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RESEARCH ARTICLE

Seasonal accumulation of acetylated triacylglycerols by a freeze-tolerant insect

Katie E. Marshall[1,*], Raymond H. Thomas[2,‡], Áron Roxin[3,4], Eric K. Y. Chen[5], Jason C. L. Brown[1], Elizabeth R. Gillies[5] and Brent J. Sinclair[1,§]

ABSTRACT

Most animals store energy as long-chain triacylglycerols (lcTAGs). Trace amounts of acetylated triacylglycerols (acTAGs) have been reported in animals, but are not accumulated, likely because they have lower energy density than lcTAGs. Here we report that acTAGs comprise 36% of the neutral lipid pool of overwintering prepupae of the goldenrod gall fly, *Eurosta solidaginis*, while only 17% of the neutral lipid pool is made up of typical lcTAGs. These high concentrations of acTAGs, present only during winter, appear to be synthesized by *E. solidaginis* and are not found in other freeze-tolerant insects, nor in the plant host. The mixture of acTAGs found in *E. solidaginis* has a significantly lower melting point than equivalent lcTAGs, and thus remains liquid at temperatures at which *E. solidaginis* is frozen in the field, and depresses the melting point of aqueous solutions in a manner unusual for neutral lipids. We note that accumulation of acTAGs coincides with preparation for overwintering and the seasonal acquisition of freeze tolerance. This is the first observation of accumulation of acTAGs by an animal, and the first evidence of dynamic interconversion between acTAGs and lcTAGs during development and in response to stress.

KEY WORDS: Lipid, Triglyceride, Acetylated triacylglycerol, *Eurosta solidaginis*, Freeze tolerance

INTRODUCTION

Lipids fulfill diverse biological functions including cell membrane structure, signalling and energy storage (Canavoso et al., 2001; Reue, 2011; Yen et al., 2008). Long-chain triacylglycerols (lcTAGs) are generally used as fuel stores by animals because of their high energy density, and are consequently the most abundant lipid class in animals (Williams et al., 2011; Yen et al., 2008). No other neutral lipid has been reported as a storage molecule in terrestrial animals.

Instead of having three long-chain fatty esters (as in lcTAGs), acetylated triacylglycerols (acTAGs) have an acetyl group esterified at the 3-position of the glycerol backbone. This distinct structure results in the reduced viscosity and the significantly lower energy density of acTAGs compared with lcTAGs (Durrett et al., 2010). Trace amounts of acTAGs have been described in a broad range of organisms, including the antlers of Japanese deer (*Cervus nippon*) (Yang et al., 2004), the udders of domestic cows (*Bos primigenius*) (Limb et al., 1999) and the seeds of the burning bush plant (*Euonymus alatus*) (Durrett et al., 2010). acTAGs constitute a very small proportion of the total lipid pool in the two mammal species investigated [<0.1% by mass (Han et al., 1999; Limb et al., 1999; Yang et al., 2004)], but predominate (92% by moles) in *E. alatus* seed oils (Durrett et al., 2010). *Euonymus alatus* synthesises acTAGs via an unusual diacylglycerol acyltransferase (DGAT) by esterifying an acetyl group, rather than a third long-chain fatty acid, to diacylglycerol (DAG) (Durrett et al., 2010). Although trace acTAGs are present in a few animals, we are not aware of any clear hypotheses for a physiological role of acTAGs in biological systems. Here we report that the goldenrod gall fly, *Eurosta solidaginis* (Fitch 1855), accumulates significant quantities of these rare acTAGs – rather than typical lcTAGs – prior to overwintering, increases acTAG concentration following repeated freezing bouts, and converts acTAGs into lcTAGs in the spring.

Eurosta solidaginis is an obligate plant parasite, feeding exclusively on galls it induces on *Solidago canadensis* (Irwin and Lee, 2003; Williams and Lee, 2005; Williams et al., 2004). Over winter, *E. solidaginis* pre-pupae are freeze tolerant, and their cold hardiness has been investigated for over 50 years (Collins et al., 1997; Lee et al., 1995; Salt, 1959). *Eurosta solidaginis* pre-pupae remodel their membrane phospholipids and lcTAGs in preparation for winter, resulting in increased membrane fluidity at low temperatures *via* increased abundance of unsaturated fatty acids and phosphatidylcholine (Bennett et al., 1997; Pruitt and Lu, 2008). Overwinter metabolism and subsequent spring reproduction are both fuelled by lipids and carbohydrates accumulated in the fat body cells during the growing season (Irwin and Lee, 2003). These fat body cells nucleate ice formation and survive intracellular freezing (Lee et al., 1993; Mugnano et al., 1996). Although the mechanism of this cryoprotection is not known, *E. solidaginis* fat body cells contain droplets that remain liquid when the cells are chilled to temperatures that would normally cause lcTAGs to solidify and the cytoplasm to freeze (Mugnano et al., 1996; Salt, 1959).

In the process of investigating overwinter energy storage in *E. solidaginis*, we examined the neutral lipid pool and found that this species accumulates acTAGs before winter, and that these acTAGs decline to trace quantities during metamorphosis. We also found that: (1) acTAGs remained liquid at temperatures that would solidify lcTAGs during overwintering conditions; (2) repeated freezing of *E. solidaginis* increased the amount of acTAGs in the neutral lipid pool; and (3) acTAGs reduced the melting point of saline while lcTAGs and free fatty acids (FFAs) had no effect.

RESULTS

acTAGs predominate in the neutral lipid pool of *E. solidaginis*

Thin-layer chromatography coupled to flame ionization detection (TLC-FID) revealed that overwintering *E. solidaginis* pre-pupae had

[1]Department of Biology, University of Western Ontario, London, ON N6A 5B7, Canada. [2]Department of Psychology, University of Western Ontario, London, ON N6A 5B7, Canada. [3]Department of Pharmaceutical Sciences, University of Toronto, Toronto, ON M5G 1L7, Canada. [4]Ontario Cancer Institute, University Health Network, Toronto, ON M5G 1L7, Canada. [5]Department of Chemistry, University of Western Ontario, London, ON N6A 5B7, Canada.
*Present address: Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. ‡Present address: Grenfell Campus, Memorial University, Corner Brook, NL A2H 5G4, Canada.

