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REVIEW

The many roles of fats in overwintering insects

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ABSTRACT

Temperate, polar and alpine insects generally do not feed over winter and hence must manage their energy stores to fuel their metabolism over winter and to meet the energetic demands of development and reproduction in the spring. In this Review, we give an overview of the accumulation, use and conservation of fat reserves in overwintering insects and discuss the ways insects modify fats to facilitate their selective consumption or conservation. Many insects are in diapause and have depressed metabolic rates over winter; together with low temperatures, this means that lipid stores are likely to be consumed predominantly in the autumn and spring, when temperatures are higher but insects remain dormant. Although there is ample evidence for a shift towards less-saturated lipids in overwintering insects, switches between the use of carbohydrate and lipid stores during winter have not been well-explored. Insects usually accumulate cryoprotectants over winter, and the resulting increase in haemolymph viscosity is likely to reduce lipid transport. For freeze-tolerant insects (which withstand internal ice), we speculate that impaired oxygen delivery limits lipid oxidation when frozen. Acetylated triacylglycerols remain liquid at low temperatures and interact with water molecules, providing intriguing possibilities for a role in cryoprotection. Similarly, antifreeze glycolipids may play an important role in structuring water and ice during overwintering. We also touch on the uncertain role of non-esterified fatty acids in insect overwintering. In conclusion, lipids are an important component of insect overwintering energetics, but there remain many uncertainties ripe for detailed exploration.

KEY WORDS: Lipid, Triglyceride, Cold tolerance, Freeze tolerance, Antifreeze, Energetics

Introduction

Insects overwintering in polar, temperate and alpine environments can be exposed to low temperatures for significant periods over winter, during which behaviour and feeding often cease. Physiological processes during this time are prioritised towards surviving extreme conditions and conserving resources for the subsequent growing season (Sinclair, 2015; Williams et al., 2015b). Although most biochemical reactions occur among polar molecules in the aqueous phase, non-polar molecules, including fats and other neutral lipids, have a range of biological roles in animals. Here, we review the neutral lipid biology of overwintering insects. We specifically do not address endocrine, membrane or cuticular lipids. Instead, we focus on fatty acid-derived neutral lipids, primarily triacylglycerols (TAGs), and their regulation and roles as fuel and functional molecules in overwintering insects.

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As small ectotherms, most insects experience body temperatures that are heavily influenced by their ambient environment. Over winter, when insects are not feeding, insects must contend with unreplaced energy consumption and water loss, as well as low temperatures and immune challenges (Sinclair et al., 2013a; Williams et al., 2015b). Of these stressors, low temperatures (which affect neutral lipid fluidity and mobilisation) and energy drain (lipids are a primary overwinter fuel source) are the most relevant here. The exponential relationship between temperature and biochemical reaction rate means that substrate use increases at higher temperatures, and (conversely) that fuel consumption over winter is reduced passively by exposure to colder temperatures (Sinclair, 2015). Many overwintering insects also enter diapause, a programmed state of developmental arrest that is almost always accompanied by the suppression of their metabolic rate beyond temperature effects (Koštál, 2006); this serves, at least in part, to dramatically reduce energy consumption (Hahn and Denlinger, 2007, 2011).

At temperatures below the melting point of their body fluids, insects risk internal ice formation. There are two main ways that insects mitigate this problem: either by surviving internal ice formation (freeze tolerance) or by maintaining their body fluids in a liquid (supercooled) state at temperatures below their melting point (freeze avoidance). Insects that adopt either of these strategies commonly accumulate hydrophilic low molecular weight cryoprotectants, such as glycerol or proline, that have colligative effects on water and may protect macromolecules, and ice-binding molecules, such as antifreeze proteins, that modify the initiation, growth and structure of ice (Lee, 2010). To explore how fats facilitate insect overwintering success, we will examine the role of fats in overwinter energy metabolism and briefly examine the putative functional roles of some fat-derived molecules at low temperatures.

A brief overview of insect lipid metabolism

The three main sources of fats in overwintering insects (see Fig. 1 for an overview of structures) are: (1) *de novo* synthesis from smaller molecules such as sugars and amino acids; (2) the incorporation or modification of ingested dietary lipids; and (3) winter-specific modifications of lipids from the first two sources. Most studies of lipid metabolism in insects have centred around lipid mobilisation and use in flight muscles or during starvation, and recent studies have used *Drosophila melanogaster* and mosquitoes as models to unravel the cellular signalling pathways that govern lipid deposition and mobilisation (see Musselman and Kühnlein, 2018, for a review). The regulation of energetics in diapausing insects (often at relatively 'warm' temperatures above 0°C) has also been investigated (Hahn and Denlinger, 2007, 2011). There are many excellent reviews of lipid metabolism and transport in insects (e.g. Blacklock and Ryan, 1994; Arrese et al., 2001; Canavoso et al., 2001; van der Horst et al., 2002; Arrese and Soulages, 2010; Van der Horst and Rodenburg, 2010; Kühnlein, 2012), so we will only briefly summarise lipid metabolism here (see Fig. 2 for a schematic diagram of lipid transport).

