

RESEARCH ARTICLE

The ecdysteroidome of *Drosophila*: influence of diet and development

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ABSTRACT

Ecdysteroids are the hormones regulating development, physiology and fertility in arthropods, which synthesize them exclusively from dietary sterols. But how dietary sterol diversity influences the ecdysteroid profile, how animals ensure the production of desired hormones and whether there are functional differences between different ecdysteroids produced *in vivo* remains unknown. This is because currently there is no analytical technology for unbiased, comprehensive and quantitative assessment of the full complement of endogenous ecdysteroids. We developed a new LC-MS/MS method to screen the entire chemical space of ecdysteroid-related structures and to quantify known and newly discovered hormones and their catabolites. We quantified the ecdysteroidome in *Drosophila melanogaster* and investigated how the ecdysteroid profile varies with diet and development. We show that *Drosophila* can produce four different classes of ecdysteroids, which are obligatorily derived from four types of dietary sterol precursors. *Drosophila* makes makisterone A from plant sterols and *epi*-makisterone A from ergosterol, the major yeast sterol. However, they prefer to selectively utilize scarce ergosterol precursors to make a novel hormone 24,28-dehydromakisterone A and trace cholesterol to synthesize 20-hydroxyecdysone. Interestingly, *epi*-makisterone A supports only larval development, whereas all other ecdysteroids allow full adult development. We suggest that evolutionary pressure against producing *epi*-C-24 ecdysteroids might explain selective utilization of ergosterol precursors and the puzzling preference for cholesterol.

KEY WORDS: *Drosophila melanogaster*, LC-MS/MS, Dietary sterols, Ecdysone, Ecdysteroids, Makisterone A

INTRODUCTION

Drosophila melanogaster is emerging as a powerful model in which to study hormonal control of growth, metabolism and development (reviewed by Padmanabha and Baker, 2014; Shim et al., 2013; Tennessen and Thummel, 2011). Ecdysteroids – the steroid hormones of arthropods – are key regulators of a network of inter-organ communication that triggers molting and regulates growth,

metabolism and fertility (reviewed by Lafont et al., 2012). Ecdysteroids comprise a large structurally diverse family of polyhydroxylated sterols (reviewed by Lafont and Koolman, 2009). In *Drosophila*, ecdysteroid levels peak just before the critical developmental transitions: mid-embryogenesis, the two larval molts, pupariation and the intra-pupal molt (Kozlova and Thummel, 2000). In larvae, ecdysteroids are synthesized from sterols in the prothoracic gland as pro-hormones and are further activated by C-20 hydroxylation in the intestine and fat body (Petryk et al., 2003). Controlling the production, activation and removal of these hormones is crucial for coupling growth and nutrition to developmental timing, but the mechanisms involved are incompletely understood. Control of ecdysteroid biosynthesis in the prothoracic gland clearly plays a key role and is regulated by signals from multiple tissues. Much less is understood about the peripheral regulation of C-20 hydroxylation and ecdysteroid turnover. To understand the behavior of this network and how it responds to genetic manipulations and environmental challenges, it is essential to be able to identify and quantify the entire complement of ecdysteroids, along with their storage forms and catabolites, here termed the ecdysteroidome. Several ecdysteroids common to other insects have been identified in *Drosophila* (Blais et al., 2010; Bownes et al., 1984) but it is unclear whether the list is exhaustive and how it depends on the developmental stage and diet (Feldlaufer et al., 1995).

Although vertebrates can produce steroid hormones from endogenously synthesized cholesterol, arthropods are sterol auxotrophs and must rely on dietary sterols for ecdysteroid biosynthesis. Carnivorous insects produce ecdysone (E) and its hydroxylated derivative 20-hydroxyecdysone (20E) from cholesterol, a C₂₇ sterol. The C₂₈ and C₂₉ plant sterols are alkylated at the C-24 position. Some plant-eating insects dealkylate plant sterols to cholesterol and use it to produce ecdysone and 20-hydroxyecdysone. Others directly convert plant sterols into 24-methylecdysone and its 20-hydroxylated derivative makisterone A (MaA). Fungi produce C₂₈ sterols (e.g. ergosterol) with an *epi*-C-24 methyl group, which has the opposite chirality to plant sterols. Leaf cutter ants, which subsist on fungus they cultivate on plant material, selectively absorb fungal sterols and produce the corresponding 24-*epi*-makisterone A (24-*epi*MaA) (Maurer et al., 1993).

In the wild, *Drosophila melanogaster* is specialized to feed on rotting plant material undergoing fermentation by yeasts, so they normally consume plant and fungal sterols that are also common in lab diets. Interestingly, lab-reared *Drosophila* produce not only MaA and 24-*epi*MaA, but also 20E (Blais et al., 2010). *Drosophila* are not thought to dealkylate plant and fungal sterols; rather, 20E is produced from trace amounts of cholesterol present in lab food, although the evolutionary significance of this remarkable selectivity remains unknown (Blais et al., 2010; Feldlaufer et al., 1995; Redfern, 1986). Are there differences in the *in vivo* functionality of

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these ecdysteroids? If so, this might be a novel mechanism by which the diet could influence organismal metabolism. However, to explore this and other fascinating problems in the physiology of ecdysteroid signaling requires further technical progress in ecdysteroid analysis and quantification.

In larvae, ecdysteroids are present at the picogram level together with hundreds of micrograms of structural and storage lipids (Kozlova and Thummel, 2000). In *Drosophila*, ecdysteroids are typically quantified by radioimmunoassay (RIA), which does not distinguish between individual molecules and only reports their total content (termed the ecdysteroid titer). To enhance analysis specificity, RIA may be performed on high performance liquid chromatography (HPLC) fractions that are collected according to the elution times of ecdysteroid standards (Feldlaufer et al., 1995). This is a laborious and imprecise solution as it assumes that all ecdysteroids are known and are well-separated by chromatography. Furthermore, measurements must be adjusted based on antibody affinity to different ecdysteroids. This has often led to conflicting reports in the past (Lafont and Koolman, 2009). Further progress in understanding ecdysteroid regulation requires a systematic ‘omics’-scale effort to tackle ecdysteroidome complexity in a quantitative and unbiased way, without recourse to any previous knowledge of the ecdysteroidome composition in other insects. Here, we developed quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based methodologies to perform an exhaustive search for all ecdysteroids present in *Drosophila* and to measure their levels at different stages of development and on different diets.

RESULTS

The composition of the *Drosophila* ecdysteroidome

Methods for LC-MS/MS detection of ecdysteroids have been developed (Blais et al., 2010; Hikiba et al., 2013; Li et al., 2006; Miyashita et al., 2011). However, crude larval extracts are difficult to analyze because of the abundance of other lipids. We developed a sample preparation protocol that reduced the total lipid content by about 100-fold down to $\sim 1 \mu\text{g}$ /per extracted third instar larva (Fig. S1A) and enabled direct analyses by microflow reversed phase HPLC. Losses of endogenous ecdysteroids were below 30% and could be normalized by adding an internal standard, muristerone A (MaA), prior to their methanol extraction (Fig. S1B).

To identify ecdysteroids, including any previously unsuspected molecules, we compiled a list of 52 masses of putative molecules that could be derived by 11 common chemical modifications (alkylation, oxidation, reduction, etc.) of the six core ecdysteroid structures: 20E, MaA, makisterone C (MaC) (Feldlaufer et al., 1991; Mauchamp et al., 1993) and their non-hydroxylated precursors (Table S1). These masses cover a chemical space of $>20,000$ putative molecules, including numerous positional isomers. We then analyzed methanol extracts of embryos, early third instar (L3) larvae, early and late pupae and female and male adults by LC-MS/MS on a Q Exactive hybrid tandem mass spectrometer and monitored these candidate masses with $>70,000$ mass resolution and low-ppm accuracy. Out of 52 masses, only 20 were detected as chromatographic peaks, and seven out of 20 were ecdysteroid candidates based on targeted MS/MS (*t*-MS2) experiments relying on facile loss of two water molecules at low (10 eV) collision energy (CE) (Fig. 1A).

To test whether these peaks corresponded to genuine ecdysteroids, we derivatized the extracts with Girard T reagent (Girard and Sandulesco, 1936), which specifically targets the C-6 ketone moiety common to all ecdysteroids (Karu et al., 2007). We

repeated LC-MS/MS analyses in *t*-MS2 mode by monitoring neutral loss of the trimethylamine group from precursors of dehydrated Girard T derivatives (Lavrynenko et al., 2013) (Fig. 1B). Thus, we base the peak identification as an ecdysteroid upon four independent criteria: expected intact mass determined with ± 1.5 ppm accuracy by targeted single ion monitoring (*t*-SIM); ecdysteroid-specific fragmentation pattern in *t*-MS2; expected mass shift upon Girard T modification also accompanied by abundant neutral loss of trimethylamine in *t*-MS2; and, finally, Girard products detectable as a doublet of chromatographic peaks of E/Z stereoisomers.

Altogether, we identified nine molecules whose elemental composition suggested seven ecdysteroids and two ecdysteroid catabolites. The ecdysteroids included E, 20E and MaA, effectively serving as positive controls. We also identified 24-methylecdysone (24-methylE) by its intact mass and comparison with the previously reported MS/MS spectrum (Blais et al., 2010). 24-MethylE and MaA were accompanied by satellite peaks of their epimers 24-*epi*-methylE and 24-*epi*-MaA: their intact masses (<1.5 ppm accuracy) and MS/MS spectra were identical to 24-methylE and MaA, respectively; however, they were eluted with different retention times (Fig. 1A).

One ecdysteroid peak remained unmatched to the known insect ecdysteroids. Its elemental composition $\text{C}_{28}\text{H}_{45}\text{O}_7$ calculated from the mass to charge ratio (m/z) 493.3159 ($[\text{M}+\text{H}]^+$; 1.5 ppm mass accuracy) and *t*-MS2 spectrum suggested that it differs from MaA ($\text{C}_{28}\text{H}_{47}\text{O}_7$) by one double bond located in its aliphatic chain at the C-24C-28 position and that both molecules share the same steroid core (Fig. 1C), which also corroborated the MS/MS spectrum of its Girard T derivative. Indeed, 24(28)-dehydromakisterone (dhMaA) has been identified in plants (Báthori et al., 1999; Kumpun et al., 2011; Thuy et al., 1998); however, to the best of our knowledge it has not been found in insects. A reference sample of dhMaA purified from plants (Báthori et al., 1999; Wessner et al., 1992) matched the retention time and MS/MS spectrum of the *Drosophila* ecdysteroid (data not shown).