§Author for correspondence (bsincla7@uwo.ca)

Received 9 November 2013; Accepted 20 January 2014

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099838

List of symbols and abbreviations

acTAG	acetylated triacylglycerol (molecules with two fatty acids greater than 12 carbons long esterified to a glycerol backbone, with an acetyl group esterified to the third position)
$CDCl_3$	deuterated chloroform
ESI-MS/MS	electrospray ionization mass spectrometry/mass spectrometry
FFA	free fatty acid
GC-MS	gas-chromatography coupled to mass spectrometry
lcTAG	long-chain triacylglycerol (molecules with three fatty acids greater than 12 carbons long esterified to a glycerol backbone)
$MgSO_4$	magnesium sulfate
NMR	nuclear magnetic resonance
R_f	retardation factor
TLC-FID	thin-layer chromatography coupled to flame ionization detection

an unusual neutral lipid composition. In mid-December, lcTAGs comprised only 29% of neutral lipids (by mass) while FFAs contributed an additional 25% (by mass). The remaining 46% of the neutral lipid pool was comprised of an unknown compound that eluted between FFAs and cholesterol [retardation factor (R_f)=0.18–0.25; Fig. 1A]. Other neutral lipids likely present in *E. solidaginis*, such as cholesterol and diacylglycerol, were not detected because of the dilution required to resolve the storage lipid peaks (e.g. cholesterol concentration is three orders of magnitude lower than that of storage lipids in *E. solidaginis*) (Yi and Lee, 2005), and we spiked samples with cholesterol to confirm that the unknown lipid did not co-elute with cholesterol (supplementary material Fig. S1B).

The unknown compound was isolated by preparative thin-layer chromatography purification, and gas chromatography-mass spectrometry (GC-MS) revealed that it was a class of lipid that contained fatty acids previously reported in *E. solidaginis*: predominantly oleic acid (C18:1), followed by palmitoleic acid (C16:1) and palmitic acid (C16:0; supplementary material Fig. S2A) (Pruitt and Lu, 2008). Electrospray ionization-mass spectrometry (ESI-MS) showed that these isolated samples comprised a mixture of several similar lipids with peaks that ranged between 654.5 and 721 *m/z* (as ammonium adducts, supplementary material Fig. S2B) (Kim et al., 1999). The ESI-MS spectrum showed a dominant peak at 680.56 *m/z* ($[M+NH_4]^+$), which ESI-MS/MS indicated was the ammonium adduct of 3-acetyl-1,2-diacyl-sn-glycerol (acTAG) with two oleic acid esters (Fig. 1B; supplementary material Fig. S2B,C). The ^1H nuclear magnetic resonance (NMR) spectrum of this purified sample exactly matched that previously reported for acTAGs (Limb et al., 1999) characterized by the presence of a singlet peak at 2.08 ppm corresponding to the terminal protons on the acetyl methyl, and corroborated the results of ESI-MS/MS. Proton NMR supported the identification by ESI-MS/MS of the unknown lipid as acTAGs, with the following peaks [deuterated chloroform ($CDCl_3$), 600 MHz]: δ 5.37–5.32 (m, 2.8H), 5.30–5.24 (m, 1H), 4.32 (t, *J*=4.3, 1H), 4.28 (t, *J*=4.3, 1H), 4.17 (d, *J*=6.1, 1H), 4.14 (d, *J*=6.1, 1H), 2.36–2.29 (m, 4H), 2.08 (s, 3H), 2.05–1.98 (m, 6.4H), 1.67–1.58 (m, 4H), 1.40–1.22 (br, m, 42H) and 0.92–0.86 (m, 6H). Fractional proton integrations confirmed that the purified acTAG samples contained a variety of fatty esters and corroborated the results of GC-MS. Thus, the unknown compound detected by TLC-FID is a mixture of an unusual class of neutral lipid, acTAG, with several distinct long-chain fatty moieties esterified at the *sn*-1- and *sn*-2-positions of the glycerol backbone (Fig. 1B; supplementary material Fig. S2A). Based on the fatty acid profiles identified by GC-MS and ESI-MS/MS analysis after purifying acTAGs by TLC, oleic acid (C18:1) is the predominant esterified fatty moiety followed by palmitic (C16:0) and palmitoleic (C16:1) acids (supplementary material Fig. S2A) (Bennett et al., 1997).

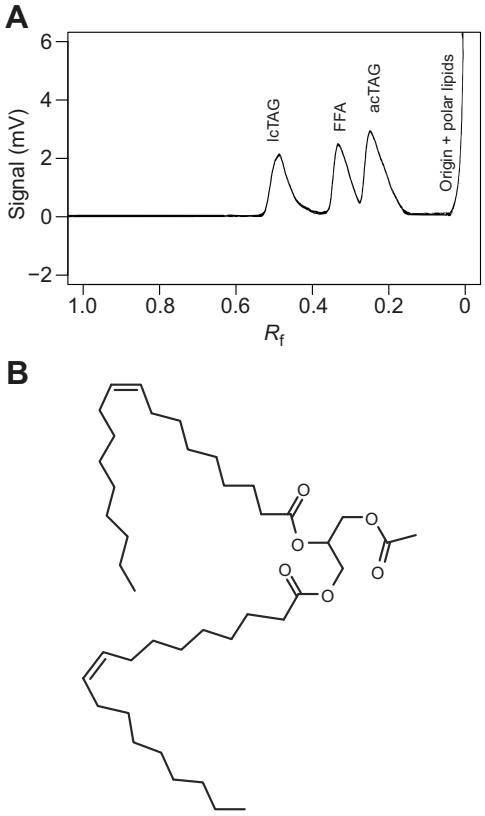

Fig. 1. Characterization of the major neutral lipid classes found in *Eurosta solidaginis*. (A) Chromatogram from TLC-FID of the neutral lipid composition of *E. solidaginis* showing: long-chain triaclyglycerols (lcTAG), free fatty acids (FFA) and acetylated triaclyglycerols (acTAG) after separation in 70:30:05 benzene:chloroform:formic acid (v/v/v). The origin includes any remaining polar lipids, which will not migrate in the solvent system. R_f, retardation factor. (B) General structure of acTAGs as determined by GC-MS, ESI-MS/MS and ^1H NMR. ESI-MS/MS and GC-MS indicated fatty acid components included mostly 18:1, 16:1 and 16:0 fatty acids.

