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Figures and figure supplements

Sex-specific triacylglycerides are widely conserved in Drosophila and mediate mating behavior

Jacqueline SR Chin, et al.

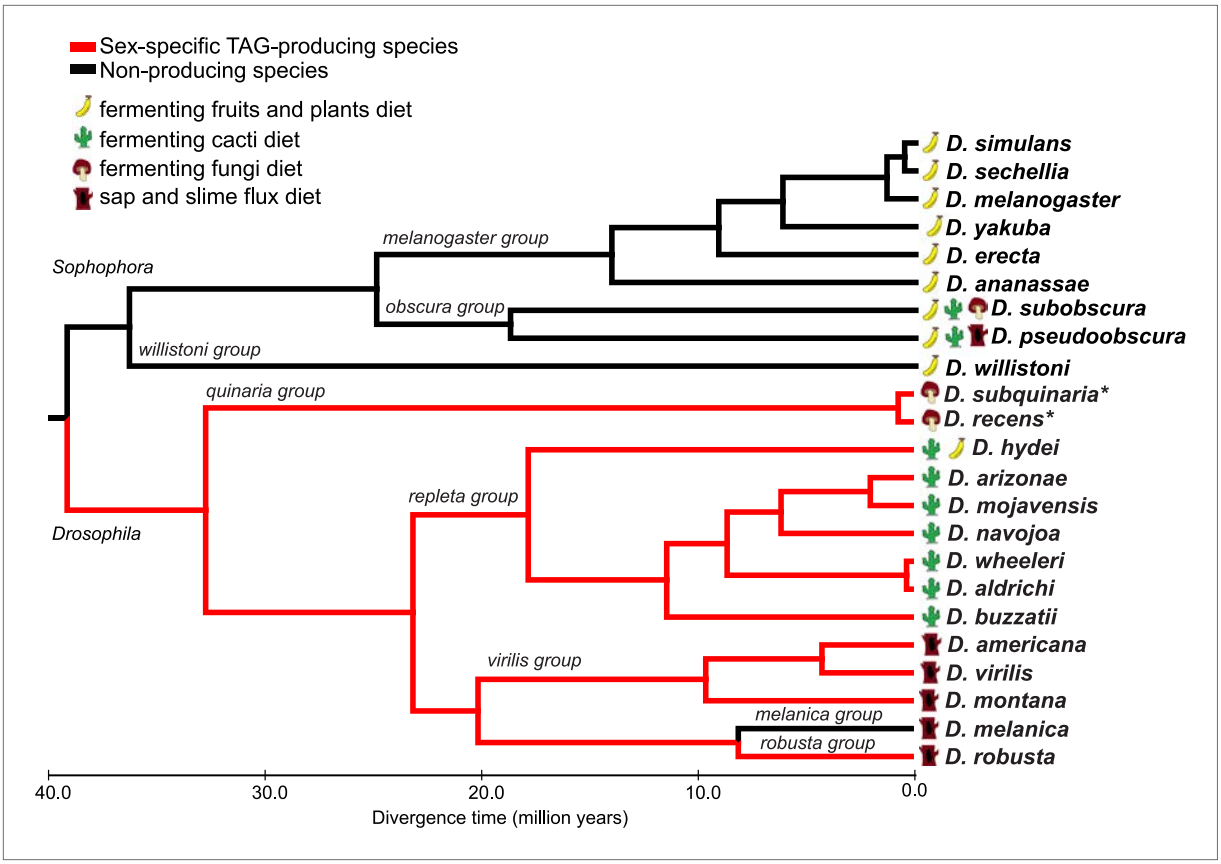

Figure 1. Male-specific TAG expression is broadly conserved across the *Drosophila* subgenus and not found in species from *Sophophora*. The primary diets of each species are indicated, based on the previous studies. Branches for TAG-producing species are colored in red. Branch lengths are proportional to evolutionary time. *Evidence for TAG-expression is based on ***Curtis et al., 2013***.

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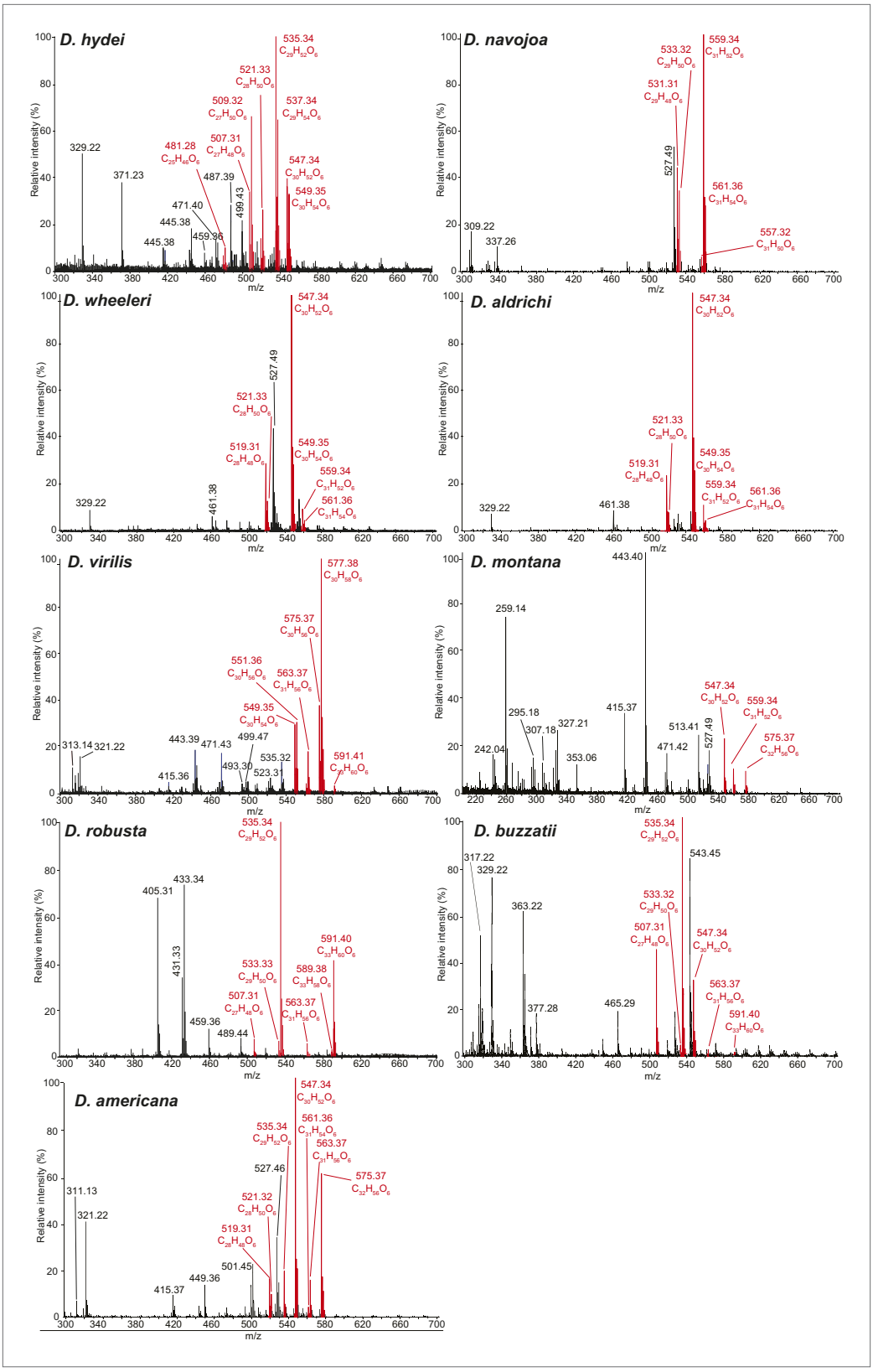

Figure 1—figure supplement 1. Representative UV-LDI spectra from distantly related drosophilids in the *Drosophila* subgenus.