LC-MS/MS screening of pupal extracts (but not those of other stages) also revealed two molecules for which m/z was consistent with the oxidized (carboxylated) catabolites of 20E (m/z 511.2907; 1 ppm) and MaA (m/z 525.3067; 1.7 ppm) (Fig. S1C,D). C-26 oxidation of 20E yields 20-hydroxyecdysoneic acid (20Eoic) and contributes to its irreversible inactivation in *Drosophila* (Guittard et al., 2011). We confirmed the identification of 20Eoic and MAoic acids by *t*-MS2: both showed facile loss of multiple water molecules at low CE as well as loss of CO_2 (Fig. S1E,F).

We also looked for ecdysteroid conjugates with glucoside, sulfate, phosphate and fatty acid moieties (see list of standards and detection methods in Table S2 and supplementary materials and methods, respectively); however, we did not identify them in embryos, larvae or pupae at concentrations comparable to ecdysteroids, in contrast to adult flies (Grau and Lafont, 1994).

Taken together, we found that the ecdysteroidome of animals reared on normal lab food contains seven major ecdysteroids: E, 20E, MaA, 24-methylE, 24-*epi*-methylE, 24-*epi*-MaA, the novel ecdysteroid dhMaA, and two catabolites – 20Eoic and MaAoic acids.

dhMaA is a functional *Drosophila* hormone

To determine whether the newly identified dhMaA was active in *Drosophila* tissues, we tested whether it could induce pre-pupal morphogenesis of cultured wing imaginal discs. Wing discs initiate morphogenesis in response to the ecdysteroid pulse that precedes pupariation. The presumptive wing epithelium everts and folds to appose the dorsal and ventral surfaces, and the entire epithelial

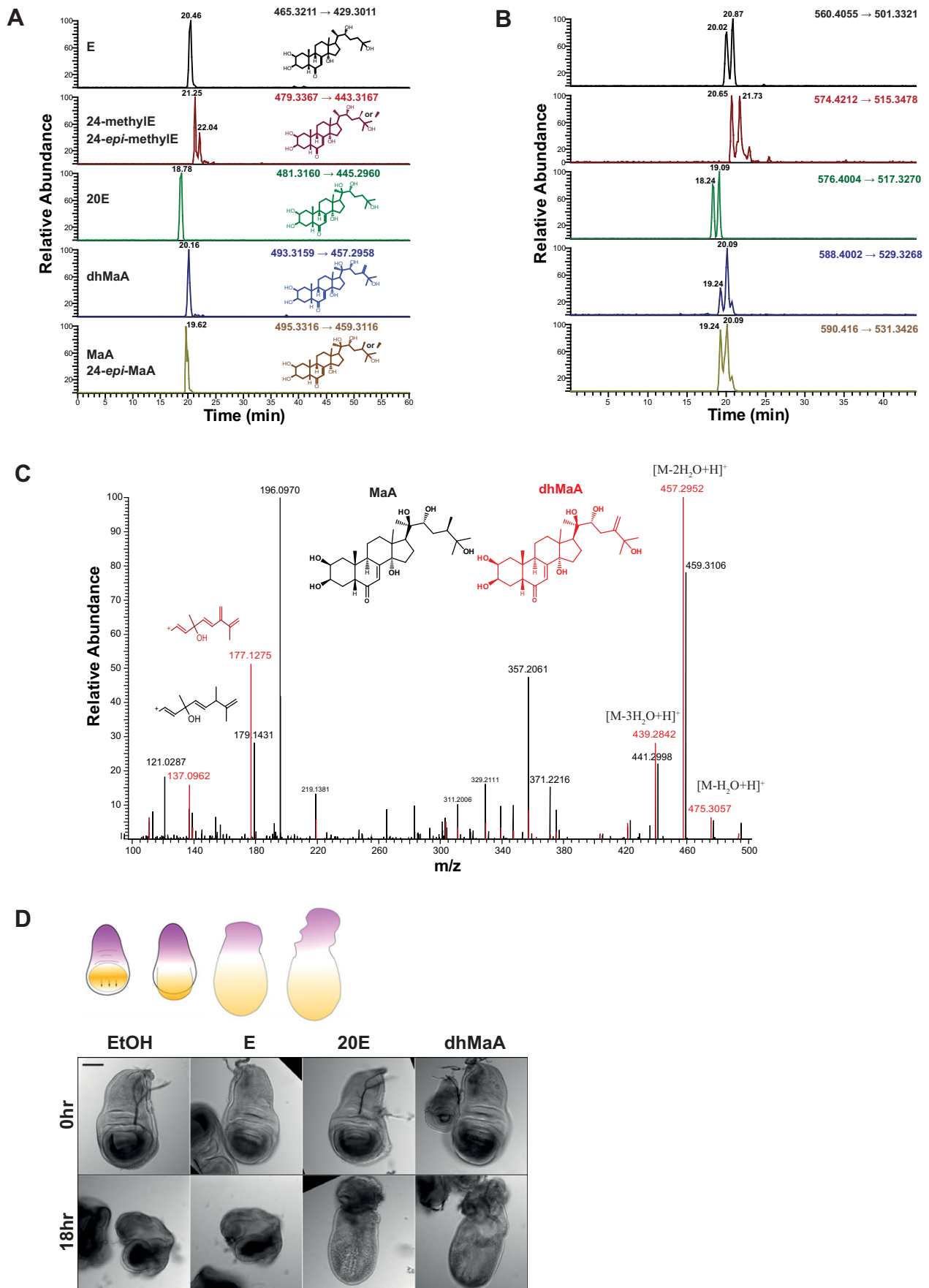


Fig. 1. See next page for legend.

Fig. 1. LC-MS screen in *t*-SIM and *t*-MS2 modes identified known ecdysteroids and discovered a novel ecdysteroid in *Drosophila*.

(A,B) Extracted ions chromatogram (XIC) of the pupa extract (A) and of the same extract modified with Girard T reagent (B). These XICs were acquired in *t*-MS2 mode; mass transitions indicated in the insets correspond to the loss of two water molecules from the intact precursor ions under CE=10 eV (A) or trimethylamine from their dehydrated Girard T derivatives under CE=40 eV (B). *Epi*-forms of ecdysteroids (*epi*-methylE; *epi*-MaA) were recognized by identical MS2 spectra and different retention times. E/Z isomers of Girard derivatives (B) are detected as partially resolved chromatographic peaks. (C) MS2 identification of the novel ecdysteroid as 24(28)-dehydromakisterone A. MS2 spectra of dhMaA (in red) and MaA (in black). *m/z* of the intact precursor, water loss fragments and the fragment of the hydrocarbon chain are all shifted by 2 Da, whereas the sterol ring fragments overlap. An abundant ion with *m/z* 196.097 is background. (D) Testing dhMaA activity using an *in vitro* wing disc eversion assay. Dissected wing discs were incubated in SSM3 media containing FBS and 0.2 μ M of corresponding ecdysteroids and imaged over time to see if the hormones were capable of stimulating eversion. In this assay, the wing pouch (yellow in diagram) should push away from the prospective notum part of the disc (pink in diagram) and flatten. Discs given 20E and dhMaA everted after 15–18 h; however, the discs given ethanol alone or E did not. Scale bar: 100 μ m.

bilayer flattens and expands (Aldaz et al., 2010). We cultured explanted wing discs from late third instar larvae in the presence of 0.2 μ M E, 20E and dhMaA and filmed them over the course of 18 h. We observed that dhMaA and 20E caused disc eversion at these concentrations, whereas E was inactive (Fig. 1D). Thus dhMaA is an active hormone *ex vivo* consistent with its *in vitro* activity in a cell culture-based assay (Clément et al., 1993).

Quantification of hormones and catabolites

Major ecdysteroids (E, 20E and MaA) for which standards are commercially available were quantified by LC-MS/MS on a triple quadrupole mass spectrometer by the method of multiple reaction monitoring (MRM). Calibration plots for 20E, E and MaA (Fig. S2A) were linear over more than a 10,000-fold concentration range, with a detection limit of 5 pg/ml and relative standard deviation below 20% at the limit of quantification and below 5% in the middle of the concentration range. To compensate for matrix effects, calibration samples of ecdysteroids were prepared in over 1,000,000-fold excess of lipids from bovine heart extract as a surrogate matrix (Fig. S2B). To the best of our knowledge, this is currently the most sensitive and accurate analytical method for ecdysteroids, enabling the quantification of the full spectrum of endogenous hormones in a single white pre-pupae (Fig. S2C).

Two ecdysteroids (dhMA and 24-methylE) and two catabolites (20Eoic and MaAoic acids) could not be quantified by MRM because of the lack of corresponding standards. In contrast to MRM, quantification by *t*-SIM relies on chromatographic peaks of intact precursor ions monitored with high mass resolution and accuracy. Similar molecules containing the same number and type of polar groups are likely to be ionized with similar efficiencies and evoke similar responses (Fig. S2D,E). This suggests that 24-methylE and dhMaA can be quantified similarly using MuA as a reference. We also applied *t*-SIM for quantifying 20Eoic and MaAoic acids, because their carboxyl groups remain protonated in the acidic eluent and probably have only a limited impact on their ionization capacity.

The ecdysteroidome in embryonic, larval and pupal development

The ecdysteroid titer peaks at the major developmental transitions (Kozlova and Thummel, 2000). To determine how different ecdysteroids contribute to these peaks during development, we

quantified the temporal profiles of E, 20E, 24-methylE, MaA and dhMaA in animals reared on normal lab food (NF) (Fig. 2A).

E, 20E and MaA were quantified by MRM in all collected samples. We also used MRM to determine relative amounts of dhMaA at different time points; however, the lack of standards for dhMaA and 24-methylE prevented their absolute quantification by this method. We therefore used *t*-SIM to quantify dhMaA and 24-methylE in a subset of 15 samples that had already been measured by MRM and used these values to extrapolate the MRM measurements at other time points by linear regression.

We plotted ecdysteroid measurements either as pg per animal (Fig. S3A) or as picograms per nanomole of phospholipids (Fig. 2A) quantified by shotgun lipidomics in a separate aliquot of a methanol extract from each sample (Carvalho et al., 2012). The latter normalization better illustrates changing ecdysteroid concentrations within animals as their size increases.