acTAGs are not found in other cold-hardy or freeze-tolerant insects

Once the presence of acTAGs was identified within the neutral lipid pool of *E. solidaginis*, we characterized the neutral lipid compositions of several cold-hardy and freeze-tolerant insects to investigate whether these animals also contained this rare class of lipid. We extracted neutral lipids from other Diptera, freeze-tolerant beetles and wasps, cold-hardy lepidopteran larvae, a parasitoid wasp of *E. solidaginis*, a caterpillar that also forms a gall on *S. canadensis*, and a beetle that feeds on *E. solidaginis* in the gall during the summer (Tables 1, 2). acTAGs were not present in any of these other insects, nor were acTAGs detected within *S. canadensis* gall tissue. These results suggested that the presence of acTAGs in *E. solidaginis* was unique, even compared with other insects capable of surviving cold conditions, and prompted further investigations of the location, pattern of accumulation and physical properties of acTAGs in *E. solidaginis*.

acTAGs are not found preferentially in the plasma membrane

To examine whether acTAGs are found in the membrane of *E. solidaginis* and thus may play a role in membrane fluidity, we used

The Journal of Experimental Biology (2014) doi:10.1242/jeb.099838

Table 1. Neutral lipid components of tissue from galls on the plant *Solidago canadensis*, and several insect species associated with the galls

Species	Association with *S. canadensis*	Order, Family	Life stage assayed	Cold tolerance strategy	lcTAG	FFA	acTAG
Solidago canadensis	NA	Asterales, Asteraceae	n.a.	n.a.	–	–	–
Eurosta solidaginis	Obligate parasitoid	Diptera, Tephritidae	Pre-pupa	Freeze tolerant (including intracellular freezing) (McMullen and Storey, 2008; Williams and Lee, 2005)	+	+	+
			Adult	Chill susceptible (McMullen and Storey, 2008)	+	Trace	Trace
Epiblema scudderiana	Obligate parasitoid	Lepidoptera, Olethreutidae	Pupa	Freeze avoiding (Baust et al., 1979)	+	Trace	–
Eurytoma obtusiventris	Parasitoid of *E. solidaginis*	Hymenoptera, Eurytomidae	Late instar larva	Freeze tolerant (Baust et al., 1979)	+	Trace	–
Mordellistina convicta	Gall inquiline	Coleoptera, Mordellidae	Late instar larva	Freeze tolerant (Baust et al., 1979)	+	Trace	–

Cold tolerance strategy indicates either the natural ability to survive extracellular freezing of body fluids (freeze tolerant), the ability to survive low temperatures without freezing (freeze avoiding), or a very limited ability to survive sub-zero temperature (chill susceptible). For the neutral lipid components [long-chain triacylglycerols (lcTAG), free fatty acids (FFA) and acetylated triacylglycerols (acTAG)], '+' indicates clear presence of the lipid, '–' indicates not detected, and 'Trace' indicates <10% of the total neutral lipid pool.

differential centrifugation to produce a plasma membrane-enriched fraction. Plasma membrane markers indicated a 9.2-fold enrichment of plasma membrane in the membrane-enriched fraction. However, there was no increase in the abundance of acTAGs relative to FFAs or lcTAGs in the membrane-enriched fraction compared with the abundance of these lipids in the un-enriched homogenate, indicating that acTAGs are not selectively localized in the membrane (Fig. 2). These results suggest that acTAGs are stored within other cellular compartments and that they likely do not contribute to modifying cell membrane fluidity.

acTAGs are accumulated prior to winter

We collected *E. solidaginis* pre-pupae from the field every month from August until mid-March and quantified acTAGs by TLC-FID. We found significant changes in the molar quantity of all three neutral lipids over this time period (FFAs: $F_{9,39}$=8.25, P<0.001; lcTAGs: $F_{9,39}$=4.48, P<0.001; acTAGs: $F_{9,39}$=9.16, P<0.001; Fig. 3A). The largest accumulation of acTAGs coincided with the onset of freeze tolerance in early autumn (Fig. 3A), indicating that *E. solidaginis* synthesized acTAGs seasonally. We also found a significant interaction between lipid type and month of collection on proportion of the neutral lipid pool ($F_{2,24}$=9.11, P=0.001), whereby acTAGs increased in proportion from August to September while lcTAGs and FFAs either remained consistent or decreased in proportion (Fig. 3B). While the quantity of both FFAs and acTAGs increased significantly between late summer and winter, the amount of lcTAG remained unchanged (Tukey's *post hoc* test, P=0.085). lcTAG abundance increased in the spring, while FFA and acTAG abundance concomitantly decreased in an equimolar ratio (Fig. 3A,C).

acTAGs increase in quantity with repeated freezing

Because increased acTAG concentration appeared to be associated with overwintering, we investigated whether acTAGs were synthesized following cold exposure. There were no differences in neutral lipid composition among individuals that received 12 h exposures every 5 days or every 10 days, so these samples were pooled in the analysis. There was also no difference between control individuals sampled at the beginning or end of the experiment, so all control individuals were pooled. Repeated freezing significantly increased the amount of acTAGs compared with unfrozen control individuals (Tukey's *post hoc* test, P=0.002), and induced a near-significant increase relative to a single prolonged freeze (Tukey's *post hoc* test, P=0.064; ANOVA, $F_{2,26}$=7.559, P=0.003, Fig. 4). There was no significant difference