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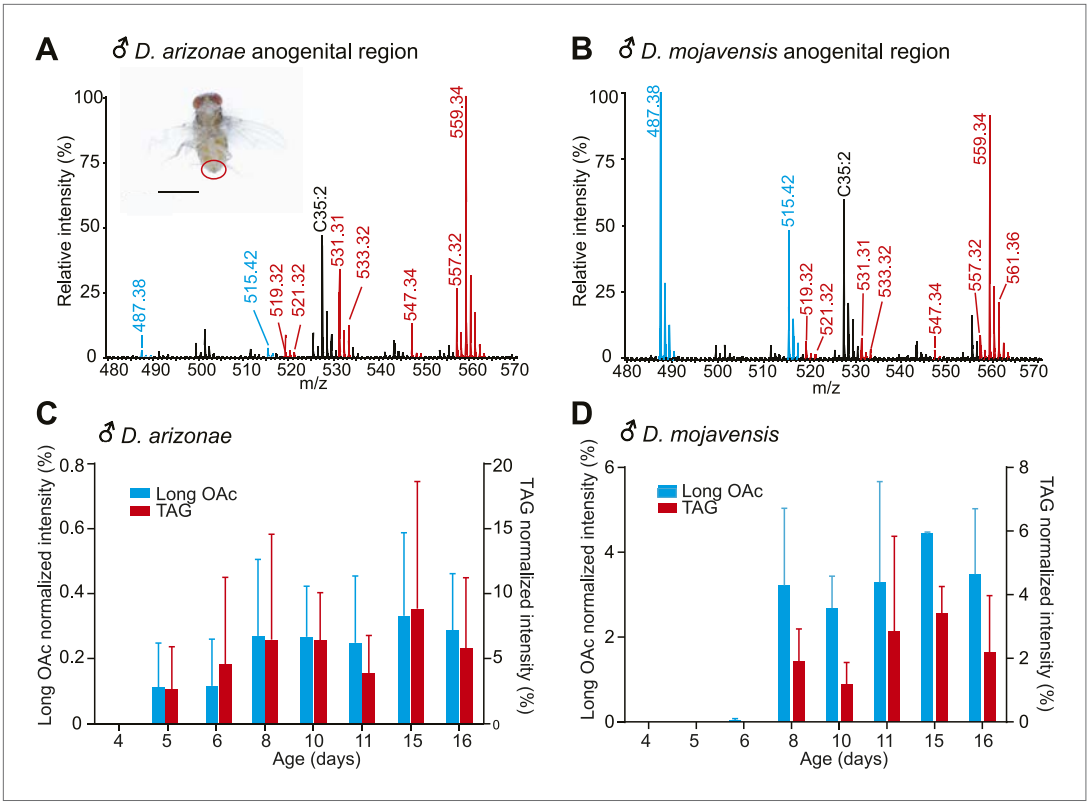

Figure 2. Pheromone profiles and age-related increase in sex-specific TAGs. (**A** and **B**) UV-LDI MS allows spatially resolved detection of high molecular weight lipids directly from intact insects, with minimal damage to the cuticle. Representative mass spectra from the anogenital region (inset) of *D. arizonae* and *D. mojavensis* males show signals corresponding to triglycerides (TAGs, red) and long chain alkadienyl acetates (long OAcs, blue). The hydrocarbon C35:2 (number of carbons: number of double bonds) is found on cuticles of males and females. Labeled signals correspond to potassiated molecules $[M + K]^+$. Scale bar: 1 mm. (**C** and **D**) Relative intensity of TAGs and long OAcs on male *D. arizonae* and *D. mojavensis*, respectively. TAGs and long OAcs increase with age, with trace quantities first appearing at 4 day old. The signal intensity for all detected TAGs or long OAcs was normalized to the signal intensity of C35:2.

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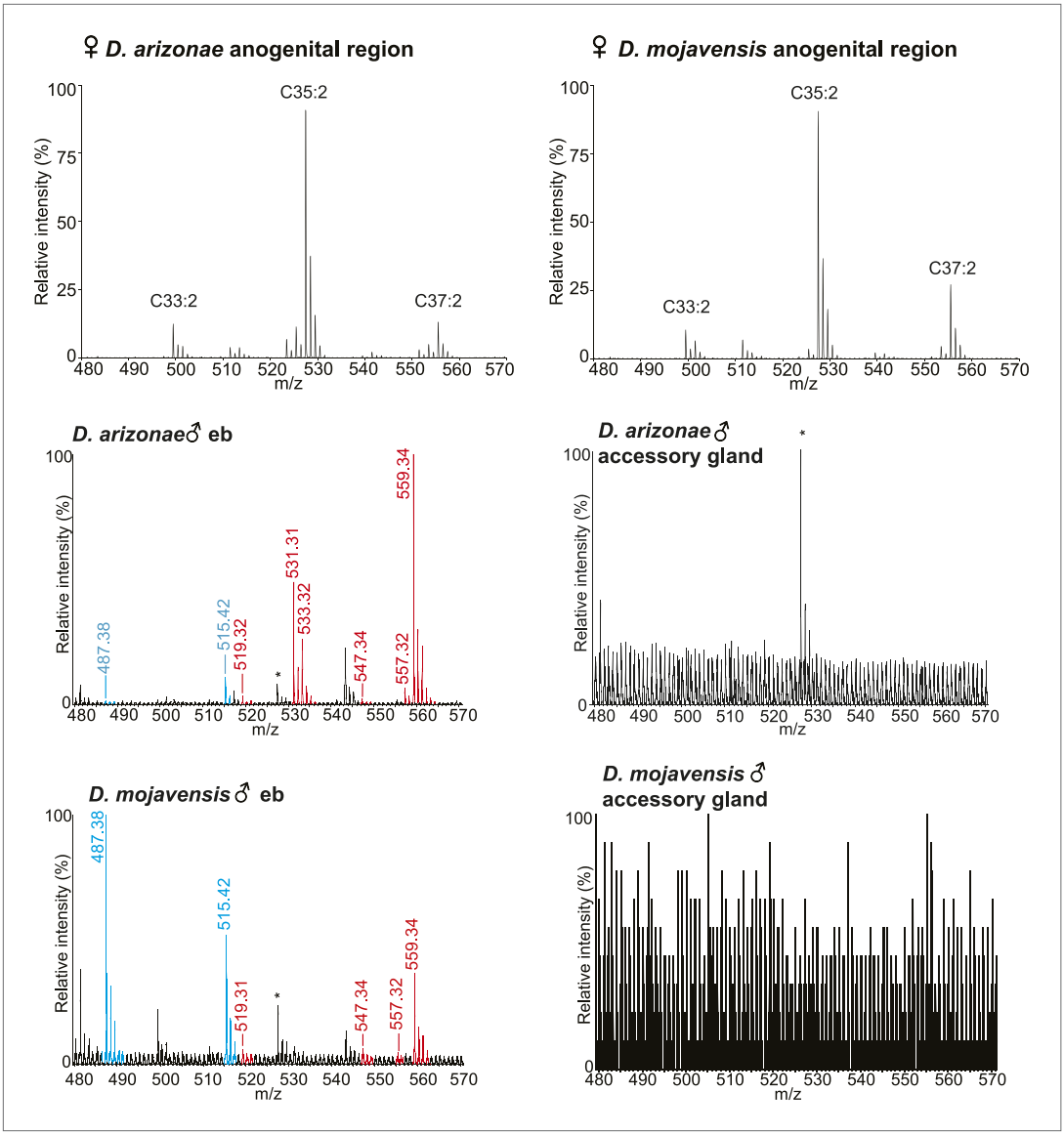

Figure 2—figure supplement 1. UV-LDI MS profiles from the anogenital region of virgin females and dissected ejaculatory bulb (eb) and accessory glands.