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List of symbols and abbreviations

AcTAG	acetylated triacylglycerol
AKH	adipokinetic hormone
AMPK	AMP-activated protein kinase
DAG	diacylglycerol
FBC	fat body cell
FFA	free fatty acid
HDLp	high-density lipophorin
lcTAG	long-chain triacylglycerol
LDLp	low-density lipophorin
NEFA	non-esterified fatty acid
PUFA	polyunsaturated fatty acid
RQ	respiratory quotient
TAG	triacylglycerol

With the caveat that the taxonomic scope of studies on insect lipid metabolism is limited, insects can generally synthesise saturated and monounsaturated fatty acids and cholesterol (from plant sterols), but cannot synthesise sterols, polyunsaturated fatty acids or carotenoids *de novo* (Arrese et al., 2001; Canavoso et al., 2001; Arrese and Soulages, 2010). There are important exceptions to these rules; for example, although members of most insect orders cannot synthesise linoleic acid (18:2*n*-6) from oleic acid (18:1), some Orthopterans, Homopterans, Isopterans and Neuropterans can (de Renobales et al., 1987), and the prevalence of Δ-12 desaturases in insects (Knipple et al., 2002) suggests that this ability could be more widespread. Similarly, although most insects can transform a wide variety of plant sterols into cholesterol, both the vinegar fly *Drosophila pachea* and the beetle *Xyleborus ferrugineus* require a source of dietary Δ^7 sterols (Heed and Kircher, 1965; Chu et al., 1970). By contrast, many groups of parasitoid wasps have no capacity for lipogenesis and must derive all their lipids from their larval hosts (Visser et al., 2010). Insects are similarly constrained in their ability to modify ingested lipids: although some transformations, such as adding or subtracting fatty acids from the glycerol backbone or shortening fatty acid chains are possible (reviewed by Stanley-Samuelson et al., 1988), transformations of the cholesterol molecule are not, and insects must prioritise ingested cholesterol to specific (e.g. endocrine) roles (Belles et al., 2005). In general, the quantity of lipid accumulated by insects is regulated by insulin-signalling pathways (DiAngelo and Birnbaum, 2009), and the molecular signalling controlling this process has been well-explored in *D. melanogaster* (Kühnlein, 2012) (see also Musselman and Kühnlein, 2018, for a review).

Long- and medium-chain fatty acids are predominantly stored as TAGs in lipid droplets in fat body cells (FBCs). FBCs form a multi-purpose tissue with roles in biochemical regulation and synthesis, energy storage and regulation, and endocrine signalling and, thus, are able to integrate and respond rapidly to lipid needs elsewhere in the insect (Arrese and Soulages, 2010). The lipid droplets are surrounded by a phospholipid monolayer with a range of inserted proteins that interact with cellular signalling pathways to regulate lipid flux from the droplets (Kühnlein, 2012). In general, adipokinetic hormone (AKH) released from the corpora cardiaca binds to receptors on the FBC surface, initiating cyclic adenosine