Table 2. Neutral lipid components of several insect species not associated with *Solidago canadensis*

Species	Order, Family	Life stage assayed	Cold tolerance strategy	lcTAG	FFA	acTAG
Choristoneura fumiferana	Lepidoptera, Tortricidae	2nd instar larva	Freeze avoiding (Han and Bauce, 1995)	+	+	–
Chrysomela aenicollis	Coleoptera, Chrysomelidae	Adult	Freeze tolerant (Boychuk, 2012)	+	Trace	–
Drosophila melanogaster	Diptera, Drosophilidae	Adult	Chill susceptible (MacMillan et al., 2009)	+	–	–
Gryllus pennsylvanicus	Orthoptera, Gryllidae	Adult	Chill susceptible (MacMillan and Sinclair, 2011)	+	–	–
Pyrrharctia isabella	Lepidoptera, Arctiidae	Final instar larva	Freeze tolerant (Layne et al., 1999)	+	–	–

Cold tolerance strategy indicates either the natural ability to survive extracellular freezing of body fluids (freeze tolerant), the ability to survive low temperatures without freezing (freeze avoiding), or a very limited ability to survive sub-zero temperature (chill susceptible). For the neutral lipid components (lcTAGs, FFAs and acTAGs), '+' indicates clear presence of the lipid, '–' indicates not detected, and 'Trace' indicates <10% of the total neutral lipid pool.

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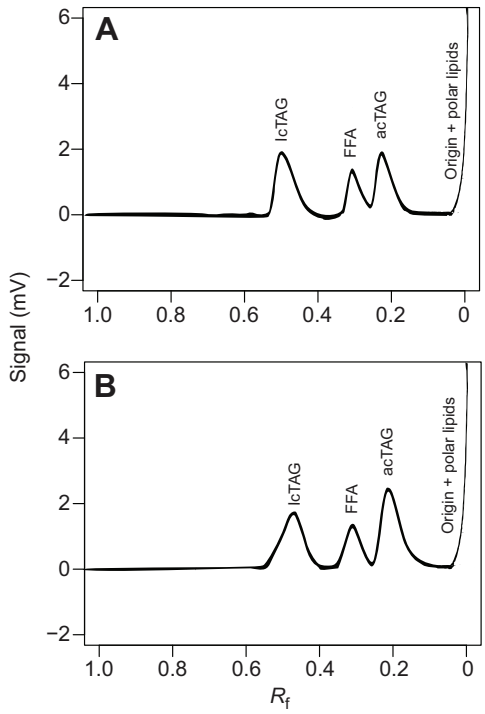

Fig. 2. Differential centrifugation shows no membrane association with acTAGs in *E. solidaginis*. (A) Lipid profile of raw homogenate from whole pre-pupae, showing lipid components including lcTAGs, FFAs and acTAGs. (B) Lipid profile of 9.2-fold enriched membrane fraction, showing no relative increase in acTAGs.

in acTAG quantity relative to control values after a single prolonged freeze (Tukey's *post hoc* test, P=0.669). While lcTAG quantity remained unchanged, there was a trend towards decreased FFA content in pre-pupae that received repeated freezing ($F_{2,26}$=2.976, P=0.069, Tukey's *post hoc* test comparing repeated freezing with control values, P=0.056).

acTAGs have low melting points and depress the melting point of saline suspensions

To explore the possibility that acTAGs in *E. solidaginis* may have unusual thermal properties, we characterized the melting point of our purified acTAG sample and compared this melting point profile with that of lcTAG mixtures present in *E. solidaginis*. Natural mixtures of lcTAGs contain a variety of fatty ester moieties and, therefore, show multiple endotherms between +5 and +35°C as individual components of these mixtures melt (Knothe and Dunn, 2009; Ribeiro et al., 2009). We used differential scanning calorimetry to investigate the thermal profile of the mixture of acTAGs isolated from *E. solidaginis,* and found a single broad endotherm between −35 and −13°C (Fig. 5). This suggests that individual acTAG compounds within the purified mixture each have low melting points compared with natural mixtures of lcTAGs within *E. solidaginis*. Specifically, the melting point of the acTAG mixture is substantially lower than the melting points of triolein (5°C) and tripalmitin (66°C) (Knothe and Dunn, 2009), which together make up 80% of the lcTAG composition of *E. solidaginis* (Pruitt and Lu, 2008).

The relatively low melting point range of the purified acTAGs and the seasonal accumulation of acTAGs in field samples of *E. solidaginis* during winter months led us to hypothesize that acTAGs could have further, potentially cryoprotective, properties. We explored the effects of acTAGs on the melting point of saline solutions using nanolitre osmometry to determine whether acTAGs had the potential to interact with aqueous solutions. We found that acTAGs (1 mol l^{-1} in 1 Osm $NaCl_{aq}$ solution) depressed the melting point of saline by 1.21±0.03°C, while the same concentrations of lcTAGs (depression of 0.02±0.05°C) and FFAs (0.02±0.02°C) had no effect ($F_{2,16}$=387, P<0.001).