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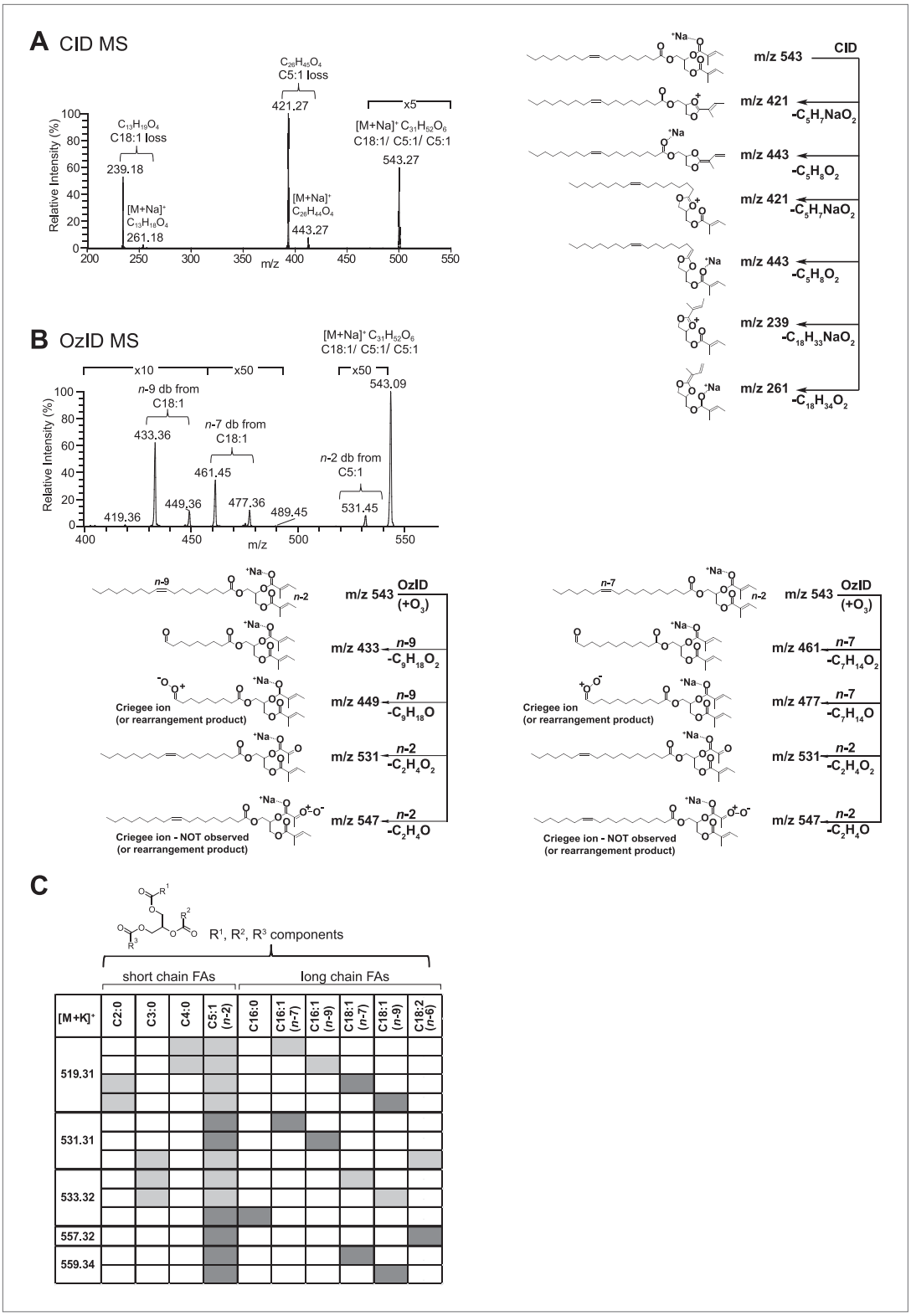

Figure 3. Structural elucidation of sex-specific TAGs. (**A**) The low energy collision-induced dissociation (CID) mass spectrum of a TAG-related signal from crude *D. arizonae* extract ([M + Na]$^+$ 543) shows fragments corresponding to losses of a 5 carbon fatty acid with a single double bond (C5:1) and an 18 carbon fatty acid with a single double bond (C18:1). Both sodiated (major peak) and protonated chain side losses are observed. The schematic rationalizes the product ions formed during CID of mass-selected [M + Na]$^+$ of unsaturated lipids. (**B**) Ozone-induced

Figure 3. Continued on next page

Figure 3. Continued

dissociation (OzID) of a TAG-related signal (shown in **A**) indicates isomers with variant double bond positions. The fragments at *m/z* 461 and *m/z* 433 are aldehyde products consistent with double bonds (db) at positions *n*-7 and *n*-9, respectively. The fragment at *m/z* 531 confirms the *n*-2 double bond position found in the tiglic acyl component. The corresponding Criegee product ions (*m/z* 477 and *m/z* 449, respectively) are also observed. The schematic rationalizes the product ions formed during OzID of mass-selected $[M + Na]^+$ of unsaturated lipids. Product ions are assigned as outlined by ***Thomas et al., 2008*** and ***Brown et al., 2011***. (**C**) CID and OzID MS analyses of the most abundant sex-specific TAGs reveal significant combinatorial complexity. A generic TAG molecule consisting of a glycerol backbone and 3 fatty acyl (FA) side chains, R1, R2, and R3, is shown. Each TAG species is comprised of 2 short chain and 1 long chain FA component. Shaded boxes indicate the composite side chains of each TAG species. The glycerol backbone positions for several TAGs are assigned based on the comparison with synthetic standards and ion product abundance patterns (dark gray boxes). Ambiguous backbone positions are in light gray.

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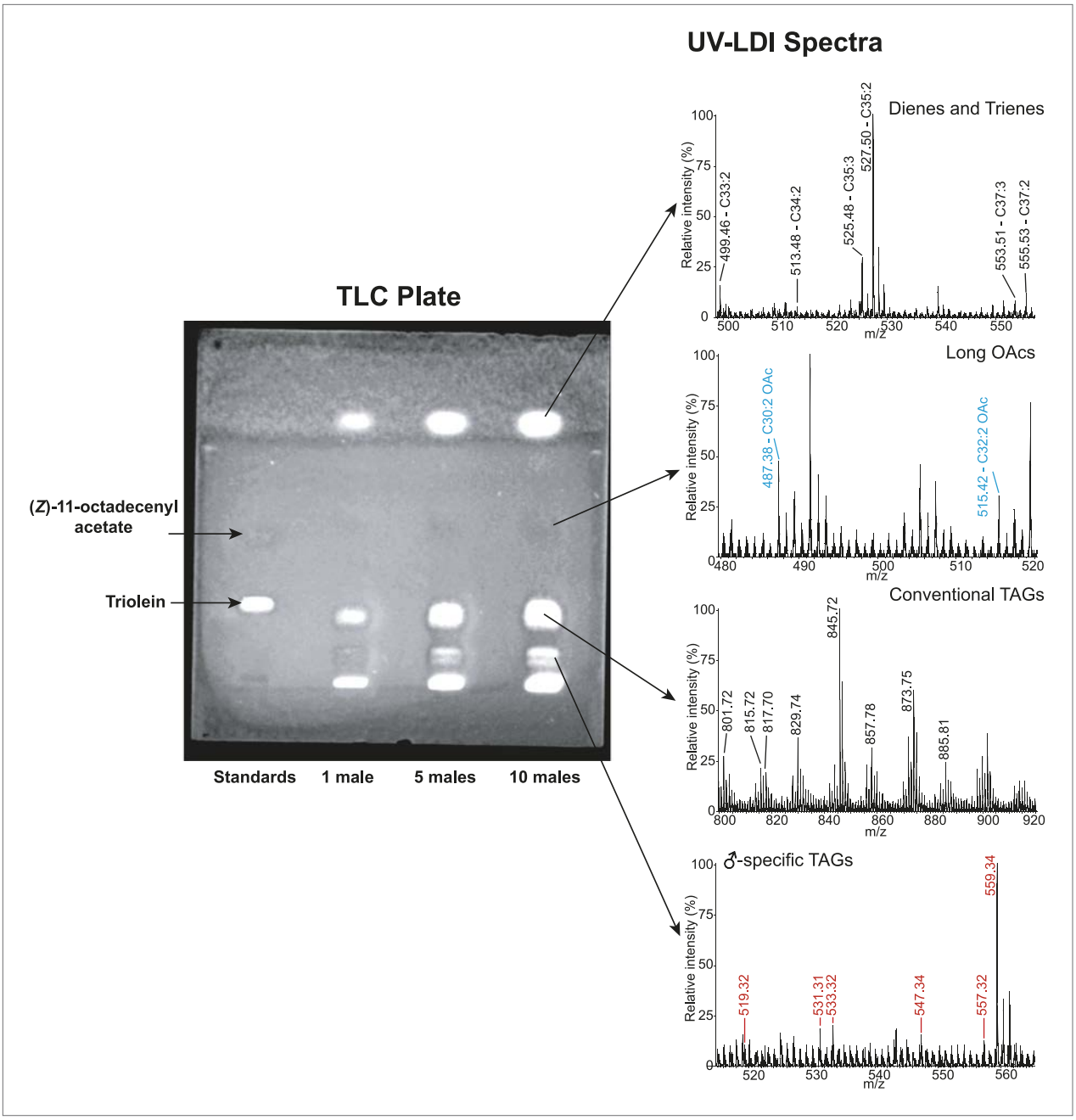

Figure 3—figure supplement 1. Thin layer chromatography (TLC) separation of *D. arizonae* male cuticular lipid extract.