The temporal concentration profiles of 20E and MaA during post-embryonic development qualitatively resemble ecdysteroid profiles determined by RIA (Handler, 1982; Parvy et al., 2005; Warren et al., 2006). Titrers peak at each developmental transition, with the highest levels occurring during pupal stages (Fig. 2A; Fig. S3A). No ecdysteroids were detected in adult males or females, consistent with extremely low RIA measurements from adults (reviewed by Schwedes and Carney, 2012). MaA is consistently about twofold more abundant than 20E throughout development, but their profiles peak at the same times, confirming that neither is a precursor for the other. We also observed a small elevation in 20E and MaA beginning at the second half of the third instar, preceding the surge in hormone production that induces pupariation. This slight increase is partly consistent with combined RIA and HPLC-based studies that report two small ecdysteroids peaks at this time (Ou et al., 2011; Warren et al., 2006). However, our quantitative profiles (Fig. 2A; Fig. S3A) are more consistent with step increases of ecdysteroid concentration, rather than time-resolved individual peaks. To investigate whether larval asynchrony might obscure such peaks, in a separate experiment we collected larvae at the molt from the second to the third instar and measured ecdysteroid levels at 4 h intervals. Although we observed no clear peaks, these measurements indeed revealed two step increases in 20E and MaA concentrations prior to the peak at pupariation (Fig. S3B).

Interestingly, MaA and 20E concentrations in embryos are much lower than expected from RIA measurements (Kozlova and Thummel, 2000; Riddiford and Truman, 1993). At the same time, the concentration profile of dhMaA peaks at the embryonic stage and is enriched 10- and 2-fold compared with its levels in larvae and pupae, respectively (Fig. 2A). Thus, dhMaA is likely to be responsible for the embryonic ecdysteroid titer measured by RIA. The fact that all three ecdysteroids are undetectable in embryos before 4 h after egg laying (AEL) confirms that they are synthesized by embryos rather than inherited from the mother (Fig. 2A). Thus, embryos and larvae/pupae synthesize specific ecdysteroids in different proportions.

Examining concentration profiles of the ecdysteroid precursors E and methylE suggests that the efficiency of their conversion to 20E and MaA may vary at different stages. The precursors are undetectable during embryonic and larval development. However, in pupae, significant peaks of E and 24-methylE precede those of 20E and MaA by about 12 h. This suggests that hormones released by the larval ring gland are extremely rapidly converted to their 20-hydroxylated forms in larval peripheral tissues, whereas hydroxylation proceeds less efficiently during pupal development.

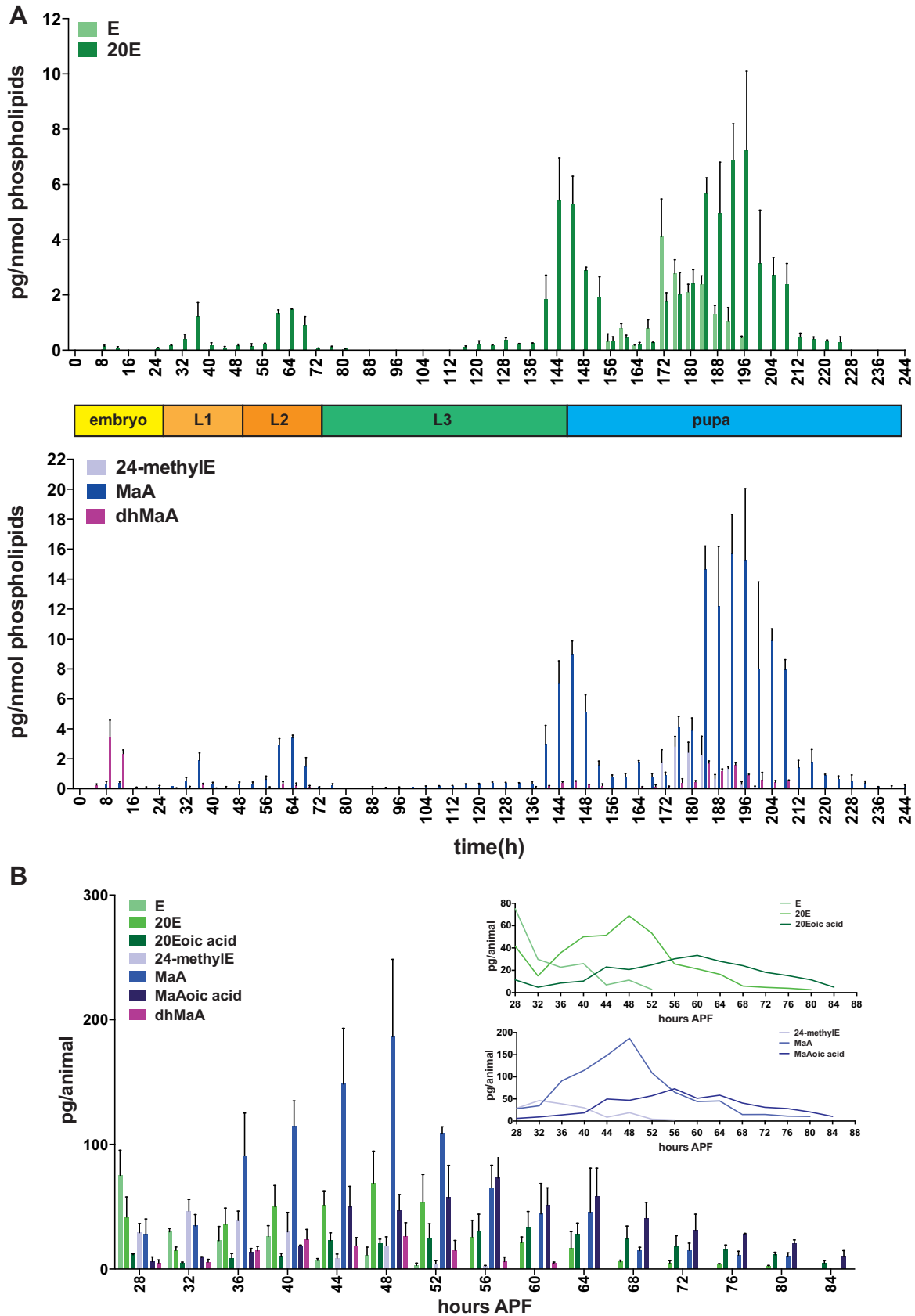


Fig. 2. Ecdysteroidome dynamics during development. (A) Profile of ecdysteroids during the full development cycle from the onset of embryogenesis until adult eclosion. Ecdysteroid content (in pg) on the y-axes is normalized to the total amount of phospholipids (in nmol); x-axes show development time (hours). (B) Time course of ecdysteroids (20E, MaA, dhMaA), their precursors (E, 24-methylE) and two major catabolites (MAoic and 20Eoic acids) at the pupal stage. The insets display changes in compound content as line graphs. Values are mean±s.d. (*n*=3).

To examine the kinetics of ecdysteroid catabolism we focused on the pupal ecdysteroid peak between 152 and 208 h AEL (Fig. 2B). Concentrations of MaA and 20E acids matched the profiles expected for MaA and 20E catabolites: their concentration maxima were observed 8 h after the maximum of both hormones. We did not detect putative precursor(s) or catabolite(s) of dhMaA, probably because of its low abundance.

Dietary sterols determine the ecdysteroidome

We wondered whether the ecdysteroidome might vary depending on the diet. The normal lab diet contains material from both plants and yeast. To investigate the role of diet, we separated the yeast and plant components and reared larvae on either plant food (PF) or yeast food (YF). PF contains mostly phytosterols (>60% sitosterol) along with minute amounts of ergosterol (~1%), whereas yeast food (YF) is enriched in yeast sterols (>70% ergosterol) with minute amounts of phytosterols. Cholesterol is not a major component of either diet, although is detectable as a trace contaminant (Carvalho et al., 2012). We quantified ecdysteroids in PF- or YF-reared animals at the white pre-pupal stage; at this time, animals can be staged to within half of an hour, limiting variation due to asynchrony. On normal food, the variation in 20E levels between individual white prepupae is <20% (Fig. S2C).

Animals raised on PF and YF produce different ecdysteroids. PF-reared animals contain MaA, 20E and small amounts of the corresponding precursors (Fig. 3A). Plant sterols would be expected to give rise to MaA; however, the large amounts of 20E are surprising. YF-reared animals produce 24-*epi*-MaA and dhMaA, in addition to MaA and 20E (Fig. 3A). Fungal sterols would be expected to give rise to *epi*-MaA, but the sterol substrate used to produce dhMaA is not clear. Overall, these data show that the dietary sterol composition influences ecdysteroid composition. However, the ecdysteroid profile does not directly reflect the abundance of different dietary sterols.

Individual sterols give rise to different ecdysteroids

We exploited the sterol auxotrophy of *Drosophila* to establish clearly which ecdysteroids can be produced from which sterols. We placed animals into single wells containing lipid-depleted (LD) food supplemented with one of ten different sterols, and quantified ecdysteroids at the white prepupal stage. Animals fed LD food alone arrest in the second larval instar, serving as a control for sterol depletion.

Only three sterols failed to support larval development: cholestanol, zymosterol and desmosterol (Fig. 3B,C). Extracts of these larvae revealed no ecdysteroids, suggesting that their structures are inconsistent with ecdysteroidogenesis. Seven sterols supported larval development. The ecdysteroids produced from these sterols revealed a direct relationship between the structures of the aliphatic sterol tail and the resulting ecdysteroid. When fed with cholesterol or 7-dehydrocholesterol, which like 20E has no methyl group at the 24 position, animals produce exclusively E and 20E (Fig. 3A). When fed with campesterol, which has a C-24 methyl group in the same chiral configuration as MaA, animals produce exclusively MaA and its precursor 24-methylE. Stigmasterol and sitosterol, which have a C-24 ethyl group in the same configuration as methyl group in campesterol, give rise mainly to MaA, suggesting that these sterols are C-29 demethylated (Fig. 3A) during ecdysteroidogenesis. Interestingly, 24-*epi*-MaA is produced in small amounts from C-24-ethyl but not methyl sterols, suggesting that demethylation partially racemizes the chiral center at the C-24 position. Brassicasterol and ergosterol both have *epi*-methyl groups at the

C-24 position, and both generate 24-*epi*-MaA and its precursor 24-*epi*-methylE (Fig. 3A). Thus, *Drosophila* cannot reverse the chiral configuration of a methyl group at the C-24 position and cannot produce MaA from brassicasterol or ergosterol. Interestingly, ergosterol-fed animals also produce dhMaA. This raises the possibility that the *epi*-C-24 methyl group in ergosterol can be converted to a C-24C-28 double bond, eliminating the chiral center. Alternatively, trace amounts of ergosterol precursors in the ergosterol preparation might give rise to dhMaA. We note that the C-24C-28 double bond is present in many ergosterol biosynthetic precursors (Daum et al., 1998) and indeed the mass spectrum of the commercial ergosterol preparation shows a very small peak (~1% compared with ergosterol) with a mass 2 Daltons smaller than the mass of ergosterol, consistent with such a molecule (data not shown).