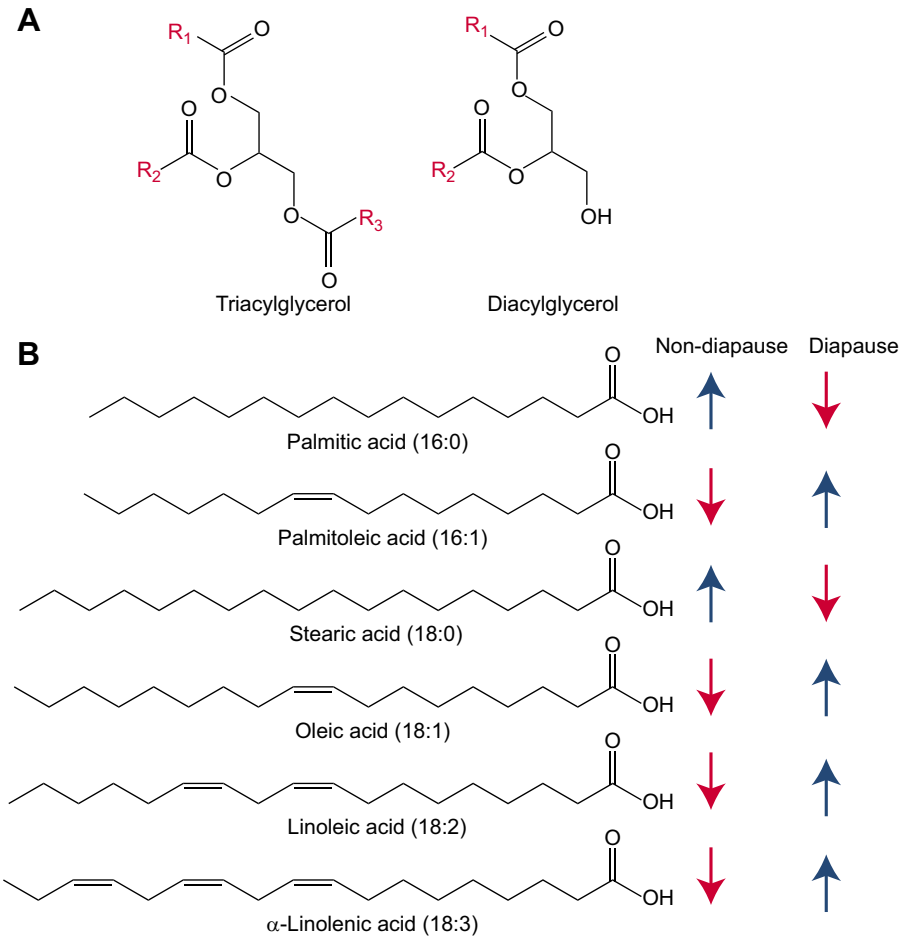

Fig. 1. Structures of storage lipids important in insect overwintering. (A) Most lipids are stored as triacylglycerols (TAGs), which have three potential fatty moieties that consist of esterified fatty acids at R_1, R_2 and R_3. During transport in the haemolymph, a fatty moiety is cleaved from TAG to form diacylglycerol (DAG). (B) Fatty acids esterified to TAGs and DAGs in overwintering insects generally include 16 and 18 carbon chain fatty acids with 0, 1, 2 or 3 double bonds. The blue and red arrows indicate a general increase or decrease, respectively, in the proportion of each fatty acid in larvae of the moth *Ostrinia nubilalis* in non-diapause and diapause states (data from Vukašinović et al., 2013).

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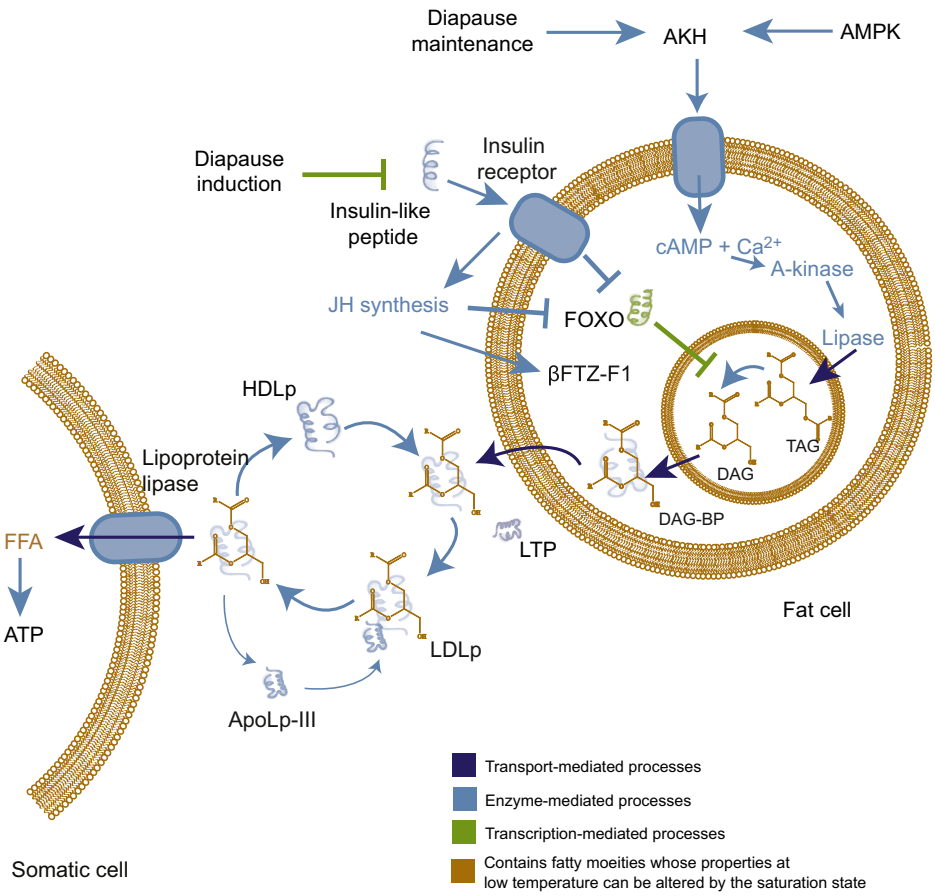