DISCUSSION

Here we show that overwintering *E. solidaginis* pre-pupae have an unusual neutral lipid composition, predominated by acTAGs (by mass) and FFAs (by moles). Although acTAGs have previously been reported as trace components (<1% by mass) of neutral lipid

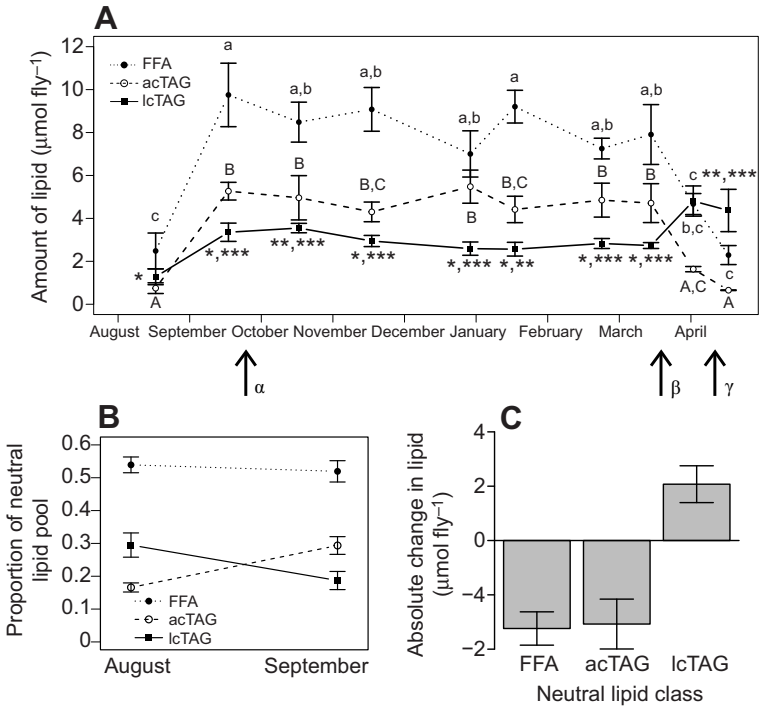

Fig. 3. Seasonal changes in acTAG concentration of *E. solidaginis*. (A) The neutral lipid pool of *E. solidaginis* shifts dramatically from late summer until early spring (2011–2012) in relation to life stage [indicated by arrows: α, onset of freeze tolerance according to Bennett et al. (Bennett et al., 1997); β, pupation; γ, eclosion]. Data are means ± s.e.m.; n=5. Within the same neutral lipid class, points with different symbols indicate statistically different quantities in an ANOVA with Tukey's *post hoc* test. Differences among months are denoted by lowercase letters for FFA, uppercase letters for acTAG, and asterisks for lcTAG (*P<0.05; **P<0.01; ***P<0.001). (B) Proportional changes in neutral lipid class composition between August and September. Data are mean proportions ± s.e.m.; n=5. (C) Quantitative changes in each neutral lipid class (μmol fly^{-1}) between mid-March and early April. Data are means ± s.e.m.; n=5.

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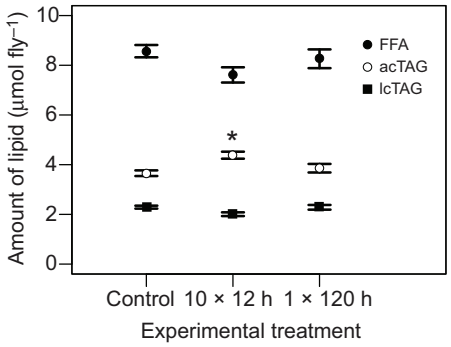

Fig. 4. Freezing *E. solidaginis* pre-pupae repeatedly (10 exposures of 12 h each at −20°C) induces additional production of acTAGs. Least square means incorporating the effect of total lipid mass are plotted. Asterisk indicates significant difference relative to control (Tukey's *post hoc* test, **P*<0.05). Data are means ± s.e.m.; *n*=5–10.

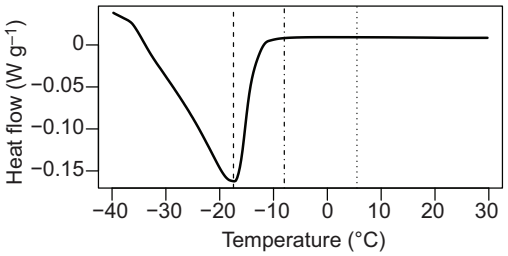

Fig. 5. Heat flow during melting of acTAGs extracted from *E. solidaginis*. Vertical lines indicate melting point of acTAGs (dashed line); whole-body supercooling point of *E. solidaginis* (dot-dash line); and melting point of triolein (lcTAG with 18:1 fatty acids esterified; dotted line).

fractions isolated from deer antlers and bovine udders (Han et al., 1999; Kim et al., 1999; Limb et al., 1999), we are unaware of any other report of an animal with a neutral lipid pool dominated by acTAGs. We observed that field-collected *E. solidaginis* accumulated large quantities of acTAGs for energy storage over winter; an unusual strategy, as animals generally use more energy-dense lcTAGs for this purpose (Canavoso et al., 2001).

We also determined that the presence of acTAGs is not a ubiquitous feature of freeze-tolerant or cold-hardy insects. Because acTAGs are absent from the galls that house *E. solidaginis*, it appears that the pre-pupae do not acquire acTAGs directly from the diet. Under temperature-controlled conditions, we found that acTAG content increased after repeated freezing, which suggests that acTAG synthesis is induced, even during mid-winter diapause, by freezing stress, and that acTAGs are synthesized from available lcTAGs. It is curious that while we observed an increase in acTAGs following repeated freezing, we did not observe a concomitant increase in FFAs. Repeated freeze–thaw cycles lead to energetic stress in *E. solidaginis* (as measured by ATP and glycogen content) (Churchill and Storey, 1989), so we suspect that additional FFAs may have been catabolized to produce ATP.