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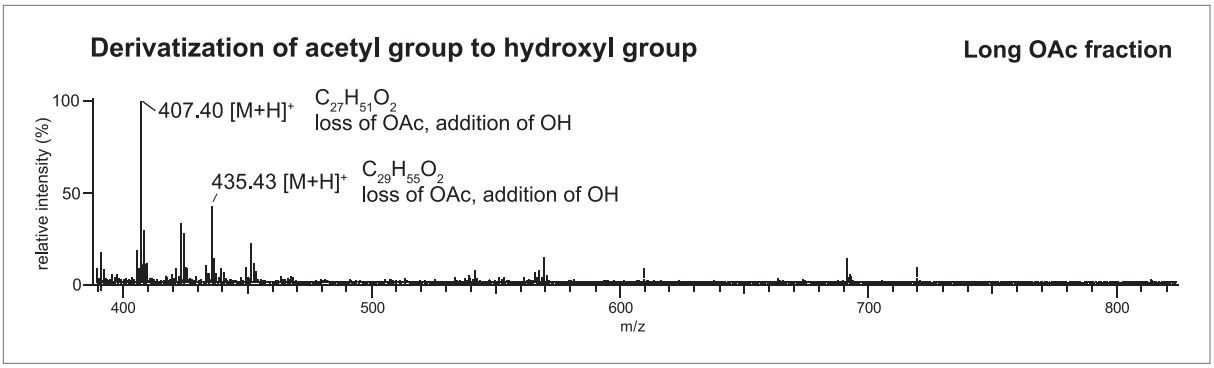

Figure 3—figure supplement 2. Direct analysis in real time (DART) MS spectrum of the TLC fraction containing long OAcs after derivatization by base hydrolysis confirms the presence of an acetyl functional group.

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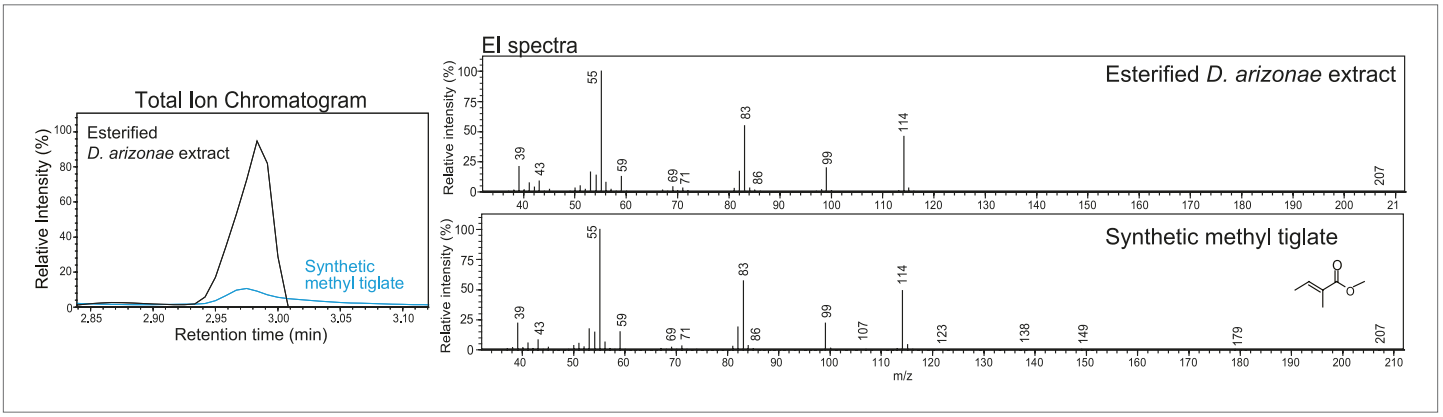

Figure 3—figure supplement 3. Structural elucidation of sex-specific TAGs using gas chromatography MS.

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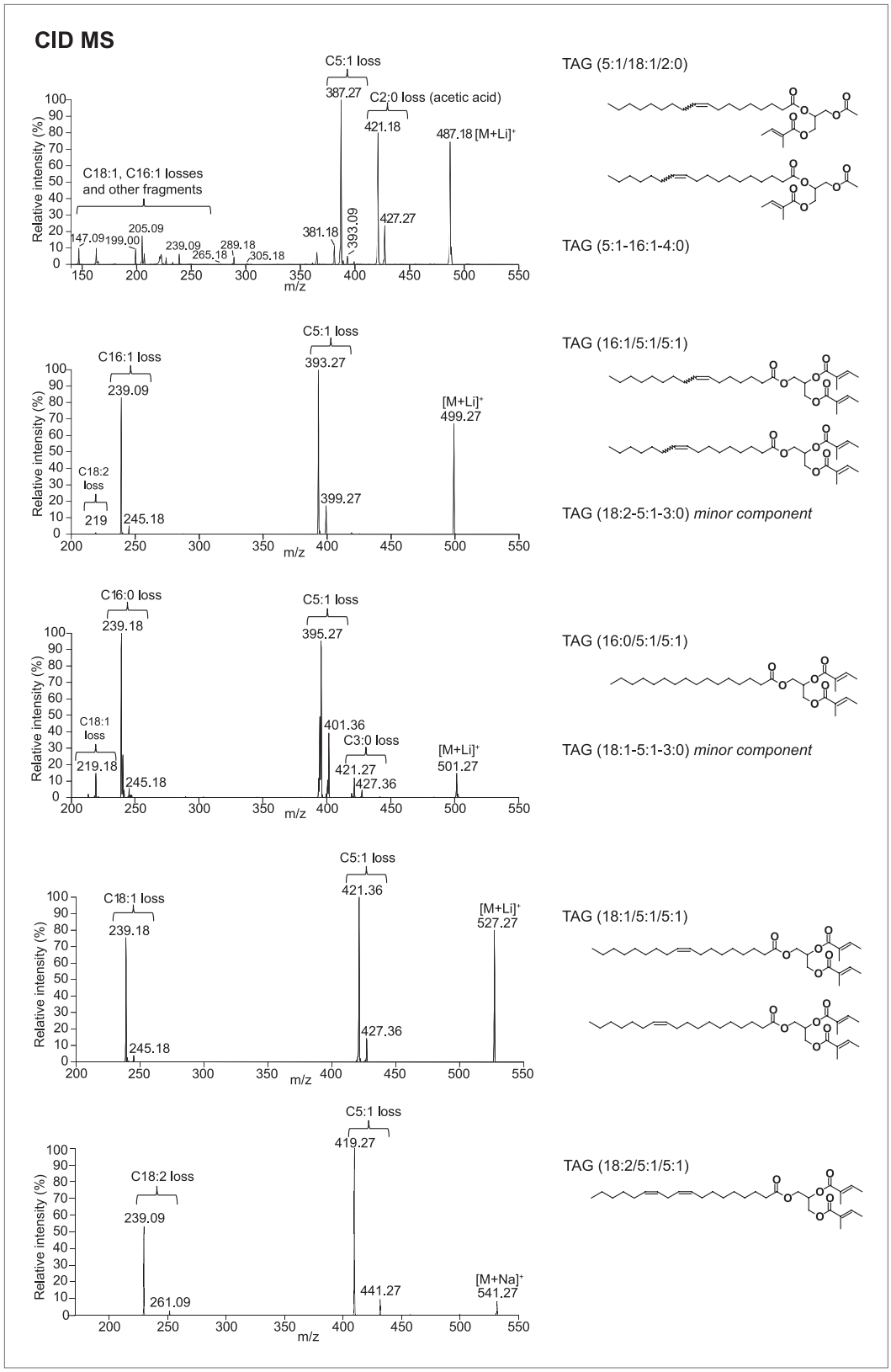

Figure 3—figure supplement 4. Structural elucidation of TAGs by CID MS reveals fatty acid (FA) components with 2, 3, 5, 16, or 18 carbons in length and 0–2 double bonds.