Fig. 2. A summary of lipid storage and mobilisation in overwintering insects. Generally, diapause induction involves the inhibition of insulin-like peptide production in insects, which removes the inhibiting effect of the insulin receptor on FOXO, allowing lipid accumulation to occur. During diapause maintenance, the adipokinetic hormone (AKH) is produced in response to AMP-activated protein kinase (AMPK) accumulation as well as other unknown factors, which stimulates the production of diacylglycerol (DAG) from triacylglycerol (TAG) via a cyclic adenosine monophosphate (cAMP) and Ca^{2+} signalling cascade. DAG is then exported from the lipid droplet and the cell is transported through the haemolymph by binding to high-density lipophorin (HDLp) using unknown factors with the help of a lipid transport particle (LTP), forming low-density lipophorin (LDLp). Abbreviations: ApopLp-III, apolipophorin 3; DAG-BP, diacylglycerol binding protein; FFA, free fatty acid; JH, juvenile hormone. Figure redrawn after Denlinger and Armbruster (2014) and Canavoso et al. (2001).

monophosphate (cAMP) and Ca^{2+} cascades that eventually activate triacylglycerol lipases, which cleave a fatty moiety from a TAG, resulting in a free fatty acid (FFA) and a diacylglycerol (DAG; Fig. 2) (Arrese and Soulages, 2010; Kühnlein, 2012). The activity of AKH is likely to be modulated by adenosine monophosphate-activated protein kinase (AMPK), which responds to low-energy conditions [a high AMP:adenosine triphosphate (ATP) ratio] to initiate the signal cascade that mobilises DAG (Braco et al., 2012). More than 60 AKH peptides have been identified in insects (Gäde et al., 2016). Different AKH peptides appear to selectively mobilise different metabolic fuels (van der Horst et al., 2002): for example, in locusts, different AKH peptides selectively mobilise particular molecular species of DAG depending on the saturation of the fatty acids (Tomčala et al., 2010). By contrast, *D. melanogaster* only has a single AKH (Schaffer et al., 1990; Braco et al., 2012), suggesting that some species lack this facility (or use different mechanisms) to select molecules within the lipid pool. To our knowledge, the effect of temperature on AKH action has not been explored.

The export of DAG from the cell is poorly understood, although the process appears to be associated with the lipid transfer particle (LTP; Fig. 2) (Arrese and Soulages, 2010). At the surface of the FBC, DAG binds to a high-density lipophorin (HDLp) with the assistance of several accessory molecules (e.g. Lp-1), and the resulting low-density lipophorin (LDLp) carries the DAG through the haemolymph to the active tissue (reviewed by Blacklock and Ryan, 1994; van der Horst et al., 2002; Arrese and Soulages, 2010). Although the insect lipophorin system can carry a range of lipids (including phospholipids), sterols and hydrocarbons, DAG is the primary circulating lipid destined for metabolic consumption in insects. Insect lipophorins are not restricted to shuttling lipids from FBCs to a consumer tissue: it is equally possible to acquire an ingested lipid at the gut and transport that directly to the metabolizing tissue or to the FBCs for storage (Canavoso et al., 2001; Palm et al., 2012). DAGs bound to LDLp are hydrolysed into FFAs+glycerol at the surface of the recipient cell by a membrane-bound lipase, and the FFAs are transported into the cell and translocated to the mitochondrion via fatty acid binding proteins (Fig. 2) (van der Horst et al., 2002; Van der Horst and Rodenburg, 2010). Unlike in vertebrates, HDLp is recycled in insects for additional lipid transport, increasing the efficiency, and reducing the overall cost, of lipid transport (van der Horst et al., 2002). Once in the cell, FFAs complex with the vitamin carnitine to cross the mitochondrial membrane for β-oxidation (Canavoso et al., 2001). Insects possess the biochemical machinery necessary for ketogenesis from fatty acids (Shah and Bailey, 1976). Ketosis remains under investigated in insects. Although β-hydroxybutyrate titre is used as a marker of ketosis in vertebrates (e.g. Guglielmo et al., 2005), in locusts, the primary ketone body is acetoacetate (Bailey et al., 1972); therefore, measures of β-hydroxybutyrate or β-hydroxybutyrate dehydrogenase activity may underestimate the extent of ketosis in insects.

Fat as fuel in overwintering insects

An increase in the accumulation of lipid stores is common in (most) diapause-destined or overwintering stages of insects (Hahn and Denlinger, 2007, 2011). Lipid accumulation in diapause-destined animals is governed by insulin signalling. This has been best explored in the mosquito *Culex pipiens*, where the FOXO transcription factor appears to initiate and maintain many aspects of the diapause phenotype, including fat deposition (Fig. 2; Sim and

Denlinger, 2008; Denlinger and Armbruster, 2014; Sim et al., 2015). In most insects, these accumulated lipid stores are the primary fuel for overwintering and post-winter activities.