The low melting point range of the acTAG mixture suggests that these lipids could remain in the liquid state *in vivo* at temperatures at which structurally similar lcTAGs would solidify. Therefore, the low melting point profile of acTAGs explains Salt's early observation of clear lipid droplets within frozen fat body cells of *E. solidaginis* (Salt, 1959). Finally, the depression in melting point of saline solutions was likely due to the relatively increased hydrophilicity of acTAGs, compared with lcTAGs, because of the presence of the acetyl group rather than a third long-chain fatty ester moiety. A modelling study of the three-dimensional structure of acTAGs indicates that the acetyl group orients in an opposite direction to the two fatty moieties and the resulting structure is similar to that of a phospholipid (Honda and Kozawa, 1992). We hypothesize that this increased hydrophilicity, in conjunction with the low melting point range of acTAGs, could allow *in vivo* interactions between acTAGs and the water molecules involved during ice formation at low temperatures.

acTAGs have two long-chain fatty acids esterified at the *sn*-1 and *sn*-2 positions and acetate-esterified at the *sn*-3 position of the glycerol moiety. This 3-acetyl ester is the key moiety that alters the properties of the resulting lipid from typical lcTAGs or DAGs (Fig. 1B) (Kim et al., 1999; Limb et al., 1999). In contrast to plants, which synthesize acTAGs *de novo*, stoichiometry of the seasonal neutral lipid changes suggests that *E. solidaginis* produces this lipid from existing lcTAGs. We suggest that identifying the metabolic pathway responsible for this conversion could yield a method to synthesize low-viscosity acTAGs from the higher-viscosity lcTAGs produced by existing oilseed crops (Durrett et al., 2010).

The biology of *E. solidaginis* has been investigated for many decades (Collins et al., 1997; Lee et al., 1995; Salt, 1959; Williams et al., 2004) and therefore it is surprising that this is the first report of these abundant acTAGs. We suspect that this oversight arises because the techniques for studying lipids (i.e. gas chromatography, vanillin or enzymatic assays) assume that lcTAGs predominate, and almost always involve hydrolysis of fatty acids from the glycerol backbone (Williams et al., 2011), masking the presence of other glycerolipids. Gravimetric techniques, by contrast, dissolve all nonpolar compounds and therefore do not distinguish among lipid species (Williams et al., 2011). Thus, previous work has likely conflated acTAGs and lcTAGs.

acTAGs contain less energy per unit mass than lcTAGs because of the inclusion of only two long-chain fatty moieties (Durrett et al., 2010). Thus, acTAGs are not an energetically favourable mode of energy storage for *E. solidaginis*, which suggests a role for acTAGs in addition to that of energy storage. Insect neutral lipid compositions are typically dominated by lcTAGs (over 90% by mass), with only small quantities of FFAs and DAGs. While we observed FFA accumulation in autumn, we do not believe that this accumulation is related to freeze tolerance. Instead, FFA accumulation may result from the synthesis of acTAGs from lcTAGs after hydrolysis of fatty esters at the *sn*-3-position on the glycerol backbone of lcTAGs. In addition, FFAs did not depress the melting point of saline, which reduces the likelihood of a cryoprotective role. The large quantity of FFAs found in *E. solidaginis* does present a toxicological riddle: in large quantity, FFAs (particularly oleic acid) are toxic to insects (Harada et al., 2000). How *E. solidaginis* resists this toxicity is unknown.

Survivable intracellular ice formation in insects has been described mainly in fat body cells, but the mechanisms involved are not yet understood (Sinclair and Renault, 2010). Given the quantity of the acTAGs, their non-polar nature, and the fact that they are not localized within cell membranes, we expect that acTAGs and lcTAGs co-localise in the lipid droplets in the fat body cells of *E. solidaginis*. Because acTAGs remain fluid at subzero temperatures and can modulate ice crystal formation, we hypothesize that acTAGs could prevent contact between ice crystals and key cellular components such as intracellular membranes as *E. solidaginis* freeze. We also note that the presence of liquid lipid droplets in fat body cells (Salt, 1959) facilitates lipid–cytosolic interactions, and

may therefore favour the evolution of lipid-based intracellular cryoprotection. Based on the physical properties of acTAGs and the increased abundance of acTAGs during the onset of freeze tolerance of *E. solidaginis*, we hypothesize a cryoprotective role for these rare lipids in *E. solidaginis*.

We identify two non-cryoprotective alternative explanations for the large accumulation of acTAGs in *E. solidaginis*. First, acTAGs may accumulate seasonally for a purpose other than cryoprotection such as maintenance of a liquid lipid pool or access to FFAs while frozen during winter. However, we suggest that the increased accumulation of acTAGs after repeated freezing stress (Fig. 1C) is inconsistent with this hypothesis. In addition, frozen *E. solidaginis* pre-pupae likely rely on carbohydrate (rather than fatty acid) metabolism (Storey and Storey, 1985). Second, acTAGs may accumulate as a byproduct of some other, unknown metabolic process during preparation for winter, and in response to the physiological stresses of freezing. While the existence of an unknown metabolic pathway that produces milligram-scale quantities of unusual lipids is unlikely, detailed investigation of acTAG biosynthesis in *E. solidaginis* could yet identify novel lipid biochemistry in animals.

Our hypothesis for a cryoprotective role for acTAGs requires further exploration. As a univoltine, gall-dependent species, *in vivo* manipulation of intracellular lipid pools of *E. solidaginis* will be difficult. However, it could be possible to develop and manipulate cell lines derived from *E. solidaginis* fat body cells to determine the role of acTAGs in survival of intracellular ice formation. Alternately, identification of the metabolic pathways associated with acTAG accumulation could allow the cryoprotective effects of acTAGs to be explored in more readily manipulated organisms, or for RNAi to be used to knock down acTAG accumulation in *ex situ E. solidaginis* larvae in the autumn if the appropriate enzyme(s) could be identified. We also suggest that the cryoprotective effects of acTAGs on individual cellular components could be explored *in vitro*.

Conclusions

Here we have shown that overwintering *E. solidaginis* is the first terrestrial animal identified to date to accumulate and consume acTAGs, and that the timing of these events coincide with the acquisition and loss of freeze tolerance. We also show that acTAGs have unusual properties for a storage lipid that may increase their utility in low-temperature environments, and that increased quantities of acTAGs are produced following repeated freezing stress.