To investigate whether ergosterol precursors with a C-24C-28 double bond might be used for synthesis of dhMaA, we fed larvae with either wild-type yeast or *erg4Δ* mutant yeast, which fails to reduce ergosta-5,7,22,24(28)-tetraenol [hereafter denoted 24(28)-dehydroergosterol] to ergosterol (Aguilar et al., 2010). Whereas animals fed wild-type yeast produce both 24-*epi*-MaA and dhMaA (and their precursors), those fed with *erg4Δ* yeast predominantly produce dhMaA and its precursor (Fig. 3D). Taken together, these data suggest that scarce ergosterol precursors are the substrates for synthesis of dhMaA, and that only 24-*epi*-MaA can be produced from ergosterol. The fact that equal amounts of dhMaA and *epi*-MaA are produced from mixtures consisting of ~1% 24(28)-dehydroergosterol and 99% ergosterol indicates that the ecdysteroidogenic machinery has at least a 100-fold preference for 24(28)-dehydroergosterol as a substrate for hormone synthesis. Thus, the high abundance of dhMaA in embryos might reflect two factors: the preference of egg-laying females for yeast, and the preferential utilization of 24(28)-dehydroergosterol for ecdysteroidogenesis.

We wondered whether an analogous preference for cholesterol might explain the presence of E and 20E in animals fed a complex diet apparently lacking significant amounts of cholesterol. To investigate this, we fed animals with stigmasterol and cholesterol in different proportions, and quantified the ecdysteroids they produced (Fig. 3E). Even when stigmasterol was present at 1000-fold molar excess, animals produce exclusively 20E and no MaA. Animals only begin to produce MaA when the molar ratio of stigmasterol:cholesterol reaches 10,000:1. Thus, vanishingly small amounts of cholesterol can saturate the ecdysteroidogenesis machinery and prevent the synthesis of MaA from stigmasterol. This probably accounts for the presence of E and 20E in animals fed lab food, consistent with previous suggestions (Feldlaufer et al., 1995).

The fact that animals fed with single sterols produce only a single ecdysteroid class allowed us to examine whether all these classes are equivalently functional in supporting development. To do so, we quantified how animals fed different sterols progressed through larval and pupal development and how often they emerged as adults. Animals fed on a lipid-depleted diet containing single sterols that give rise E/20E or 24-methylE/MaA progressed through these developmental stages at the same rate and almost all completed adult development (Fig. 3B,C). However, we noticed that animals fed cholesterol or dehydrocholesterol (giving rise to E and 20E) formed smaller pupae and adults than those fed plant sterols (Fig. 4A,B). We wondered whether this reflected different effects of 20E and MaA on growth, or whether the properties of membranes containing these sterols might differ. To distinguish these possibilities, we fed larvae with a 100:1 ratio of stigmasterol:cholesterol, which should produce animals with stigmasterol in their membranes that make exclusively 20E. We compared their growth

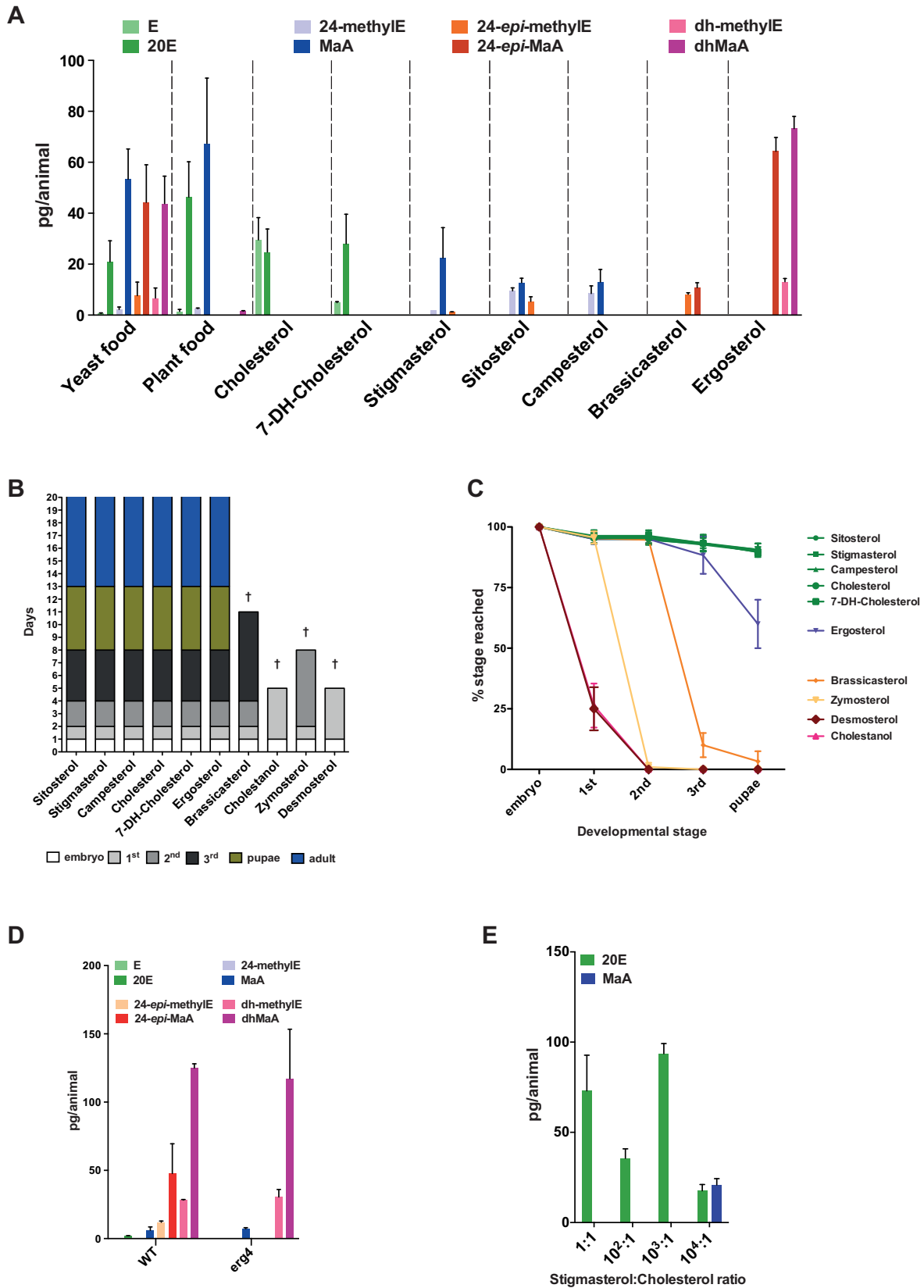


Fig. 3. Ecdysteroidome composition and development of *Drosophila* reared on diets with controlled composition of sterols. (A) Ecdysteroidome of white pupae reared on lipid-depleted food supplemented with individual sterols; complex ‘yeast’ and ‘plant’ foods are shown for comparison. Ecdysteroids were quantified by *t*-SIM. (B,C) stages and timing of animal development (B) and percentage of animals reaching the indicated development stage (C) on the same foods as shown in A. †Indicates lethality at the corresponding development stage. (D) Ecdysteroidome of white pupae fed on the WT yeast and $\Delta erg4$ yeast mutant. (E) Ecdysteroidome of white pupae reared on lipid-depleted food supplemented with the mixture of stigmasterol and cholesterol at the indicated molar ratios. Values are mean \pm s.d. ($n=3$ for A, D and E; $n=6$ for B and C).

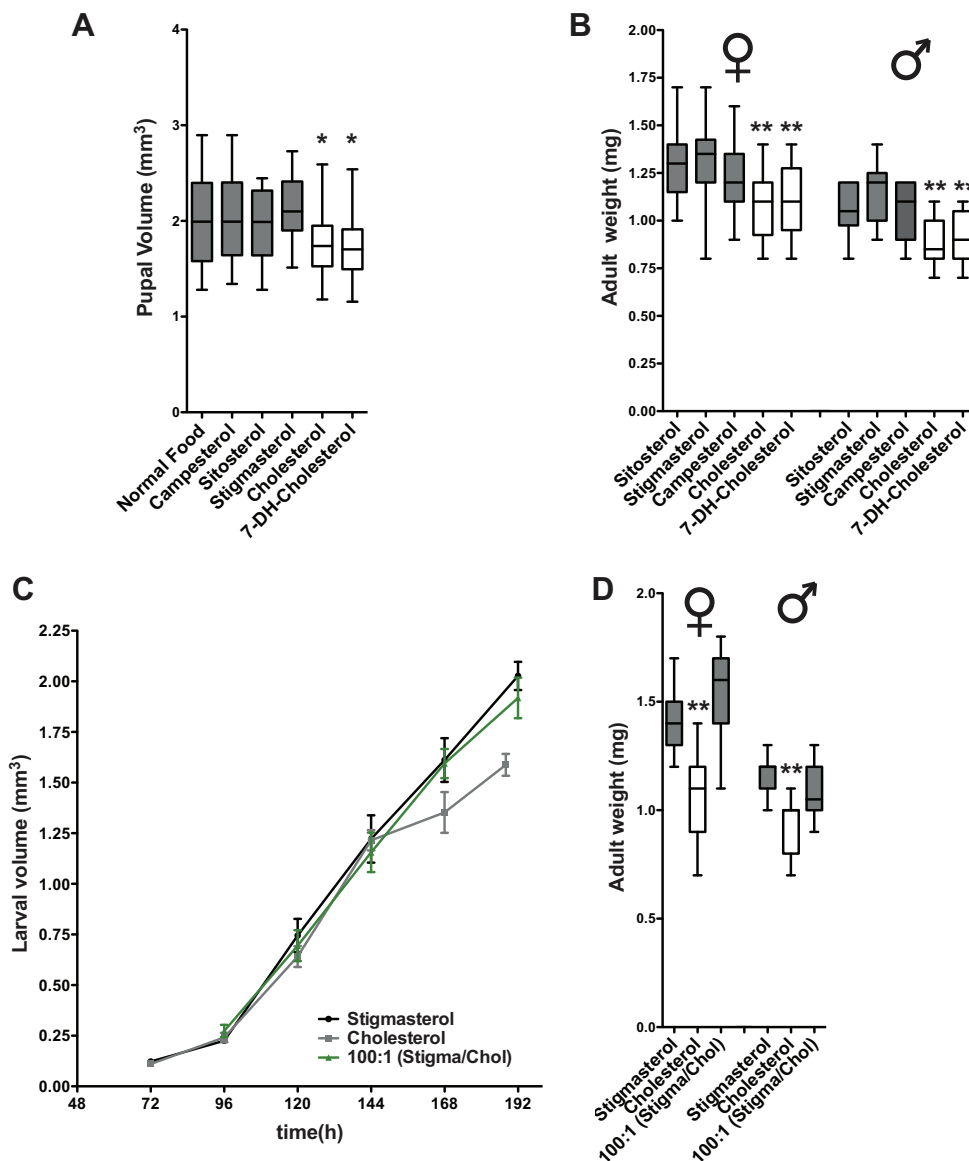


Fig. 4. The bulk of dietary sterols, but not ecdysteroids, affect the size and weight of developing animals.