Most overwintering insects end winter with substantially fewer lipid stores than they began with, which suggests that lipids are a primary source of overwintering fuel (Table 1; see also Wipking et al., 1995; Hahn and Denlinger, 2011; Sinclair, 2015). Most evidence of lipid use comes from studies that infer fuel use by measuring the lipid content of insects through the winter (Table 1). Only a few studies have determined fuel use using the respiratory quotient (RQ; Table 1) – such measurements are challenging because the small body size, actively suppressed metabolic rate and low body temperatures of overwintering insects impede the collection of oxygen consumption measurements that are accurate enough to estimate the RQ (Lighton, 2008). Although we are not aware of any studies that have used stable isotope-based approaches (e.g. McCue et al., 2015; Levin et al., 2017), we suggest that these approaches could be used to determine overwinter fuel use. Alternatively, enzyme activities should give some clues to overwinter fuel use: for example, lipolysis-related enzymes of the freeze-avoidant gall moth *Epiblema scudderiana* show increased activity in winter, indicating a shift to lipid metabolism (Rider et al., 2011). By contrast, the activity of lipolysis enzymes decreases over winter in the freeze-tolerant goldenrod gall fly *Eurosta solidaginis* (Joanisse and Storey, 1996), which indicates either a shift to non-lipid fuels or very low rates of lipid-fuelled metabolism. An upregulation of AMPK in both these species during winter also implies a shift towards lipolysis over winter (Rider et al., 2011).

Our assumption here has been that overwintering insects consume fatty acids solely via β-oxidation. However, insects can catabolise ketone bodies (Bailey et al., 1972) and ketosis is a source of energy for the brain in mammalian hibernation (Rauch and Behrisch, 1981). β-Hydroxybutyrate dehydrogenase activity is significantly upregulated in overwintering *E. scudderiana* moths (Joanisse and Storey, 1996), suggesting the potential for fatty acid catabolism via ketosis. To our knowledge, this possibility has not been explored further.

Because overwintering insects do not eat, they are effectively fasting and need to enhance their starvation resistance to survive for an extended period – sometimes more than six months; it is therefore useful to compare our understanding of the energetics of overwintering insects to the broader literature on starvation and fasting. Based on starvation and fasting theory, there are three main ways that insects can increase their starvation resistance: (1) increase their energy stores; (2) reduce energy consumption; and/or (3) tolerate lower energy reserves (Rion and Kawecki, 2007). However, as there appears to be little evidence for this latter strategy in either studies of starvation tolerance or overwintering insects, we shall focus on the first two strategies. Larger insects (within a species) have larger stores of lipids (and other energy sources), and individuals with more energy stores survive starvation for longer (Marron et al., 2003; Rion and Kawecki, 2007; McCue, 2010). This is consistent with increased lipid reserves in diapause-destined and overwintering insects (Hahn and Denlinger, 2007). There is some evidence that (some) starved insects reduce their metabolic rates, i.e. they reduce their energy consumption, which increases starvation survival (e.g. Djawdan et al., 1997; Marron et al., 2003; Casas et al., 2015). However, because of changing fuel use during starvation, reports of decreased metabolic rate need to be interpreted with caution (Sinclair et al., 2011). By contrast, a suppressed metabolic rate is a hallmark of diapause and overwintering (Tauber et al., 1986; Wipking et al., 1995; Danks, 2002; Hahn and Denlinger, 2011), and this depressed metabolism will be exacerbated by low temperatures overwinter. Thus, we argue that overwintering insects employ a combination of increased energy reserves and decreased metabolism to reduce starvation stress, and that the polyphenism associated with differences in these properties between overwintering and non-overwintering insects may be a suitable model for the evolution of starvation resistance in general. A more specific comparison regarding the relationship between starvation and overwintering energetics is necessary to examine the role of lipid consumption in each. In general, starved insects seem to switch from carbohydrate- to lipid- (and protein-)fuelled metabolism during the early stages of starvation (Hill and Goldsworthy, 1970; Auerswald and Gäde, 2000), and variation in the timing and thresholds for that switch appear to be associated with variations in starvation tolerance (McCue et al., 2015). This observation may apply directly to overwintering insects (although temporal shifts in the fuel source have not been well-explored), but other strategies, such as the resorption of oocytes seen in some grasshoppers (Lim and Lee, 1981), are unlikely to be used by overwintering insects.

The rate of lipid consumption by the insect over winter depends on both the mean and the variability of the temperature of the overwintering microhabitat (Sinclair, 2015). Jensen's inequality (Denny, 2017) means that highly variable temperatures in the autumn and spring can lead to disproportionately high lipid

Table 1. Examples of some common insect models in overwintering biology and their primary overwintering life stages and fuels