MATERIALS AND METHODS

Field collection of *E. solidaginis*

Spherical galls containing *E. solidaginis* were collected from *Solidago* spp. plants in old field habitats in London, ON, Canada (43°00′N, 81°15′W). A seasonal profile consisting of monthly collections of *E. solidaginis* taken directly from the field was performed. Collections took place in late autumn 2009 for the repeated freezing experiment, and winter 2011–2012 for the seasonal profile. Pre-pupae for the seasonal profile were extracted from galls within 2 h of collection, snap-frozen by direct immersion in liquid nitrogen and stored in 0.2 ml Eppendorf microcentrifuge tubes at −80°C. Pre-pupae destined for laboratory manipulations (see 'acTAG quantity following repeated freeze–thaw cycles', below) were kept in 0.2 ml Eppendorf tubes (with an air hole) in constant darkness at 0°C for 2 months prior to experimentation. For the spring collections in the seasonal profile, pre-pupae were collected and then incubated in 0.2 ml Eppendorf tubes in constant light at 20°C. Pupation occurred in all individuals within 7 days, and then pupae were snap-frozen after 2 weeks. All individuals eclosed within 28 days of incubation, at which time adults were snap-frozen. The acTAGs of each individual were quantified by thin layer chromatography coupled to flame ionization detection (TLC-FID) (Williams et al., 2011).

TLC-FID

Folch extractions were conducted by grinding whole bodies of *E. solidaginis* in 2.5 ml of 2:1 chloroform:methanol (v/v) containing 0.1% butylated hydroxytoluene (v/v). Next, 0.25% $KCl_{(aq)}$ was added and the solution was heated (to 70°C). The organic layer was collected and dried under nitrogen while heating at 70°C. The crude lipid extracts were resuspended in 1.7 ml chloroform and the components were separated on chromarods (Shell USA) with 70:30:0.5 benzene:chloroform:formic acid (v/v/v) (Williams et al., 2011). TLC-FID was performed using an Iatroscan MK-6 TLC-FID Analyzer (Shell USA) at the following settings: flow rate of 2 l min^{-1} for atmospheric air, 160 ml min^{-1} for hydrogen, and scanning speed of 3.0 cm s^{-1}, and neutral lipids were identified by comparing peak retention times to known standards (supplementary material Fig. S1A). A sample of *E. solidaginis* lipids was also spiked with cholesterol to confirm that the unknown lipid peak was not cholesterol (supplementary material Fig. S1B). Quantification of neutral lipid components was based on integrated peak area and standard curves of known concentrations of lcTAG and FFA standards (60% triolein, 20% tripalmitin, 20% tripalmitolein for lcTAGs, 60% oleic acid, 20% palmitic acid, 20% palmitoleic acid for FFAs). To quantify acTAGs, the acTAGs from 10 individual *E. solidaginis* were purified, pooled, and then weighed. A standard curve was then produced on a mass/volume basis.

Purification of acTAGs

The acTAGs were purified from Folch lipid extracts using 200 µm silica plates eluted with the described solvent system. A band of silica was removed at R_f=0.18–0.25 and was suspended in chloroform. Trace water was removed with $MgSO_4$. The suspension was filtered and the purified acTAGs were dried under nitrogen while heating at 70°C. Purity was assessed using TLC-FID as above.

Other insect species assayed

During collections of *E. solidaginis* in September 2011, *Epiblema scudderiana*, *Eurytoma obtusiventris*, *Mordellistina convicta* and *Pyrrharctia isabella* were collected from the same old field habitats and immediately assayed for lipid content. *Choristoneura fumiferana* (diapausing strain) was obtained from Insect Production Services (Canadian Forest Service, Sault Ste. Marie, Ontario, Canada) and kept in a temperature-controlled incubator in constant darkness at 2°C for 1 month before lipids were measured. *Drosophila melanogaster* and *Gryllus pennsylvanicus* were obtained from a laboratory colony maintained at 22°C. In all cases except for *G. pennsylvanicus*, the assayed life stage was the overwintering life stage.

Gas chromatography coupled to mass spectrometry

Gas chromatography coupled to mass spectrometry (GC-MS) was conducted according to the combined methods of Chow et al. (Chow et al., 1989) and Bicalho et al. (Bicalho et al., 2008). Briefly, 200 µl of 0.5 mol l^{-1} methanolic-HCl (Sigma, St Louis, MO, USA) was added to the dried acTAG samples (4 mg), and the solution was heated at 90°C for 30 min. After cooling, distilled water (0.8 ml) was added, and the non-polar components were extracted with 3×500 µl of hexane. The hexane was dried under nitrogen, and the residue was resuspended in 50 µl of hexane. Samples (both aqueous and organic, 1 µl each) were injected in splitless mode on a Varian GC 3500 equipped with a Finnigan MAT 8400 MS fitted with a DB-23 high resolution column, and run on the following temperature profile: injection at 80°C (held for 2 min), temperature ramped to 180°C at 5°C min^{-1}, then to 200°C at 1.5°C min^{-1}, then to 240°C at 10°C min^{-1} and held for 3 min. The resolved peaks were identified from literature references (Bicalho et al., 2008) and by comparison of retention times and mass spectra obtained from commercial standards (Supelco 37 component mix, Supelco PUFA No. 3, Supelco FAME mix C8-C24).

ESI-MS/MS

The structure of purified acTAGs (in methanol) was examined by infusion with a triple quadruple mass spectrometer (Micromass QuattroMicro; Waters, Milford, MA, USA) equipped with a Z-spray source operated in the positive ion mode, at a flow rate of 50 µl min^{-1}. Ammonium or sodium adducts of acTAG species were determined using full scan mode (200–1800 *m*/*z*). The fatty acid composition of each acTAG species identified from the full scan analysis was determined via electrospray ionization mass spectrometry/mass spectrometry (ESI-MS/MS; 80–1000 *m*/*z*) with reference to the literature (Kim et al., 1999). The cone voltage was set at 30 V, the collision energy at 30-50 V, source temperature at 80°C and the collision gas at $2\times e^{-3}$ Mbar. Calibration was performed with sodium iodide and mass error was less than 0.05 Da.