(A,B) Pupal volume (A) and adult weight (B) of animals reared on lipid-depleted foods supplemented with individual sterols. * $P < 0.05$, ** $P < 0.01$.

(C,D) Changes in larval volume (C) and adult weight (D) of animals reared on the food supplemented with stigmasterol, cholesterol or their 100:1 mixture.

** $P < 0.01$. Values in C are mean \pm s.d. ($n > 30$).

rates with animals fed either sterol alone (Fig. 4C). Animals fed cholesterol alone initially grow at the same rate as those fed stigmasterol or 1:100 stigmasterol:cholesterol; however, in the middle of the third larval instar their growth rate slows. They pupariate slightly earlier (8 h) than control animals and their pupae and adults are smaller (Fig. 4C,D). These observations suggest that membrane cholesterol, rather than 20E, is responsible for the slow growth of cholesterol-fed larvae. Thus, 20E and MaA function interchangeably to support growth and development.

We next turned our attention to dhMaA and 24-*epi*-MaA. Most animals fed with the 24-*epi*-MaA precursor brassicasterol arrest in the third larval instar. The few white prepupae that develop contain only 24-*epi*-methylecdysone and 24-*epi*-makisterone. By contrast, animals fed commercial ergosterol [containing ~1% of 24(28)-dehydroergosterol(s)] reach adulthood. These white prepupae contain both dhMaA and 24-*epi*-MaA. Taken together, these data suggest that *epi*-MaA cannot support pupal development, but that dhMaA can. Furthermore, larvae fed with *erg4Δ* mutant yeast (which produce almost entirely dhMaA) pupariate at normal rates and reach adulthood (Fig. 3D). Thus, dhMaA is a fully functional

ecdysteroid *in vivo*, whereas 24-*epi*-MaA suffices for larval stages, but cannot support pupal development, consistent with its lower activity in tissue culture assays (Clément et al., 1993; Ravi et al., 2001). Thus, the ability of yeast-fed animals to selectively utilize 24(28)-dehydroergosterol(s) for dhMaA synthesis is likely to be crucial for survival when feeding on yeast in the wild.

As dhMaA is the most abundant ecdysteroid in embryos, we wondered whether 20E or MaA could replace it at this stage. To address this question, we raised larvae on lipid-depleted food supplemented with cholesterol, and fed the emerging female adults the same diet. Embryos produced by these females contained exclusively 20E and had no obvious developmental defects (not shown). Furthermore, flies can be maintained for multiple generations on PF, which does not support synthesis of dhMaA. Thus, 20E, MaA and dhMaA, but not *epi*-MaA, are all equally capable of supporting the *Drosophila* life cycle.

Interestingly, white prepupae formed by larvae fed with single sterols on lipid-depleted medium contained much smaller amounts of 20-hydroxylated ecdysteroids than animals fed on more complex lipid-rich diets (NF, YF, PF) (Fig. 3A,B; Fig. S3C). This suggests

that diet exerts strong quantitative effects on the level of ecdysteroids that are unrelated to their role as molting hormones because these widely different amounts of ecdysteroids are sufficient to initiate puparium formation.

Taken together, these experiments show that the structure of available dietary sterol directly determines the class of ecdysteroid produced. Nevertheless, the ecdysteroidogenesis machinery has very strong competitive preferences for particular sterol substrates, utilizing cholesterol over plant sterols and dehydroergosterols over ergosterol. Not all ecdysteroids are equally functional, although a variety of ecdysteroid compositions can support adult development.

DISCUSSION

Analytical tools for ecdysteroidomics

The key advance in our approach is the use of high-resolution *t*-SIM instead of MRM for profiling the ecdysteroidome composition. We took advantage of the ability of Q Exactive instruments to determine the masses of intact molecular ions with low-ppm accuracy even in samples with complex biological matrices. As the mass is unequivocally linked to the molecules' elemental composition, a limited number of unique masses cover a variety of plausible structures, which is a clear advantage in screening experiments. The absence of peaks with a given mass rules out the presence of all structural variants sharing the same elemental composition. Further validation and detailed structural characterization of a small number of returned candidates is performed at the next step by *t*-MS2, chemical derivatization and other analytical means. We also observed that, in contrast to MS2/MRM, the instrument response towards intact ions is rather homogeneous for structurally related molecules, which enables the quantification of newly discovered molecules using already available standards of the same class.

We identified all previously known and one novel *Drosophila* ecdysteroid, as well as two previously proposed catabolites. The dynamic profile of total ecdysteroids measured by *t*-SIM/*t*-MS2 was in good agreement with estimates previously reported using RIA (Riddiford and Truman, 1993). However, the ability to discover and separately quantify distinct ecdysteroid species revealed an important new ecdysteroid, dhMaA, as well as unexpected differences in profiles of individual ecdysteroids. Equally importantly, our screen ruled out the presence of many plausible ecdysteroid structures and demonstrated that conjugated forms of ecdysteroids are unlikely to play a major role in ecdysteroidome dynamics in *Drosophila*. Together with already available lipidomics technology, this provides a methods platform to address exciting problems in insect endocrinology in a quantitative, systematic manner.

Structural features of dietary sterols determine the ecdysteroidome

With respect to ecdysteroidogenesis, 11 tested dietary sterols fall into three large groups. Some do not support ecdysteroidogenesis at all (desmosterol, cholestanol and zymosterol). The others give rise to four different ecdysteroid classes, depending on the structure of their hydrocarbon side chain. Comparison of chemical structures of sterols of the 'lethal' group prompts interesting considerations regarding the structural requirements for entering the ecdysteroidogenic pathway. The presence of a hydroxyl group at C-25 is a conserved feature in ecdysteroids of arthropods, including *Drosophila*. However, the 'terminal' double bond at C-24C-25 in zymosterol and desmosterol might prevent C-25 hydroxylation. The 'internal' double bond C-22C-23 in brassicasterol and ergosterol is reduced during ecdysteroidogenesis; however, this reaction is

strictly regiospecific and, apparently, does not occur at the branched terminus of the hydrocarbon chain.

Cholesterol is not enantiomeric at the C-24 position. However, plant and fungal sterols contain chiral C-24 methyl or ethyl groups whose stereochemical configuration influences the structure of the resulting ecdysteroid class – producing either MaA or *epi*-MaA. The fungal sterol ergosterol gives rise exclusively to *epi*-MaA whereas C-24 methyl plant sterols give rise only to MaA. Interestingly, C-24 ethyl plant sterols can be used to produce both *epi*-MaA and MaA. This suggests that de-methylation of the C-24 ethyl group may lead to partial racemization at this position.

Preferential utilization of sterol substrates for ecdysteroidogenesis

These studies have revealed that *Drosophila* exhibit a remarkable selectivity in their choice of sterol substrates for ecdysteroidogenesis. Cholesterol is used preferentially for production of 20E even when present at 10,000-fold lower concentration than other sterols. Furthermore, flies preferentially utilize the ergosterol precursor 24(28)-dehydroergosterol to synthesize dhMaA even in the presence of an overwhelming excess of ergosterol and plant sterols. Which mechanisms might underlie this specificity? It seems clear that more efficient dietary uptake of cholesterol and 24(28)-dehydroergosterol cannot completely account for their biased utilization. Although *Drosophila* larvae do enrich for some sterols over others, tissues of larvae fed with plant food or yeast food accumulate predominantly plant sterols and ergosterol, respectively. Levels of cholesterol and dehydroergosterol in these animals are below detection limits (Carvalho et al., 2012). Indeed, when animals are forced to accumulate the non-native cholesterol in cell membranes, growth rate and body size is reduced. The fact that cholesterol and 24(28)-dehydroergosterol are not correspondingly enriched in the animal overall suggests that they must interact much more efficiently with transporters or enzymes dedicated to ecdysteroid biosynthesis in the prothoracic gland. Interestingly, when larvae on complex diets produce multiple ecdysteroids, all of them appear and disappear with indistinguishable kinetics. This may suggest that sterols compete for selection at an early step in the pathway, but that subsequently they are processed with similar efficiencies. It would be interesting to examine the affinities of these different sterols for transporters such as NPC1a and StAR (Huang et al., 2005, 2007).

What sort of selective pressures might have driven the preference for cholesterol and 24(28)-dehydroergosterol over other sterols? In the wild, *Drosophila* feed on a mixture of yeast and plant material that is undergoing fermentation. Although 24(28)-dehydroergosterol is likely to be available in small amounts from yeast, there is no apparent source of cholesterol in this environment. Thus, it is difficult to understand how the ability to utilize cholesterol preferentially could have been selected in the wild. Furthermore, *in vitro* studies utilizing reporter assays for ecdysone receptor activity show that 20E is less than twofold more active than MaA (Clément et al., 1993), and we have shown that animals producing exclusively MaA or 20E have indistinguishable rates of growth, pupariation and emergence. Thus, it is not obvious what difference in their function could have driven the selective use of cholesterol for ecdysteroid production. By contrast, we have shown that the most abundant sterol in yeast, ergosterol, is converted to 24-*epi*-MaA, a hormone that does not support development after larval stages. However, yeast also contain much lower levels of 24(28)-dehydroergosterol, which flies use to produce dhMaA, a potent ecdysteroid that fully supports development through adulthood.

Clearly, this difference could have driven the emergence of mechanisms that allow the preferential utilization of 24(28)-dehydroergosterols. Reduced incorporation of sterols containing methyl or *epi*-methyl groups in favor of ‘flat’ achiral C-24 methylene may incidentally have promoted selective utilization of cholesterol as well.

Ecdysteroidogenesis and development control

Ecdysteroids have been primarily thought of as hormones controlling developmental transitions and fertility. However, our findings suggest that they may also have more basic physiological functions. We have noted surprising diet-dependent differences in levels of 20-hydroxylated ecdysteroids present at pupariation, even between diets that all support normal rates of pupariation. Whereas ecdysteroid concentrations vary less than 20% between individual white prepupae fed the same diet, they vary over tenfold when larvae are fed different diets. On normal laboratory food, white prepupae contain >300 pg/animal. However, <25 pg/animal is sufficient to induce pupariation when larvae are fed on a lipid-depleted diet supplemented with a variety of single sterols. As these diets contain similar numbers of calories (Brankatschk et al., 2014), this seems to suggest that larvae produce higher levels of 20E ecdysteroids when nutritional lipids are available. Therefore, it will be interesting to investigate whether insect ecdysteroids have metabolic functions like mammalian steroid hormones. The quantitative tools we have developed here will allow us to address these and other emerging questions in ecdysteroid physiology.