Order	Family	Species	Primary overwintering fuel	Life stage	Reference
Diptera	Calliphoridae	*Calliphora vicina*	Lipid	Larva	Saunders, 2000
	Sarcophagidae	*Sarcophaga crassipalpis*	Lipid, late switch to carbohydrate	Pupa	Adedokun and Denlinger, 1985
	Tephritidae	*Eurosta solidaginis*	Carbohydrate and/or lipid	Prepupa	Joanisse and Storey, 1996
		Rhagoletis cerasi	Lipid	Pupa	Papanastasiou et al., 2011
Hemiptera	Pyrrhocoridae	*Pyrrhocoris apterus*	Lipid (RQ=0.6±0.1)	Adult	Koštál et al., 2008
Hymenoptera	Megachilidae	*Megachile rotundata*	Lipid (RQ=0.6–0.7)	Prepupa	Yocum et al., 2005
		Osmia lignaria	Lipid (RQ=0.6–0.8)	Adult	Bosch et al., 2010
Lepidoptera	Crambidae	*Diatraea grandiosella*	Lipid	Larva	Chippendale, 1973
	Hesperiidae	*Erynnis propertius*	Lipid	Larva	Williams et al., 2012
	Nymphalidae	*Aglais io*	Lipid	Adult	Pullin, 1987
		Aglais urticae	Lipid	Adult	Pullin, 1987
		Danaus plexippus	Lipid	Adult	Alonso-Mejía et al., 1997
	Sphingidae	*Manduca sexta*	Lipid	Pupa	Siegert, 1986
	Tortricidae	*Choristoneura fumiferana*	Carbohydrate	Larva	Han and Bauce, 1993
		Epiblema scudderiana	Lipid	Larva	Joanisse and Storey, 1996

Most overwintering insects appear to fuel their metabolism over winter with lipid. RQ, respiratory quotient (the ratio between oxygen consumption and carbon dioxide production; conventionally a value of approximately 0.7 indicates lipid metabolism).

consumption during these seasons (Williams et al., 2012). Microhabitat selection can also determine the quantity of energy consumed – in mid-western North America, gall flies exposed to (cold) winter air temperatures consume less energy than those insulated beneath a layer of snow (Irwin and Lee, 2003). In the case of the freeze-tolerant woolly bear caterpillar, a substantial component of the lipid savings appears to be explained by caterpillars overwintering above the snow layer; these caterpillars are frozen, which presumably suppresses lipid consumption (see below) and the metabolic rate (Marshall and Sinclair, 2012). Thus, most lipid consumption by insects over the winter period probably occurs during periods of relatively warm conditions, and during early spring and late autumn when insects are not feeding but are subject to elevated metabolic rates during warm spells.

Insects use lipids remaining at the end of winter to fuel post-winter activities. Many holometabolous insects overwinter as late-instar larvae, prepupae or pupae. Some of these species can regain energy post-winter from adult feeding. For example, in the case of nectarivorous insects, nectar sugars are used for amino acid synthesis (O'Brien et al., 2002, 2004; Fischer et al., 2004); however, whether any of these sugars are allocated to lipogenesis is unclear. Other insects have little or no adult feeding – indeed, adult fall webworms (*Hyphantria cunea*) lack mouthparts, which means that adult activities are fuelled entirely by post-winter energy stores derived from larval feeding (Williams et al., 2015a). Larval lipids (and FBCs) persist through adulthood in *D. melanogaster* (Aguila et al., 2007), and eggs of many species have a significant lipid component that may be derived from these larval stores in some species. These findings suggest that energy (and particularly lipid) conservation is a key component of overwintering success and post-winter fitness in these insects (Sinclair, 2015). Fecundity is correlated with the end-of-winter lipid mass of *E. solidaginis* (Irwin and Lee, 2003) and (indirectly via body size: larger individuals have more fat) *H. cunea* (Williams et al., 2015a). Post-winter lipid stores may be used for activities other than direct investment in eggs; for example, the initial flight by monarch butterflies (*Danaus plexippus*) from overwintering sites to their first breeding site in southern USA appears to be fuelled by lipid (Brower et al., 2006). Thus, there is a close link between overwinter lipid conservation and post-winter fitness; however, this connection has not been made directly in many species. Given that the persistence of many insect populations has been linked to overwintering success (e.g. Lynch et al., 2014), investigation of this linkage may be crucial for understanding drivers of species range in insects.

