	E-22-oleat
9	dehydro-E-sulphate
10	dehydro-E-phosphate

[1] Four glycoside conjugates of 20-hydroxyecdysone at C2; C3; C25 position and silenoside A were kind gifts from Dr. Juraj Harmatha (Institute of Organic Chemistry and Biochemistry, Prague) and Dr. Z Saatov (Institute of Chemistry of Plant Substances, Tashkent), respectively. Dehydro-ecdysone-sulfate and dehydro-ecdysone-phosphate were synthesized from ecdysone as described (Ikeda et al., 1995; Pis et al., 1995); 22-palmitate – and 22-oleate- according to (Dinan, 1988).

Supplementary Table S3

Mass transitions and instrument settings for the detection of major ecdysteroids by the method of multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer TSQ Vantage.

Ecdysteroid	Precursor ion, *m/z*	Fragment ion, *m/z*	CE, eV	S-lens, V
E	465.32	109.23	28	116
	465.32	429.43	13	116
20E	481.32	371.29	17	138
	481.32	445.43	14	138
MaA	495.32	371.29	15	148
	495.32	459.43	14	148
MU	497.32	297.21	21	145
	497.32	425.36	16	145
MethE[1]	479.32	359.22	15	140
	479.32	443.32	14	140
dhMaA[1]	493.32	439.28	20	148
	493.32	457.29	14	148

[1] Settings were not optimized using standards

Supplementary Table S4

Number of animals collected at different time points for quantifying ecdysteroids during the development time course (Figure 2 and Supplementary Figure 3A)

Time of collection, hours[1]	Number of collected animals[2]
0-12	200
16-20	150
24-28	100
32-36	80
40-44	50
48-52	30
56	20
60	15
64-72	10
76 und later	6

[1]hours after embryos hatching
[2] the volume of collected embryos was approximately equivalent to the volume of 200 larvae at the 1st instar

SUPPLEMENTARY METHODS

Separation of ecdysteroids by microflow LC

HPLC was performed on Agilent 1200 system (Agilent, Santa Clara CA) equipped with a trap column (OPTI-PAK, 1µL, C18) from Dichrom GmbH (Marl, Germany) that was mounted in-line to a 0.5 mm x 150 mm analytical column packed with Zorbax SB-C18 5µm from Agilent. The mobile phase delivered at the flow rate of 10 µl/min consisted of solvent A: 0.1% aqueous formic acid; solvent B: 0.1% formic acid in neat acetonitrile. Gradient elution program delivered in 60 min was: holding 5% of B for10 min until 30 µl sample was loaded and concentrated at the trap column; then computer-steered valve switched the system from on-line concentration to gradient elution and the concentration of solvent B ramped from 15% to 30% of B between 11 and 30 min; step increased to 100% of B in one min and hold for 9 min; stepped down to 5% in one minute and hold for 19 min for equilibrating the system to starting conditions. After 5 successive analyses of extracts 30 µL of ecdysteroid standards mixture of MuA, E, 20E, MaA, each with the concentration of 2.5 nM was injected as a quality control.

MRM quantification of ecdysteroids

LC-MS/MS was performed on a TSQ Vantage triple quadrupole instrument (Thermo Fisher Scientific, San Jose CA) equipped with a H-ESI ion source operated under spray voltage of 2.7 kV; 5 psi of sheath gas pressure of 5 psi; auxiliary gas set at 2 arbitrary units; vaporizer temperature 50^0C; transfer capillary temperature 200^0C. S-lens offset and collision energy were optimized for detecting E, MaA, 20E and MuA using standards solution in the direct infusion mode. Quantification relied on the two simultaneously monitored mass transitions between $[M+H]^+$ precursor and the characteristic fragment ions. One transition acquired at the low collision energy (CE) (<10 eV) used the abundant fragment produced from the precursor ion by the loss of two water molecules and served as a quantifier. Another transition was acquired at higher (>25 eV) CE and either used steroid rings fragment in 20E, MaA, MuA or aliphatic chain fragment in E and served as a qualifier (Supplementary Table S3).