MATERIALS AND METHODS

Chemicals and standards

20-Hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, polypodine B and Girard T reagent [1-(carboxymethyl)trimethylammonium chloride hydrazide] were purchased from Sigma-Aldrich Chemie (Munich, Germany); ecdysone from A. G. Scientific (Göttingen, Germany); muristerone A from Merck Biosciences (Bad Soden, Germany); makisterone A and ponasterone A from Enzo Life Sciences (Lörrach, Germany); and ecdysteroid conjugates were from various sources (Table S2). All solvents were LC-MS grade. Acronyms of ecdysteroids names are in accordance with current guidelines (Lafont et al., 1993).

Growing and collection of larvae

Drosophila melanogaster WT (Oregon-K) embryos were treated as described (Carvalho et al., 2012, 2010). Dechorionated embryos were transferred to plates containing standard lab food consisting of malt, soy, cornmeal and yeast. Embryos, larvae or pupa were collected at specific time points, washed twice with water and frozen in liquid nitrogen and stored at -80°C .

For time course experiments, groups of animals were collected in triplicate at 4 h intervals immediately after the beginning of embryonic stage and up to adult eclosion (Table S4).

For the detailed time course experiments (Fig. S3B), third larval instar *phm-Gal4/+* (Ono et al., 2006) animals were staged at the second to third instar transition and collected in triplicate at 4 h intervals up to early pupal stage.

Identification and quantification of ecdysteroids by LC-MS/MS

Frozen animals were smashed in 1.5 ml plastic tubes (Eppendorf) with 0.2 ml of cold methanol using plastic pestles attached to a cordless motor; the homogenate was diluted to a total volume of 1 ml and vortexed at 4°C overnight. Then samples were centrifuged for 5 min at 13,400 rpm (12,000 g) and the supernatant was collected. The residual pellet was twice extracted with 1 ml methanol for 40 min. The combined extracts were dried down in a vacuum concentrator, re-dissolved in 1 ml of methanol containing 0.25 pmol MuA (internal standard) and twice extracted with 3 ml hexane for 20 min. The collected lower methanol fraction was dried in a vacuum concentrator and re-dissolved in 180 μl

70% aqueous methanol. Samples were loaded on C18 MicroSpin columns from Nest group (Southborough, MA, USA). Columns were pre-washed with 180 μl methanol and water. Upon sample loading, columns were twice washed with 180 μl 70% methanol and centrifuged for 1 min at 2000 rpm (370 g). Eluates were dried down, re-dissolved in 15% aqueous methanol and analyzed by microflow LC-MS/MS by the methods of MRM on a Vantage or by *t*-SIM on Q Exactive mass spectrometers, both from Thermo Fisher Scientific (Bremen, Germany). Method details and instrument settings are provided in supplementary materials and methods and Tables S1 and S3. Every sample series included a blank sample of equal aliquot of methanol that was processed according the same protocol.

Eversion of *Drosophila* wing discs

The wing disc eversion assay was performed in SSM3 media (Sigma, S3652) supplemented with 5% fetal bovine serum, Penicillin/Streptomycin (Sigma 4333) and 0.2 μM hormone. Wandering third instar larvae were washed in water, surface sterilized for 1–2 min in 70% ethanol, washed again with water and then dissected at room temperature in media lacking hormone. Discs were then transferred to wells of an 8-well glass-bottom μ -Slide (Ibidi 80826) containing media with the specified hormones and imaged simultaneously with an Olympus IX81 microscope using brightfield illumination, a 10 \times UplanSApo phase objective and a Hamamatsu ORCA ER camera.

Rearing larvae on single sterol diets

Lipid-depleted media (LDM) was added to 24-well plates (1 ml to each well) as described (Carvalho et al., 2010). Sterols in ethanol (15 μl of 1 mM stock solutions) were spiked on top of dried media and evaporated under air stream. *Drosophila melanogaster* WT (Oregon-K) embryos were prepared as described by Carvalho et al. (2012, 2010). Dichorionated embryos were transferred to 24-well plates with LD foods spiked with different sterols, allowed to develop to white pupa stage, collected and snap-frozen. Growth rate and developmental timing analyses were performed as described previously (Colombani et al., 2005; Delanoue et al., 2010). The weight of individual adult was determined for 2-day-old male or female animals on a fine-balance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

O.L. developed analytical methods and quantified the ecdysteroidome in all analyzed samples. O.L., J.R. and M.C. collected samples for the developmental time course. J.R. performed developmental, growth and survival analysis of animals reared on different diets. N.A.D. performed the dhMaA functional assay. R.L. provided key compounds and reviewed the data. A.S. and S.E. planned the experiments. O.L., J.R., S.E. and A.S. analyzed the data and wrote the manuscript.

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Supplementary information

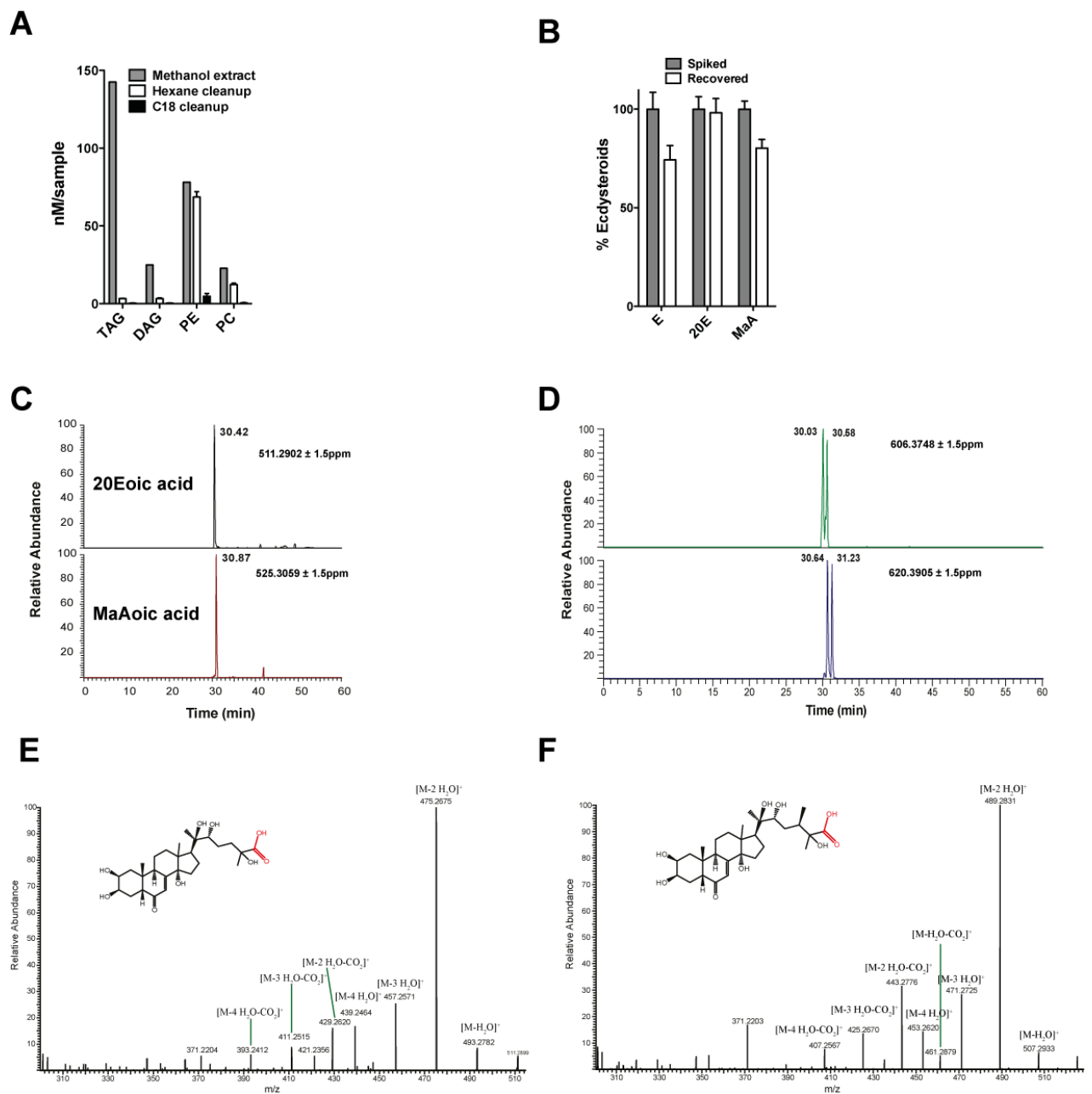
Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.124982/-/DC1>

References

- Aguilar, P. S., Heiman, M. G., Walther, T. C., Engel, A., Schwudke, D., Gushwa, N., Kurzchalia, T. and Walter, P. (2010). Structure of sterol aliphatic chains affects yeast cell shape and cell fusion during mating. *Proc. Natl. Acad. Sci. USA* **107**, 4170–4175.
- Aldaz, S., Escudero, L. M. and Freeman, M. (2010). Live imaging of *Drosophila* imaginal disc development. *Proc. Natl. Acad. Sci. USA* **107**, 14217–14222.