Finally, although there is a clear argument based on energy density that overwintering insects should prefer lipids as a fuel, some species primarily consume carbohydrates, sometimes even when lipids are abundant. For example, overwintering 2nd instar spruce budworm (*Choristoneura fumiferana*) larvae use carbohydrates as a winter fuel source (Han and Bauce, 1993), and even *E. solidaginis* depends, at least in part, on carbohydrates (Storey and Storey, 1986). Overwinter fuel selection by insects is subject to inherent life-history trade-offs that are dependent on the role of overwintering in the lifetime energy budget of the insect. Species that cannot replenish lipid reserves post-winter may conserve them for reproductive investment, leading to the relationships with fitness that we described in the previous paragraph. By contrast, if carbohydrate stores are reserved to produce low-molecular-weight cryoprotectants such as glycerol and sorbitol (Storey, 1997), then the insect may prioritise lipid consumption to fuel overwinter energetics, making carbohydrates available for cryoprotectants (however, *C. fumiferana* produces large quantities of glycerol while overwintering; Han and Bauce, 1993). Furthermore, it appears that lipid and carbohydrate metabolism are interrelated: lipids can provide both the fuel and precursors for the production of low molecular weight cryoprotectant molecules. Lipid metabolism fuels synthesis of the disaccharide (and common cellular protectant) trehalose via β-oxidation (McDougall and Steele, 1988). Similarly, not all cryoprotectants are derived from carbohydrates: the free amino acid proline is emerging as an important cryoprotectant in freeze-tolerant insects (Koštál et al., 2011, 2016). Although protein or carbohydrates are the most likely source for this proline, in exercising insects, proline is derived from fatty acid precursors via acetyl Co-A in the fat body (Arrese and Soulages, 2010) and, therefore, it is possible that lipids could be an important precursor in overwintering insects as well, although this has not been explored. In spite of these exceptions, the majority of overwinter (and post-winter) metabolism in the majority of temperate insects appears to be fuelled by fat.

Using fats at subzero body temperatures

Thus far, we have focused on whether insects use fat to fuel their overwinter energy demands. As small ectotherms in sub-freezing temperatures, low temperatures are likely to modify the use and utility of lipids in the cold. Although metabolic rates decrease markedly in the cold, it is possible to detect metabolism in cold – and even frozen – animals (Sinclair et al., 2004), suggesting that cellular metabolism continues, albeit at a low rate. Here, we will examine how cryoprotectants, ice formation and the physical properties of lipids interact with this metabolism.

At low temperatures, fats and other neutral lipids solidify, with the melting point dependent primarily on the saturation and length of fatty acyl chains. The melting point of triolein (three 18:1 *n*-9 fatty moieties esterified to a glycerol backbone, Fig. 1) is +5°C (Hagemann et al., 1972): even monounsaturation is not sufficient for lipids to remain liquid under mildly cold conditions. Because lipids must be liquid for lipases to function, insects must therefore maintain fluid lipids if they are to be metabolically accessible below zero. Thus, just as for phospholipids, where changes in saturation are associated with maintaining fluidity in the cold (Somero et al., 2017), insects modify the fatty acid composition of their lipids prior to overwintering. For example, both *E. solidaginis* and *E. scudderiana* have more unsaturated lipids in winter than in summer (Joanisse and Storey, 1996) and, as a consequence of this, *E. solidaginis* has lipid droplets that remain sufficiently liquid to coalesce when the cytoplasm of the cell freezes (Salt, 1959; Lee et al., 1993). Similarly, the proportion of polyunsaturated (18:2) fatty acids in the storage lipids of codling moth (*Cydia pomonella*) larvae increases during winter, with a consequent 5–10°C decrease in the melting point of the storage lipids (Rozsypal et al., 2015). Although vertebrates generally cannot synthesise polyunsaturated fatty acids (PUFAs), or desaturate monounsaturated fatty acids to generate PUFAs, several groups of insects (notably excluding *Drosophila*) appear able to do so (de Renobales et al., 1987; Stanley-Samuelson et al., 1988). The biochemical machinery involved in PUFA synthesis has not been explicitly explored in overwintering insects; however, moths, for example, usually possess a Δ12-fatty-acid-desaturase (Knipple et al., 2002), which is likely to explain observations of winter shifts towards PUFAs. However, unsaturated fatty acids are particularly susceptible to peroxidation at the hydrogen next to the double bond, causing the eventual production of reactive aldehydes such as malondialdehyde.

Indeed, repeated freezing of *E. solidaginis* has been shown to increase lipid peroxidation (Doelling et al., 2014).

The variety of methods used to characterise lipids in overwintering insects provide very different information and, hence, determining which lipids are accumulated and used by insects over winter has been a challenge (Williams et al., 2011). The main quantitative methods make important assumptions: measurements obtained using gravimetric methods (which involve extraction using a non-polar solvent) include not only FFAs and acyl glycerols but also sterols and waxes, which are not energy sources. Similarly, the widely used vanillin assay quantifies only the presence of double bonds on fatty acids; the vanillin assay is suitable as an index under the assumption that there is no change in fatty acid saturation among treatments and seasons – such an assumption seems particularly inappropriate for studies of overwintering insects. A common enzymatic approach is to use lipases to cleave fatty acids from the glycerol backbone and then measure the glycerol spectrophotometrically; however, this provides poor information about the energy content of the fatty acids, and does not account for the heavy use of DAGs in insect lipid transport (Tennessen et al., 2014). Thin-layer chromatography coupled to flame ionisation detection (Iatroscan) enables neutral lipid species to be separated; however, this method provides little information about fatty acid composition and, therefore, fluidity of the molecules. Gas chromatography coupled to mass spectrometry (GC-MS) may solve some of these difficulties because it enables the fatty moiety identity in each of the major lipid classes to be determined (Borrull et al., 2015; Li et al., 2015). Although researchers have chosen each of these methods for good reasons, the wide range of methods used has made it difficult to ascertain whether there is a 'general' picture of lipid accumulation and use in overwintering insects.