^{1}H NMR characterization

The ^{1}H NMR spectrum of the purified acTAG mixture was obtained using a Varian Inova 600 MHz NMR with acTAGs dissolved in $CDCl_3$ (NMR chemical shifts are reported in ppm and are calibrated against the residual solvent signal of $CDCl_3$ at δ=7.26 ppm). Coupling constants are expressed in Hz.

Subcellular localization of acTAGs

Crude subcellular fractions of *E. solidaginis* (15–30 individuals in 15 ml of homogenization buffer) were purified by differential centrifugation (Graham, 2002). All purified fractions were assayed for 5′-nucleotidase, a plasma membrane marker (Butters and Hughes, 1980; Norris and Cary, 1981), by incubating 50 µl aliquots of crude fraction (and homogenate) in a solution of 200 mmol l^{-1} glycine (pH 8.5), 200 mmol l^{-1} $MgSO_4$ and 66 mmol l^{-1} 5′-AMP for 60 min at 37°C. An aliquot of the incubation mixture was removed, added to 10% trichloroacetic acid (w/v), and centrifuged at 1000 ***g*** for 15 min. Inorganic phosphate production was measured according to Taussky and Shorr (Taussky and Shorr, 1953), and expressed relative to protein concentration measured by Bradford assay.

The fraction with the highest enrichment of 5′-nucleotidase (~3-fold), was further purified using a discontinuous sucrose gradient (McKeel and Jarett, 1970) with four density layers (1.02, 1.08, 1.14 and 1.18 g ml^{-1}) and centrifugation at 28,000 ***g*** for 90 min at 4°C. Following centrifugation, bands at each density boundary were removed, resuspended in homogenization buffer, and centrifuged twice at 8700 ***g*** for 10 min at 4°C to pellet the purified plasma membrane. The band at 1.14 g ml^{-1}, where plasma membrane should accumulate based on its density (McKeel and Jarett, 1970), showed 5′-nucleotidase activity 9.2-fold higher than homogenate, suggesting considerable enrichment of the plasma membrane. Lipids from the raw homogenate and the 1.14 g ml^{-1} band were extracted using the described Folch method. Samples were profiled by TLC-FID, as described above.

acTAG quantity following repeated freeze–thaw cycles

Freezing experiments commenced in January 2010. Pre-pupae were subjected to one of two experimental freezing regimes: frozen at −20°C for 12 h every 5 or 10 days, or frozen at −20°C for a single period of 120 h. All freezing experiments were conducted in refrigerated baths (Lauda Proline 3530, Wurzburg, Germany) with 1:1 methanol:water (v/v) with a cooling rate of 0.1°C min^{-1}. After the freezing treatment, pre-pupae were incubated for 24 h at 0°C, before being snap-frozen by direct immersion in liquid nitrogen and stored at −80°C. The acTAGs were then quantified in pools of three individuals using TLC-FID.

Differential scanning calorimetry

The melting point of a 10 mg sample of the purified acTAGs was measured using a 822e Mettler Toledo DSC based on duplicate melt curves of −100°C to +40°C at 5°C min^{-1} (Ribeiro et al., 2009).

Nanolitre osmometry

Melting point depression was determined in a Clifton nanolitre osmometer using droplets of 1 mol l^{-1} triolein, oleic acid or acTAG mix in 1 Osm saline [$NaCl_{(aq)}$] in 20–30 nl droplets suspended in Type B immersion oil. Samples were vigorously vortexed immediately prior to loading in wells. Melting point was reported as the temperature at which the final ice crystal in each solution melted after snap-freezing and slow heating (0.11°C min^{-1}).

Statistical analyses

The average molar mass of each neutral lipid class was estimated by assuming that relative fatty acid compositions were identical in each neutral lipid class, and in the same proportions as previously reported in whole-animal overwintering *E. solidaginis* (60% oleic acid, 20% palmitic acid and 20% palmitoleic acid) (Bennett et al., 1997). In the seasonal profile, the concentration (µmol fly^{-1}) of each neutral lipid component was compared among months by ANOVA, followed by two-tailed Tukey's *post hoc* tests, with month treated as a fixed factor as it was expected there would be non-linearity in response. In the repeated freezing experiment, because there was a large (but non-significant) effect of experimental treatment on total lipid mass, so all analyses were conducted as ANCOVAs with total lipid mass (minus the component of interest) as a covariate (using total lipid mass as a covariate does not alter the conclusions). Residuals of a regression between total lipid mass and the neutral lipid were obtained and compared between experimental groups by ANOVA with two-tailed Tukey's *post hoc* tests. Population means (least-squares means) were obtained for plotting using the popMeans function in the doBy package in R (Højsgaard et al., 2012). In all tests, alpha was set to 0.05.

Acknowledgements

We thank Caroline Williams, Mark Bernards, John Steele, David Wharton and numerous anonymous referees for constructive criticism of earlier versions of the paper; Chris Guglielmo and Jim Staples for use of laboratory space and equipment; and Evelyn Boychuk, Annegret Nicolai, Ryan Guterman, Paula Pittiock and Doug Hairsine for technical assistance.

Competing interests

The authors declare no competing financial interests.

Author contributions

K.E.M., A.R. and B.J.S. designed the research. K.E.M., R.H.T., A.R., E.K.Y.C. and J.C.L.B. performed the research. R.H.T., J.C.L.B. and E.K.Y.C. contributed new reagents or analytic tools. K.E.M., R.H.T., A.R., E.K.Y.C., J.C.L.B., E.R.G. and B.J.S. analyzed the data. K.E.M. and B.J.S. wrote the paper.

Funding

This research is supported by a Natural Sciences and Engineering Research Council (NSERC) post-graduate scholarship to K.E.M., and NSERC Discovery grants to B.J.S. and E.R.G.

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.099838/-/DC1

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