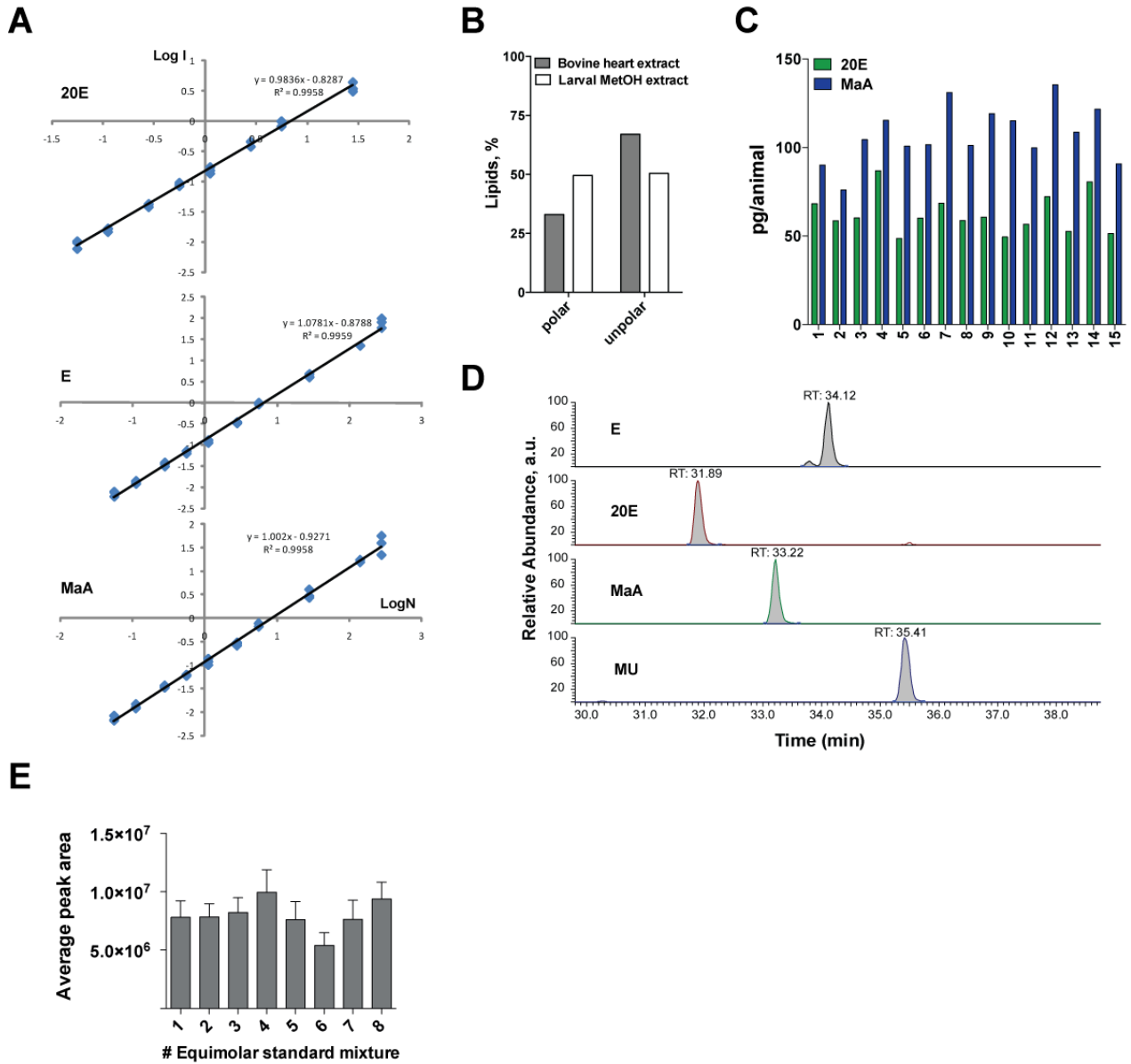
- Báthori, M., Girault, J.-P., Kalasz, H., Mathé, I., Dinan, L. N. and Lafont, R. (1999). Complex phytoecdysteroid cocktail of *Silene otites* (Caryophyllaceae). *Arch. Insect Biochem.* **41**, 1–8.
- Blais, C., Blasco, T., Maria, A., Dauphin-Villemant, C. and Lafont, R. (2010). Characterization of ecdysteroids in *Drosophila melanogaster* by enzyme immunoassay and nano-liquid chromatography–tandem mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **878**, 925–932.
- Bownes, M., Dübendorfer, A. and Smith, T. (1984). Ecdysteroids in adult males and females of *Drosophila melanogaster*. *J. Insect Physiol.* **30**, 823–830.
- Brankatschk, M., Dunst, S., Nemetschke, L. and Eaton, S. (2014). Delivery of circulating lipoproteins to specific neurons in the *Drosophila* brain regulates systemic insulin signaling. *eLife* **3**, e02862.
- Carvalho, M., Schwudke, D., Sampaio, J. L., Palm, W., Riezman, I., Dey, G., Gupta, G. D., Mayor, S., Riezman, H., Shevchenko, A. et al. (2010). Survival strategies of a sterol auxotroph. *Development* **137**, 3675–3685.
- Carvalho, M., Sampaio, J. L., Palm, W., Brankatschk, M., Eaton, S. and Shevchenko, A. (2012). Effects of diet and development on the *Drosophila* lipidome. *Mol. Syst. Biol.* **8**, 600.
- Clément, C. Y., Bradbrook, D. A., Lafont, R. and Dinan, L. (1993). Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiectysteroid activities. *Insect Biochem. Mol. Biol.* **23**, 187–193.
- Colombani, J., Bianchini, L., Layalle, S., Pondeville, E., Dauphin-Villemant, C., Antoniewski, C., Carré, C., Noselli, S. and Léopold, P. (2005). Antagonistic actions of ecdysone and insulins determine final size in *Drosophila*. *Science* **310**, 667–670.
- Daum, G., Lees, N. D., Bard, M. and Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **14**, 1471–1510.
- Delanoue, R., Slaidina, M. and Léopold, P. (2010). The steroid hormone ecdysone controls systemic growth by repressing dMyc function in *Drosophila* fat cells. *Dev. Cell* **18**, 1012–1021.
- Feldlaufer, M. F., Weirich, G. F., Lusby, W. R. and Svoboda, J. A. (1991). Makisterone C: a 29-carbon ecdysteroid from developing embryos of the cotton stainer bug, *Dysdercus fasciatus*. *Arch. Insect Biochem. Physiol.* **18**, 71–79.
- Feldlaufer, M. F., Weirich, G. F., Imberski, R. B. and Svoboda, J. A. (1995). Ecdysteroid production in *Drosophila melanogaster* reared on defined diets. *Insect Biochem. Mol. Biol.* **25**, 709–712.
- Girard, A. and Sandulesco, G. (1936). On a new series of reactants of the carbonyl group, their use for the extraction of ketonic substances and for the microchemical characterisation of aldehydes and ketones. *Helv. Chim. Acta* **19**, 1095–1107.
- Grau, V. and Lafont, R. (1994). Metabolism of ecdysone and 20-hydroxyecdysone in adult *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **24**, 49–58.
- Guittard, E., Blais, C., Maria, A., Parvy, J.-P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P. J. and Dauphin-Villemant, C. (2011). CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis. *Dev. Biol.* **349**, 35–45.
- Handler, A. M. (1982). Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster*. *Dev. Biol.* **93**, 73–82.
- Hikiba, J., Oghihara, M. H., Iga, M., Saito, K., Fujimoto, Y., Suzuki, M. and Kataoka, H. (2013). Simultaneous quantification of individual intermediate steroids in silkworm ecdysone biosynthesis by liquid chromatography–tandem mass spectrometry with multiple reaction monitoring. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **915–916**, 52–56.
- Huang, X., Suyama, K., Buchanan, J., Zhu, A. J. and Scott, M. P. (2005). A *Drosophila* model of the Niemann-Pick type C lysosome storage disease: *dnpc1a* is required for molting and sterol homeostasis. *Development* **132**, 5115–5124.
- Huang, X., Warren, J. T., Buchanan, J., Gilbert, L. I. and Scott, M. P. (2007). *Drosophila* Niemann-Pick type C-2 genes control sterol homeostasis and steroid biosynthesis: a model of human neurodegenerative disease. *Development* **134**, 3733–3742.
- Karu, K., Hornshaw, M., Woffendin, G., Bodin, K., Hamberg, M., Alvelius, G., Sjøvall, J., Turton, J., Wang, Y. and Griffiths, W. J. (2007). Liquid chromatography–mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols. *J. Lipid Res.* **48**, 976–987.
- Kozlova, T. and Thummel, C. S. (2000). Steroid regulation of postembryonic development and reproduction in *Drosophila*. *Trends Endocrinol. Metab.* **11**, 276–280.
- Kumpun, S., Maria, A., Crouzet, S., Evrard-Todeschi, N., Girault, J.-P. and Lafont, R. (2011). Ecdysteroids from *Chenopodium quinoa* Willd., an ancient Andean crop of high nutritional value. *Food Chem.* **125**, 1226–1234.
- Lafont, R. and Koolman, J. (2009). Diversity of ecdysteroids in animal species. In *Ecdysone: Structures and Functions* (ed. G. Smagghe), pp. 47–72. New York: Springer.
- Lafont, R., Koolman, J. and Rees, H. (1993). Standardized abbreviations for common ecdysteroids. *Insect Biochem. Mol. Biol.* **23**, 207–209.
- Lafont, R., Dauphin-Villemant, C., Warren, J. T. and Rees, H. (2012). Ecdysteroid chemistry and biochemistry. In *Insect Endocrinology* (ed. L. I. Gilbert), pp. 106–176. London, MA: Elsevier/Academic Press.
- Lavrynenko, O., Nedielkov, R., Moller, H. M. and Shevchenko, A. (2013). Girard derivatization for LC-MS/MS profiling of endogenous ecdysteroids in *Drosophila*. *J. Lipid Res.* **54**, 2265–2272.
- Li, Y., Warren, J. T., Boysen, G., Gilbert, L. I., Gold, A., Sangaiah, R., Ball, L. M. and Swenberg, J. A. (2006). Profiling of ecdysteroids in complex biological samples using liquid chromatography/ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **20**, 185–192.
- Mauchamp, B., Royer, C., Kerhoas, L. and Einhorn, J. (1993). MS/MS analyses of ecdysteroids in developing eggs of *Dysdercus fasciatus*. *Insect Biochem. Mol. Biol.* **23**, 199–205.
- Maurer, P., Girault, J.-P., Larchevéque, M. and Lafont, R. (1993). 24-Epi-makisterone A (not makisterone A) is the major ecdysteroid in the leaf-cutting ant *Acromyrmex octospinosus* (Reich) (hymenoptera, formicidae: Attini). *Arch. Insect Biochem. Physiol.* **23**, 29–35.
- Miyashita, M., Matsushita, K., Nakamura, S., Akahane, S., Nakagawa, Y. and Miyagawa, H. (2011). LC/MS/MS identification of 20-hydroxyecdysone in a scorpion (*Liocheles australasiae*) and its binding affinity to in vitro-translated molting hormone receptors. *Insect Biochem. Mol. Biol.* **41**, 932–937.
- Ono, H., Rewitz, K. F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J. T., Marques, G., Shimell, M. J. et al. (2006). Spook and Spookier code for stage-specific components of the ecdysone biosynthetic pathway in Diptera. *Dev. Biol.* **298**, 555–570.
- Ou, Q., Magico, A. and King-Jones, K. (2011). Nuclear receptor DHR4 controls the timing of steroid hormone pulses during *Drosophila* development. *PLoS Biol.* **9**, e1001160.
- Padmanabha, D. and Baker, K. D. (2014). *Drosophila* gains traction as a repurposed tool to investigate metabolism. *Trends Endocrinol. Metab.* **25**, 518–527.
- Parvy, J.-P., Blais, C., Bernard, F., Warren, J. T., Petryk, A., Gilbert, L. I., O'Connor, M. B. and Dauphin-Villemant, C. (2005). A role for betaFTZ-F1 in regulating ecdysteroid titers during post-embryonic development in *Drosophila melanogaster*. *Dev. Biol.* **282**, 84–94.
- Petryk, A., Warren, J. T., Marques, G., Jarcho, M. P., Gilbert, L. I., Kahler, J., Parvy, J.-P., Li, Y., Dauphin-Villemant, C. and O'Connor, M. B. (2003). Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl. Acad. Sci. USA* **100**, 13773–13778.
- Ravi, M., Hopfinger, A. J., Hormann, R. E. and Dinan, L. (2001). 4D-QSAR analysis of a set of ecdysteroids and a comparison to CoMFA modeling. *J. Chem. Inf. Comput. Model.* **41**, 1587–1604.
- Redfern, C. P. F. (1986). Changes in patterns of ecdysteroid secretion by the ring gland of *Drosophila* in relation to the sterol composition of the diet. *Experientia* **42**, 307–309.
- Riddiford, L. M. and Truman, J. W. (1993). Hormone receptors and the regulation of insect metamorphosis. *Receptor* **3**, 203–209.
- Schwedes, C. C. and Carney, G. E. (2012). Ecdysone signaling in adult *Drosophila melanogaster*. *J. Insect Physiol.* **58**, 293–302.
- Shim, J., Gururaja-Rao, S. and Banerjee, U. (2013). Nutritional regulation of stem and progenitor cells in *Drosophila*. *Development* **140**, 4647–4656.
- Tennessen, J. M. and Thummel, C. S. (2011). Coordinating growth and maturation – insights from *Drosophila*. *Curr. Biol.* **21**, R750–R757.
- Thuy, T. T., Porzel, A., Ripberger, H., van Sung, T. and Adam, G. (1998). Chalcones and ecdysteroids from *Vitex leptobotrys*. *Phytochemistry* **49**, 2603–2605.
- Warren, J. T., Yerushalmi, Y., Shimell, M. J., O'Connor, M. B., Restifo, L. L. and Gilbert, L. I. (2006). Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: Correlations with changes in gene activity. *Dev. Dyn.* **235**, 315–326.
- Wessner, M., Champion, B., Girault, J.-P., Kaouadji, N., Saidi, B. and Lafont, R. (1992). Ecdysteroids from *Ajuga lva*. *Phytochemistry* **31**, 3785–3788.