The width of transmission mass windows of Q1 and Q3 quadrupoles was set to 0.7 Da; duty cycle 3s; collision cell gas pressure (argon) 1 mTorr. Spectra were processed by QualBrowser software from Xcalibur 2.2; chromatographic peaks were integrated using Genesis algorithm with 5-points Gaussian smoothing.

To prepare the ecdysteroid calibration samples, we used more than a million-fold excess of commercial bovine heart lipid extract as an ecdysteroid-free surrogate matrix (see Supplementary Figure S1).

Profiling ecdysteroidome composition on a Q Exactive mass spectrometer

Extracts from embryos, early 3rd instar larvae, early pupa (40 h APF), late pupa (68 h APF) and female adults were prepared and processed as above, however from 10-fold higher amount of starting material. The extract was first analyzed in high resolution MS mode and

candidate masses were retrieved by matching against the full list of masses of putative ecdysteroids (Supplementary Table S1) with ±1.5 ppm accuracy. Then the LC-MS/MS analysis was repeated such that these candidate masses were detected by *t*-SIM mode and subjected to *t*-MS2 fragmentation under 10 eV collision energy (CE). The abundance of fragment ions yielded by the loss of two water molecules was monitored at the extracted ion chromatogram (XIC) profiles.

A separate portion of each extract was treated with Girard T reagent as described in (Lavrynenko et al., 2013) and analyzed as described above, however in MS and *t*-SIM experiments the abundance of precursor ions corresponding to dehydrated Girard T-derivatization products (Supplementary Table S1), and in *t*-MS2 experiments the abundance of trimethylamine loss fragments (CE = 40 eV) was monitored.

Lipid quantification in larvae extracts

Aliquot of 5 µl of the methanol extracts were re-dissolved in 45 µl of 5 mM ammonium acetate in *iso*-propanol/methanol/chloroform 4/2/1 (v/v/v) containing 0.56 µM of the lipid standards PE 34:0 and PC 34:0. Lipids were identified and quantified by shotgun analyses on a Q Exactive tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described in (Carvalho et al., 2012) using LipidXplorer software (Herzog et al., 2011).

Ecdysteroids quantification on a hybrid tandem mass spectrometer Q Exactive

Extracts from animals corresponding to 162 h to 268 h were re-injected subjected to LC-MS analyses on Q Exactive in *t*-SIM mode using inclusion list in Supplementary Table S1. The target mass resolution of the Orbitrap $R_{m/z=200}$ of 70,000; automated gain control (AGC) was 10^5 and maximum injection time of 200 ms; the width of transmission window of the quadrupole was 1.0 Da; *m/z* 445.1200 ($[C_2H_6SiO]_6$) (Keller et al., 2008) was used as a lock mass. XIC traces of individual ecdysteroids were produced by Xcalibur 2.2 software using 2 ppm mass accuracy; peak areas were calculated using 5-points Gaussian smoothing.

Monitoring polar ecdysteroids by LC-MS/MS

Polar conjugates of ecdysteroids (including acidic conjugates with phosphate and sulphate moieties) were detected by the same LC-MS/MS method as native unconjugated ecdysteroids as $[M+H]^+$ molecular ions at the similar level of sensitivity. The sample preparation and detection methods were validated by analyzing 10 standards of various ecdysteroid conjugates (Supplementary Table S2).

Quantification of the total content of ecdysteroids conjugates with fatty acid moieties

Larvae were extracted with chloroform / methanol by the method of Folch *et al* (Folch, 1957) and dried extracts were subjected to mild alkaline hydrolysis with 50 mM methanolic NaOH, which

quantitatively cleaves fatty acid conjugates, leaving MaA and 20E intact. Alkaline treatment did not noticeably increase ecdysteroid concentration compared to untreated methanol extracts.

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