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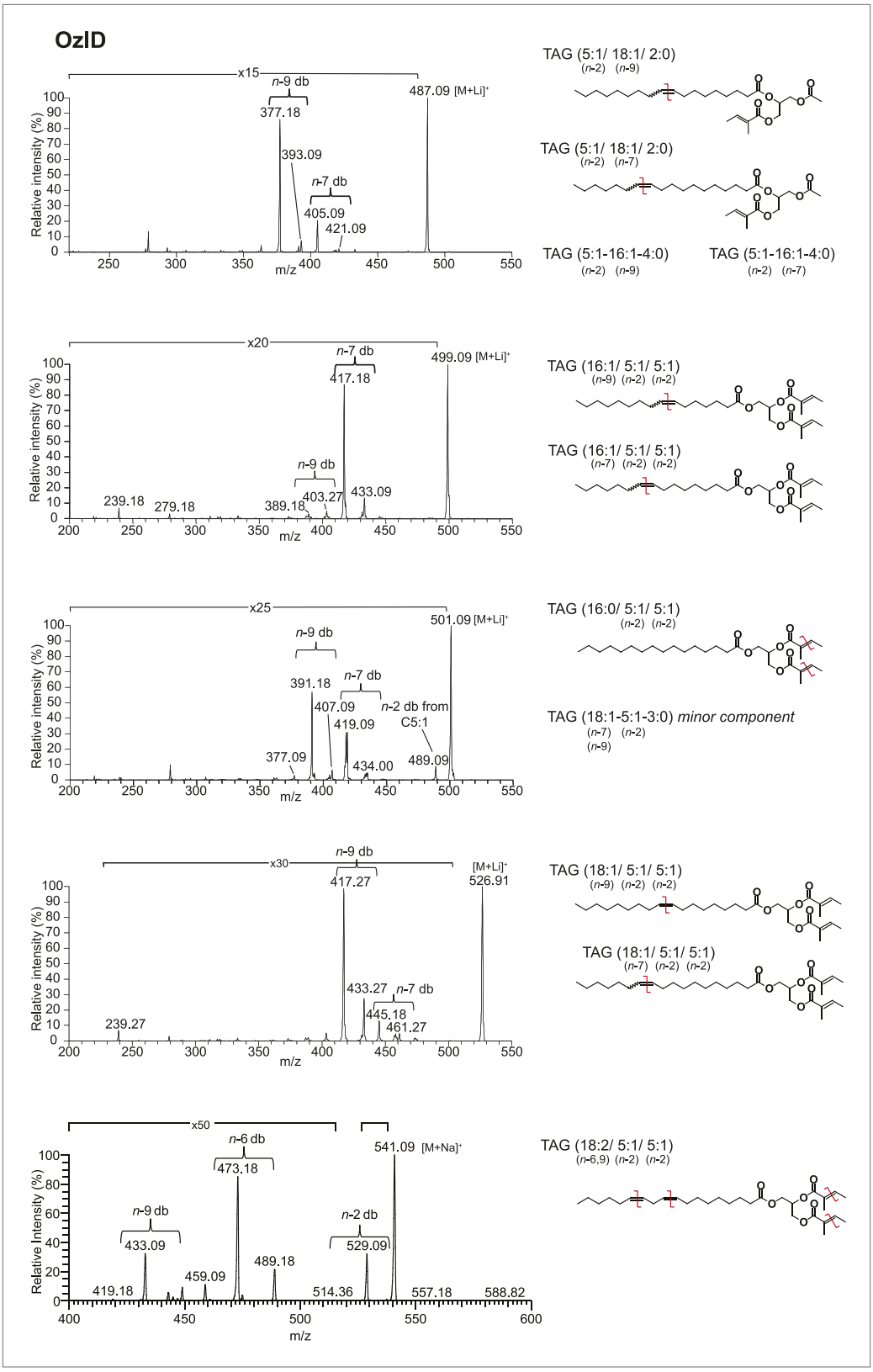

Figure 3—figure supplement 5. Analysis of TAGs by OzID reveals double bond positions of acyl side chains.
DOI: 10.7554/eLife.01751.012

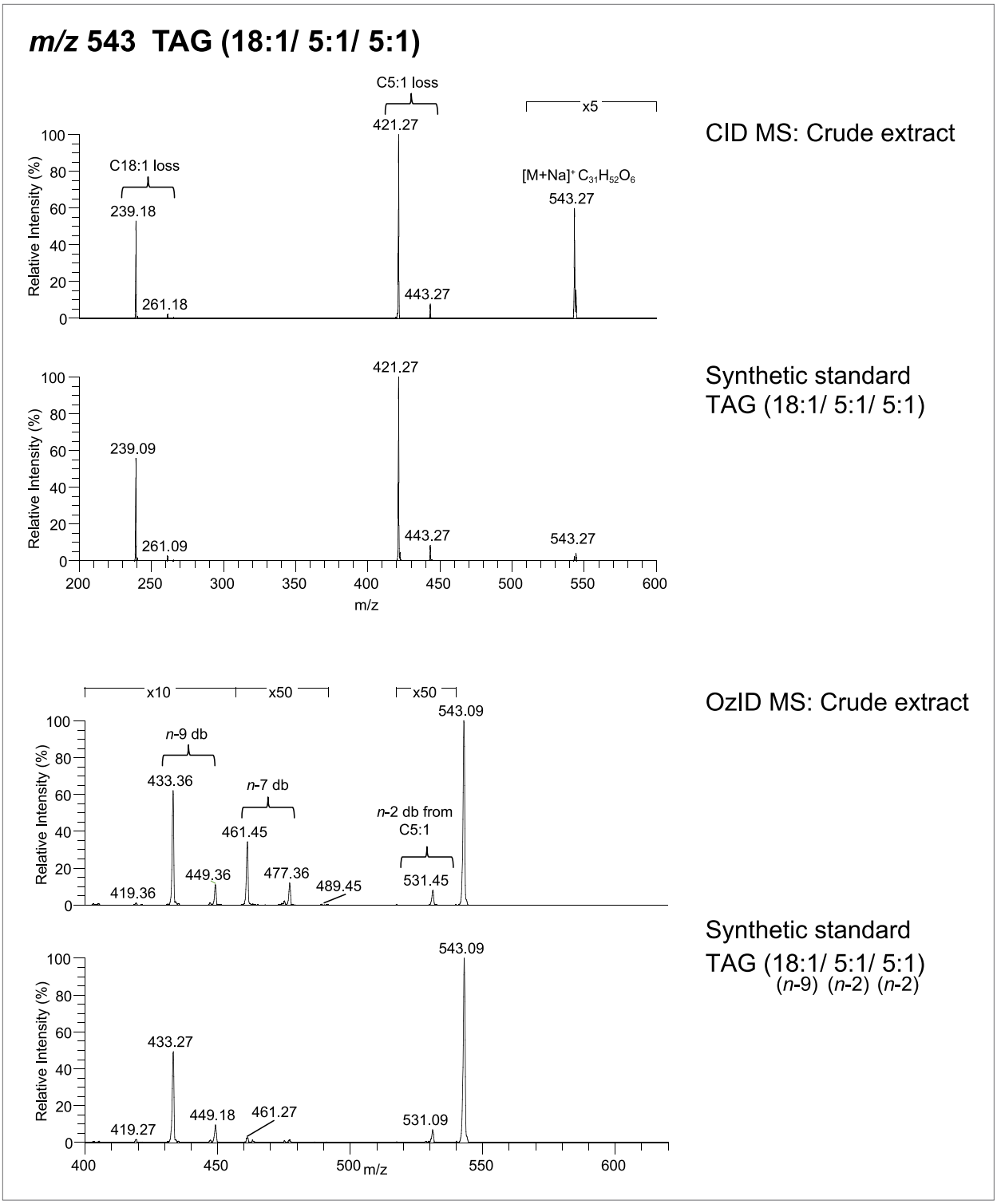

Figure 3—figure supplement 6. Spectra obtained from CID MS and OzID analyses of a synthetic TAG comprised of an oleic acid (*cis*-9-Octadecenoic acid) and tiglic acid side chains are consistent with the analysis of a TAG molecule with identical *m/z* found from crude extract.