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-axis. **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 CO₂.

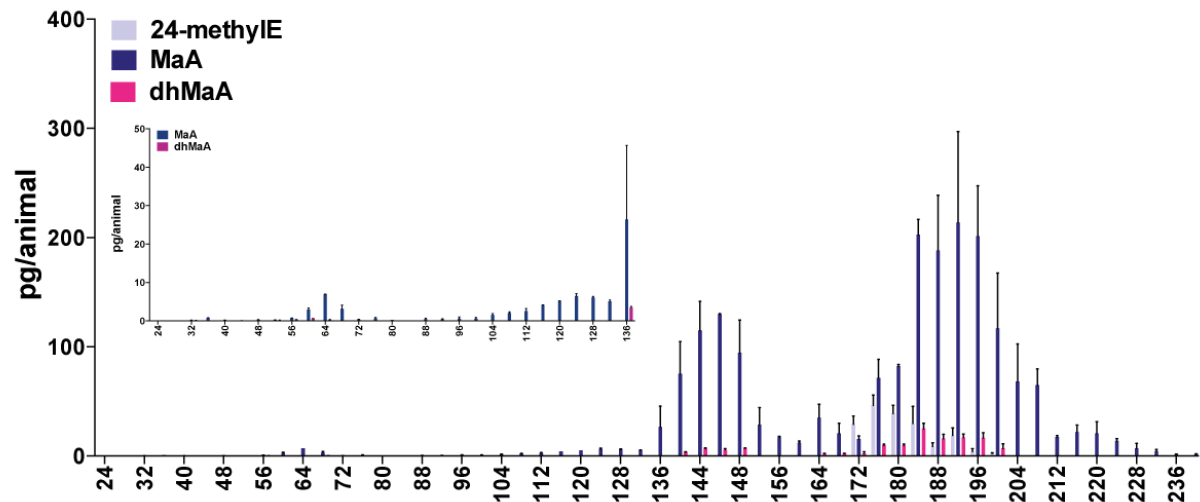
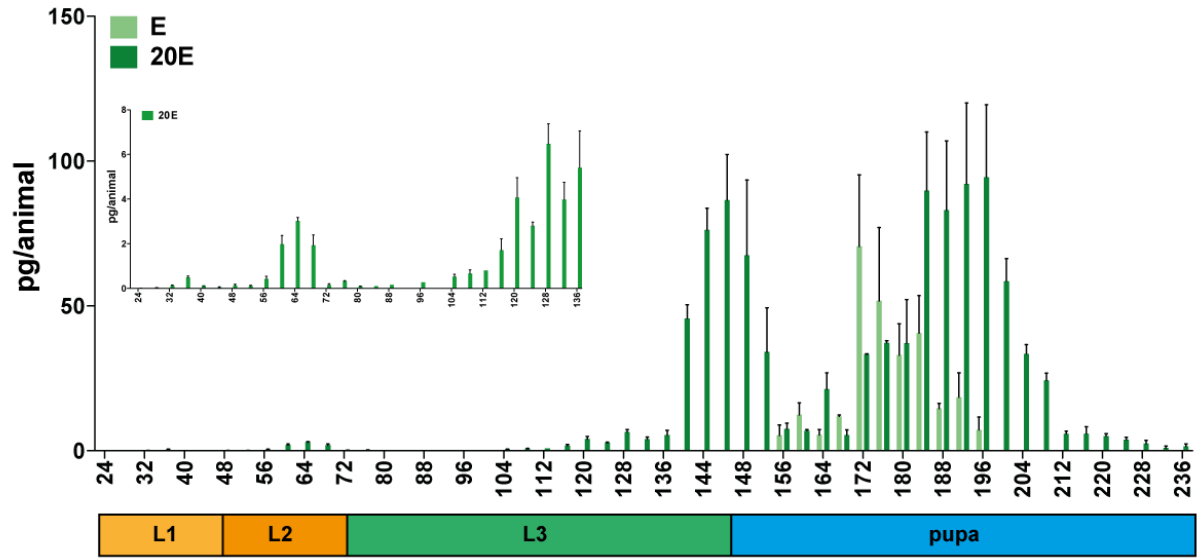
Supplementary Figure 2.



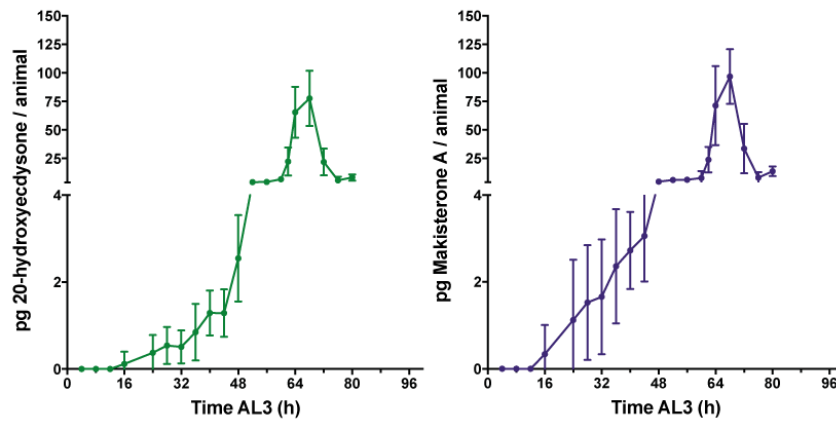
Supplementary Figure 2 Quantification of endogenous ecdysteroids. **A**: calibration curves for 20E, E and MaA obtained by the method of MRM on a triple quadrupole mass spectrometer. For calibration ecdysteroid standards were mixed into one million fold excess of polar lipid extract from bovine brain 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.

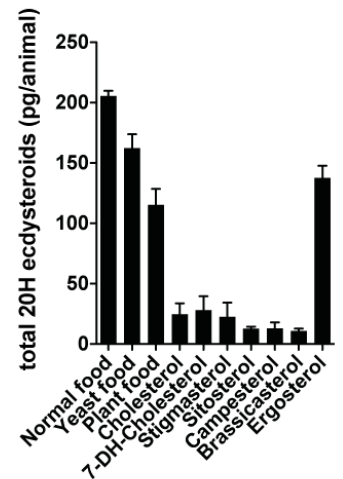
A



B



C



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 ₂ O+H] ⁺	GiT ¹	GiT-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/MS¹

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

¹hours after embryos hatching

²the 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, 1 μ L, 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 5 μ m from Agilent. The mobile phase delivered at the flow rate of 10 μ l/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 for 10 min until 30 μ l 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 μ L 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 50⁰C; transfer capillary temperature 200⁰C. 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 μ l of the methanol extracts were re-dissolved in 45 μ l of 5 mM ammonium acetate in *iso*-propanol/methanol/chloroform 4/2/1 (v/v/v) containing 0.56 μ M 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.

SUPPLEMENTARY REFERENCES

Carvalho, M., Sampaio, J. L., Palm, W., Brankatschk, M., Eaton, S. and Shevchenko, A. (2012). Effects of diet and development on the *Drosophila* lipidome. *Mol Syst Biol* **8**, 600.

Dinan, L. (1988). The chemical synthesis of ecdysone 22-long-chain fatty acyl esters in high yield. *J Steroid Biochem* **31**, 237-245.

Folch, J. M., Lees, M., Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226**, 497-509.

Herzog, R., Schwudke, D., Schuhmann, K., Sampaio, J. L., Bornstein, S. R., Schroeder, M. and Shevchenko, A. (2011). A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. *Genome Biol* **12**, R8.

Ikeda, M., Fujita, T., Naoki, H., Naya, Y., Mamiya, Y., Kamba, M. and Sonobe, H. (1995). Charge-remote fragmentation of ecdysteroids conjugated with phosphoric acid. *Rapid Commun Mass Spectrom* **9**, 1480-1483.

Keller, B. O., Sui, J., Young, A. B. and Whittal, R. M. (2008). Interferences and contaminants encountered in modern mass spectrometry. *Anal Chim Acta* **627**, 71-81.

Pis, J., Girault, J. P., Grau, V. and Lafont, R. (1995). Analysis of ecdysteroid conjugates: chromatographic characterization of sulfates, phosphates and glucosides *Eur J Entomol* **92**, 41-50.