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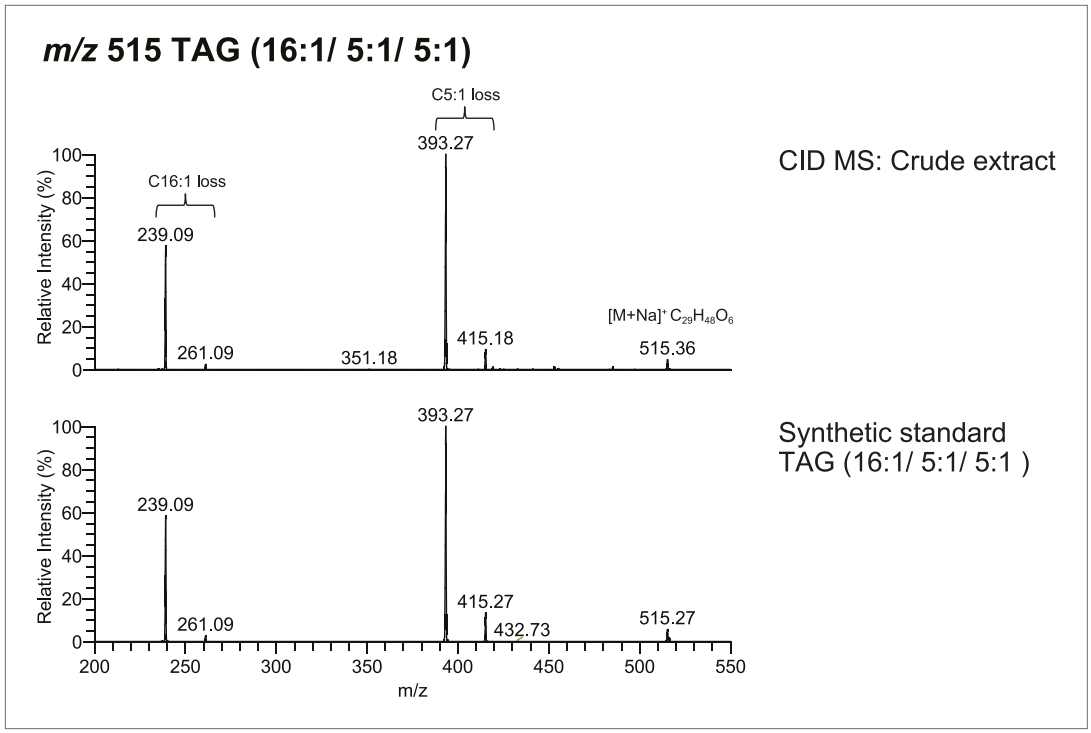

Figure 3—figure supplement 7. The spectrum obtained from CID MS analysis of a synthetic TAG (16:1/5:1/5:1) is consistent with the spectrum from a TAG molecule with identical *m/z* found from crude extract.
DOI: 10.7554/eLife.01751.014

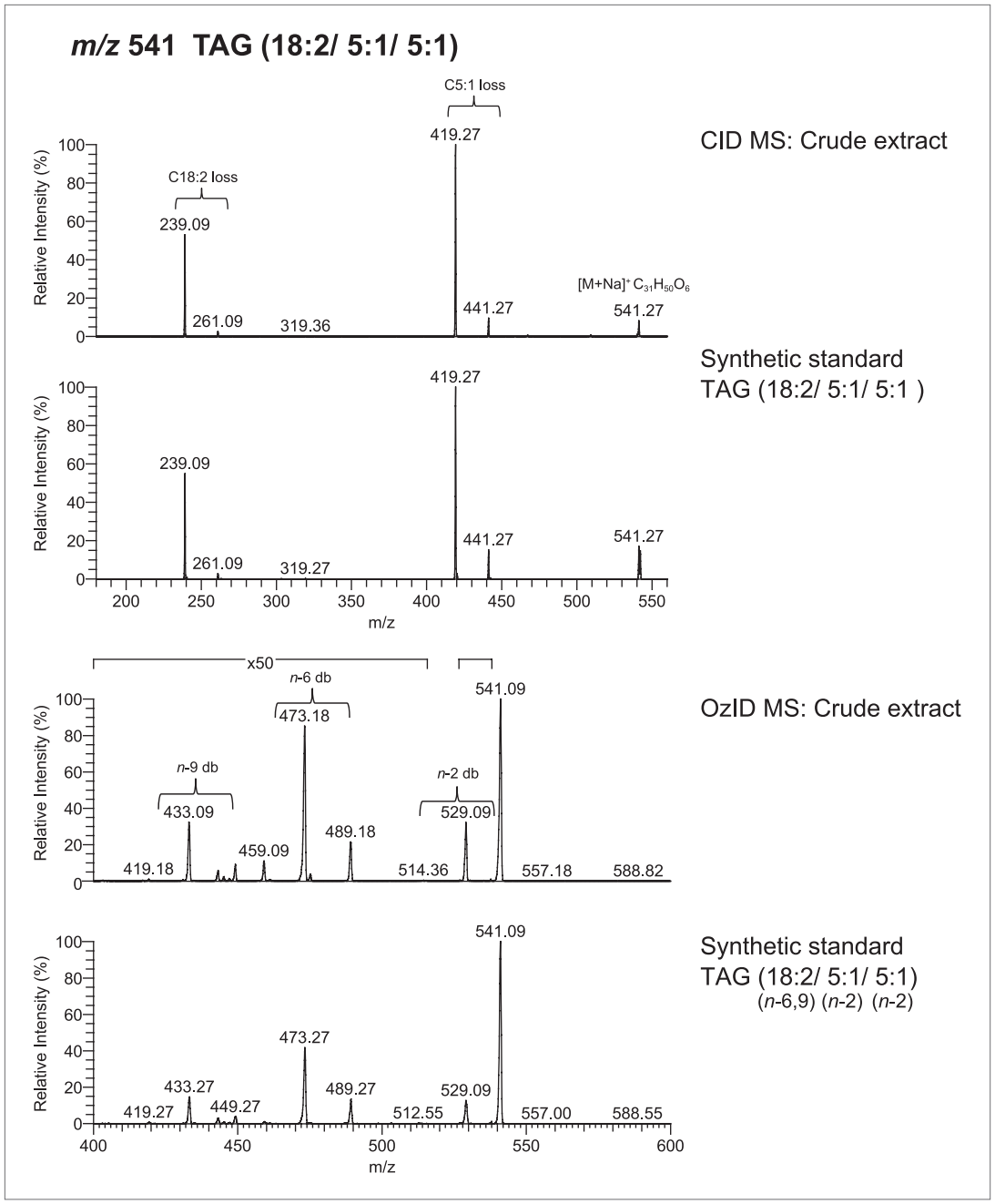

Figure 3—figure supplement 8. Spectra obtained from CID MS and OzID analyses of a synthetic TAG consisting of linoleic acid (*cis, cis*-9,12-Octadecadienoic acid) and tiglic acid side chains are consistent with spectra from analysis of a TAG molecule with identical *m/z* found from crude extract.

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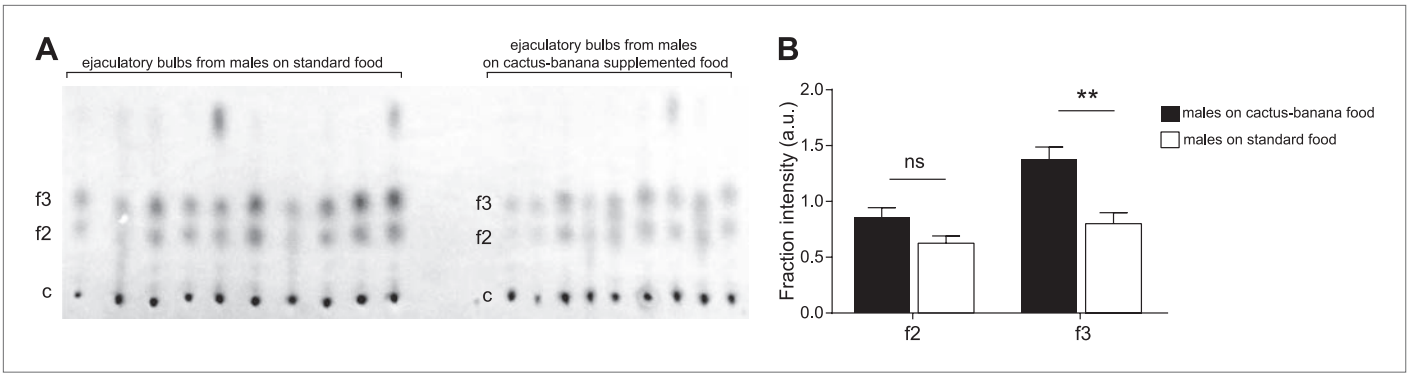

Figure 4. Diet changes the quantity but not composition of sex-specific TAGs. (**A**) TAGs from individual ejaculatory bulbs of males raised on standard fly food (n = 10) or cactus-banana supplemented food (n = 9) were quantified using direct tissue thin layer chromatography. Each lane contains a single bulb. c: control band (point of origin) used for normalization; f2 and f3: fractions containing sex-specific TAGs. (**B**) The amount of TAGs in f3 from ejaculatory bulbs of males raised on standard food is significantly lower than supplemented food conditions (Student's t-test, two-tailed, p=0.0016). TAGs found in f2 were not significantly different (p=0.062). Error bars indicate SEM. **: p<0.005; ns: not significant; a.u.: arbitrary units.

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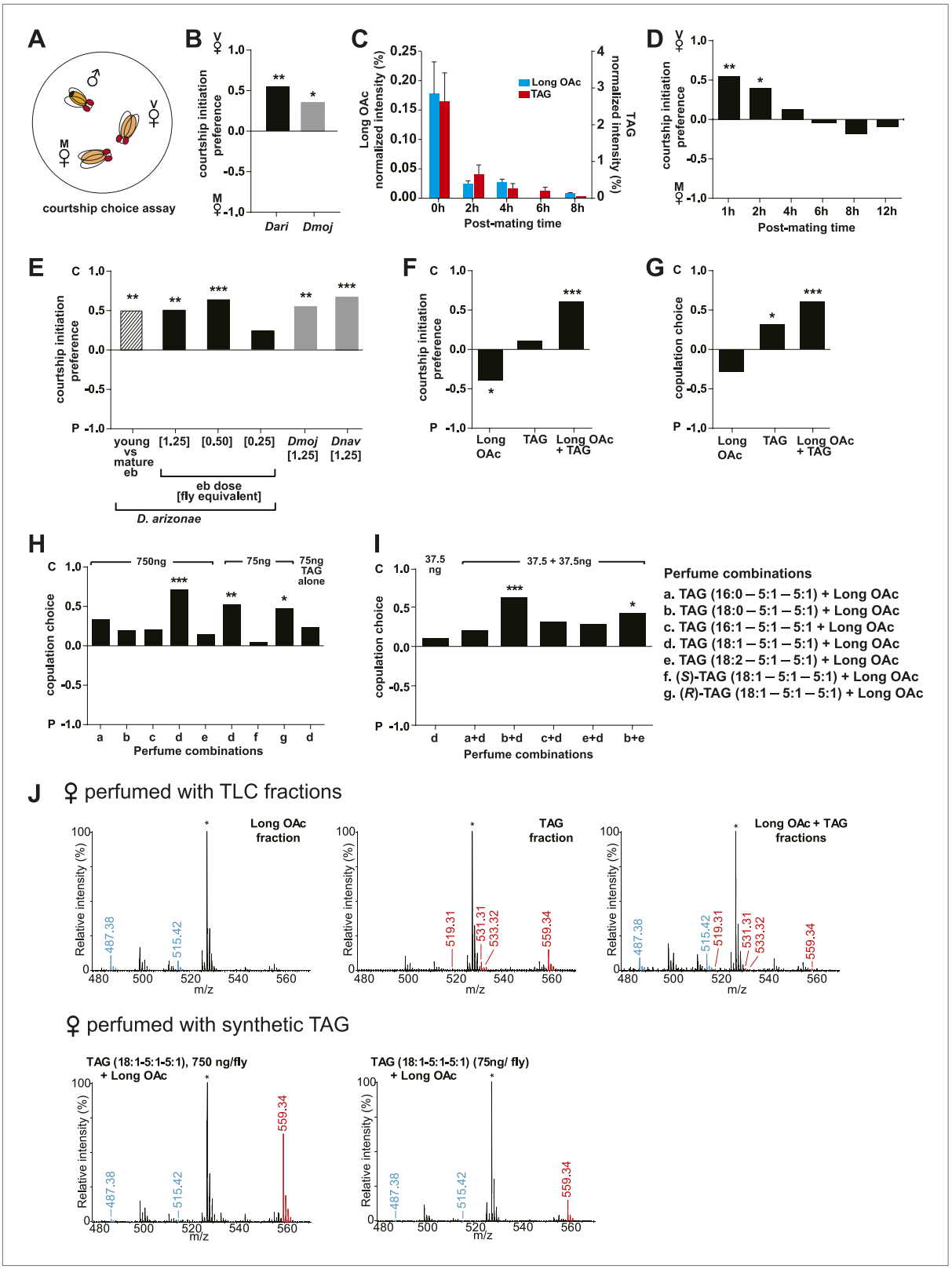

Figure 5. Sex-specific lipids suppress male mating behavior. (**A**) To measure male courtship behavior, one male fly is placed with 2 females, one mated (M), and one virgin (V). (**B**) *D. arizonae* (*Dari*) and *D. mojavensis* (*Dmoj*) prefer to court virgin females over recently mated females (n = 20, Fisher's exact test, p=0.00123; n = 31, p=0.0105). **: p<0.01; ns: not significant. A preference score of 1 indicates all males initiate courtship first with the virgin female; −1 indicates all males initiated courtship first with the mated female. (**C**) Levels of male-transferred TAGs and long OAcs on the female cuticle

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Figure 5. Continued

after first mating decreases by 2 hr post-mating. (**D**) Females are significantly less attractive for up to 2 hr after mating. By 4 hr, males do not exhibit significant preference for courting mated vs virgin females. **: Fisher's exact test, p=0.00123; *: p=0.0256. (**E**) *D. arizonae* males are more reluctant to initiate courtship with females perfumed with the contents of [1.25] ejaculatory bulb (eb) (n = 27, Fisher's exact test, p=0.000624) or [0.5] eb (n = 28, p<0.0001) but not [0.25] eb (n = 28, p=0.176). Extracts from immature male ebs were ineffective at inhibiting male courtship (n = 28, p=0.000389). *D. mojavensis* and *D. navojoa* (*Dnav*) males also avoided virgin females perfumed with eb contents (*Dmoj*: n = 21, p=0.00160; *Dnav*: n = 21, p<0.0002). **: p<0.002; ***: p<0.0001. C: solvent control; P: perfumed. (**F**) Suppression of *D. arizonae* courtship initiation is elicited only when TAGs and long OAcs are combined (n = 28, Fisher's exact test, p<0.0001). TAGs alone are ineffective (n = 28, p=0.593). Long OAcs on their own could be attractive to males (n = 28, p=0.006). *: p<0.05; ***: p<0.0001. (**G**) *D. arizonae* copulation is suppressed in the presence of TAGs alone (n = 28; Fisher's exact probability test, p=0.0287) or TAGs combined with long OAcs (n = 28; p<0.0001), but not long OAcs alone (n = 28; p=0.0543). *: p<0.05; ***: p<0.0001. A copulation choice score of 1 indicates all males copulated with solvent-perfumed females; −1 indicates all males copulated with TAG-perfumed females. (**H**) Perfuming with synthetic TAGs recapitulates copulation suppression. Oleic acid (C18:1)-containing TAGs produced significant effects at high and low doses (750 ng: n = 21, Fisher's exact test, p<0.0001; 75 ng: n = 21, p=0.00167). Only the (*R*)-18:1 stereoisomer was bioactive (75 ng: n = 21, p=0.00480); the (*S*)-18:1 stereoisomer did not elicit a significant behavioral response (75 ng: n = 21, p=1). *: p<0.01; **: p<0.002; ***: p<0.0001. (**I**) Two combinations of TAGs produced synergistic effects on copulation suppression: oleic acid-TAG paired with stearic acid-TAG (n = 21, p=0.000139) and stearic acid-TAG paired with linoleic acid-TAG (n = 19, p=0.022). The oleic acid-containing TAG is not bioactive at a dose of 37.5 ng/fly (n = 20, Fisher's exact test, p=0.751). *: p<0.05; ***: p<0.0005. (**J**) UV-LDI MS spectra of females perfumed with TLC fractions or synthesized TAGs. Signals for TAGs and long OAcs are indicated in red and blue, respectively. *: C35:2 Pentatriacontadiene reference peak, m/z 527.5 $[M + K]^+$.

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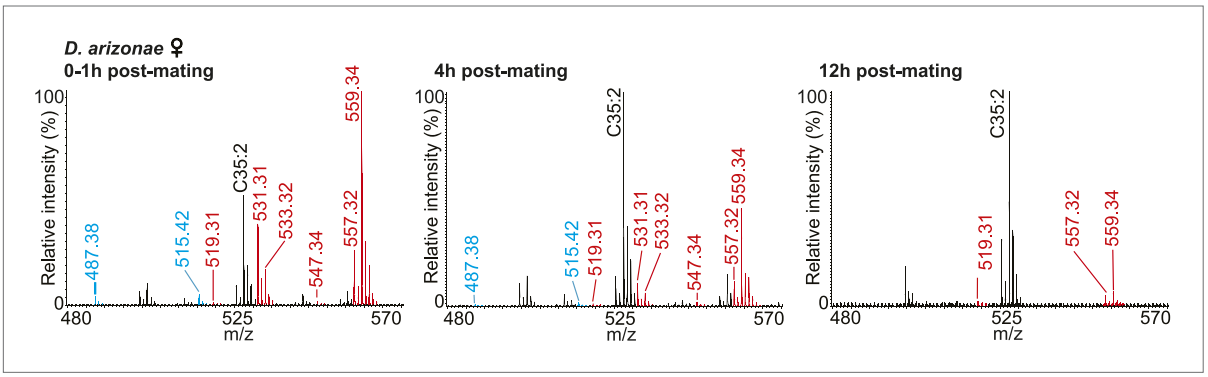

Figure 5—figure supplement 1. UV-LDI spectra of mated *D. arizonae* female anogenital regions reveal signals corresponding to male-specific TAGs (red) and long OAcs (blue) for up to 12 hr post-mating.

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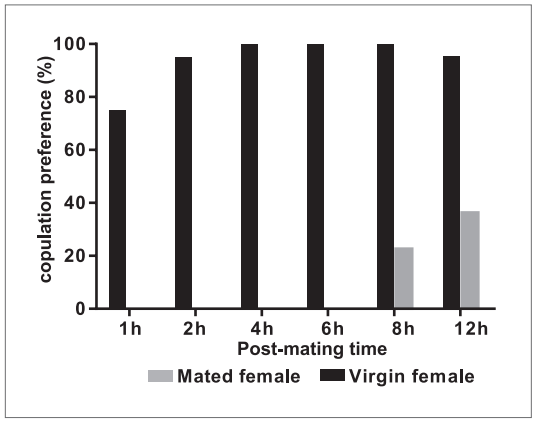

Figure 5—figure supplement 2. *D. arizonae* mated females are receptive to copulation starting at 8 hr after the first mating.

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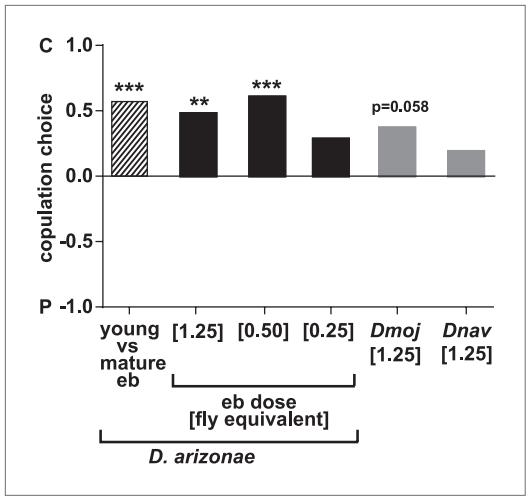

Figure 5—figure supplement 3. *D. arizonae* males avoided copulating with females perfumed with the contents of male ejaculatory bulbs (eb) at a concentration of [1.25] eb (n = 27, Fisher's exact test, p=0.0003) or [0.5] eb (n = 28, Fisher's exact test, p<0.0001) but not [0.25] eb (n = 28, p=0.176).

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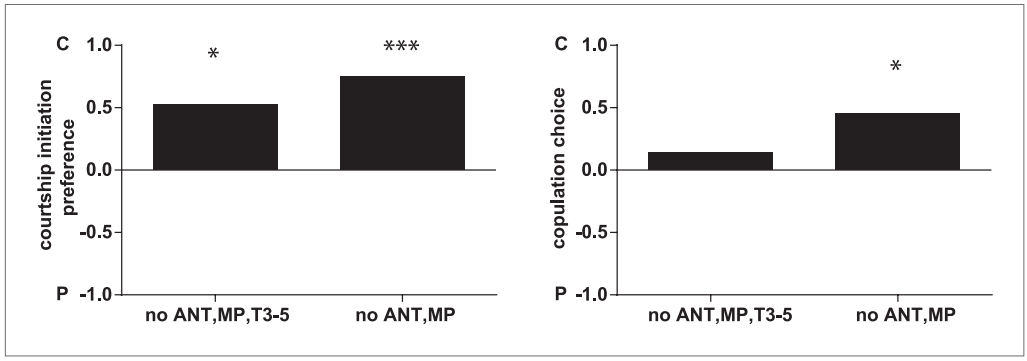

Figure 5—figure supplement 4. *D. arizonae* courtship and copulation preferences towards partly olfactory and gustatory perception-deficient virgin females that are perfumed with control or ejaculatory bulb extract.

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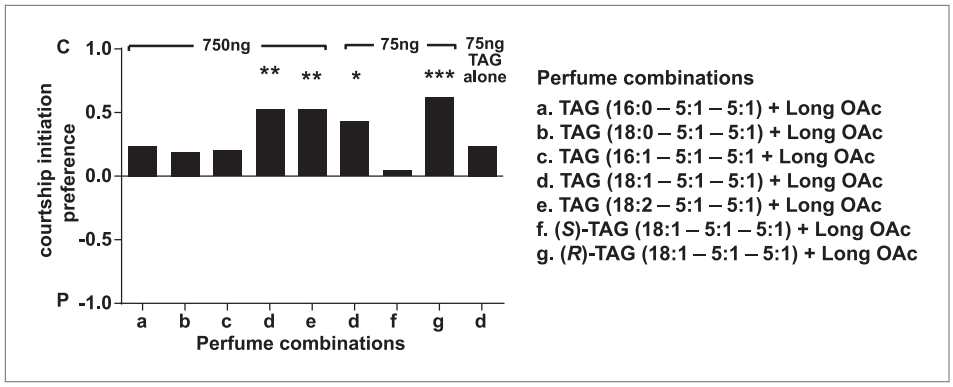

Figure 5—figure supplement 5. *D. arizonae* courtship preferences towards virgin females perfumed with synthetic TAGs together with extract-purified long OAcs.

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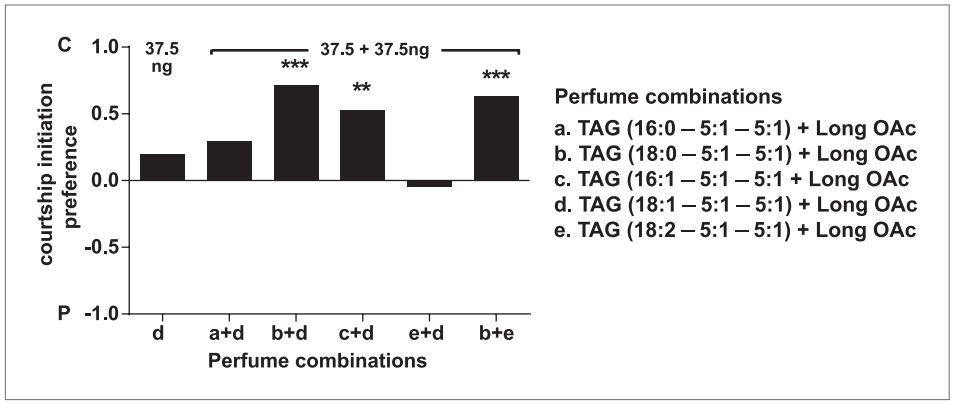

Figure 5—figure supplement 6. *D. arizonae* courtship preferences towards virgin females perfumed with different combinations of synthetic TAG together with extract-purified long OAcs.

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