Even if lipid droplets remain liquid and biochemically accessible, the enzymes associated with lipid mobilisation and metabolism also need to remain functional in the cold for insects to fuel low-temperature activities via lipolysis. To our knowledge, the sub-zero activity of lipolytic enzymes has not been investigated. Furthermore, cold-hardy insects often accumulate very high extra- and intra-cellular concentrations of low molecular weight cryoprotectants [e.g. >1 mol l^{-1} glycerol in some species (Lee, 2010)]. The resulting haemolymph viscosity, combined with slow or no heart pumping below the critical thermal minimum (Overgaard and Macmillan, 2017), leads us to speculate that the transport of both AKH signals to mobilise lipids, and the transport of DAG from FBCs to consumer cells, will be greatly reduced if not completely halted (Fig. 2). Thus, at subzero temperatures, cells are likely to rely on endogenous supplies of fuel. The extent to which lipids are stored in tissues other than FBCs and, therefore, are available to fuel metabolism in the absence of transport has not been investigated.

Transport is likely to be further curtailed in the (frozen) haemolymph of frozen freeze-tolerant insects. The standard model of insect freeze tolerance suggests that ice is confined to the haemolymph (Sinclair and Renault, 2010) and although a fraction of the haemolymph remains unfrozen under this model, this concentrated fraction is likely to exacerbate any viscosity problems with transport. There is evidence for at least some intracellular ice formation [specifically, in the FBCs of *E. solidaginis* (Salt, 1959; Lee et al., 1993)], which should further prevent lipid metabolism. The impact of high concentrations of solutes on enzyme structure and function in frozen insects has not been explored. Although increased substrate and enzyme concentrations could potentially increase reaction rates (a problem that may occur in frozen foods; Frelka et al., 2015), this may also increase the rate of non-specific binding – reducing reaction rates. Similarly, whether the function of some enzymes is preserved in overwintering insects under these conditions is unknown.

β-Oxidation of fatty acids requires oxygen (which does not permeate ice) to finally produce ATP by shuttling high-energy electrons through the electron transport chain and, hence, oxygen supply may be an additional problem associated with lipid metabolism while frozen. The larger tracheae do not appear to be crushed during ice formation in freeze-tolerant fly larvae (Sinclair et al., 2009), and there is at least some evidence of continued gas exchange in frozen caterpillars (Sinclair et al., 2004). However, *E. solidaginis* accumulates anaerobic end products while frozen (Storey and Storey, 1986), suggesting a shift to anaerobic, carbohydrate-fuelled glycolysis. Thus, we do not know whether lipid metabolism occurs in frozen insects; however, the most parsimonious interpretation is that although lipids are likely to fuel the bulk of overwinter energy demands (in spring and autumn, when temperatures are relatively high), metabolism at very low temperatures and while frozen is likely to be carbohydrate fuelled. We have previously used this interpretation in a model of the metabolism of overwintering frogs (Sinclair et al., 2013b), and it could explain the lipid savings we observed in frozen caterpillars in the field (Marshall and Sinclair, 2012).

Unusual lipids and lipid derivatives

Although fats are clearly important as a fuel source for many overwintering insects, there is a diverse range of neutral lipids and their derivatives, and some of these can have functional roles during winter. Here, we outline two examples: antifreeze glycolipids and acetylated triacylglycerols (acTAGs), and we speculate about potential roles for FFAs in overwintering insects.

Preventing ice formation or managing the structure and distribution of ice that does form is a key component of freeze-avoidance and freeze-tolerance strategies (respectively) for overwintering insects (Zachariassen and Kristiansen, 2000). Antifreeze proteins and glycoproteins are perhaps the most celebrated of the molecules associated with low-temperature tolerance and have been well-characterised in insects (Doucet et al., 2009; Duman, 2015; Bar Dolev et al., 2016). Recently, a xylomannan glycolipid with activity similar to that of well-known antifreeze proteins was identified in a freeze-tolerant Alaskan beetle (Walters et al., 2009); these glycolipid antifreeze proteins have subsequently been found in a range of overwintering insects (Walters et al., 2011). These glycolipids consist of a repeated xylose-mannose backbone with fatty acyl moieties attached (Fig. 3A). Although the exact structure is uncertain, a related patent (Walters et al., 2013) suggests several possible functional conformations. The discovery of these antifreeze glycolipids demonstrates that lipids and their derivatives may have functional roles in overwintering, and (as -omics data become increasingly available for overwintering insects) cautions against the wholesale characterisation of differentially regulated lipid metabolism genes as solely representing changes in lipid metabolism at the expense of considering other functional responses.

acTAGs (Fig. 3B) form the majority of the neutral lipid pool (by mass) of overwintering *E. solidaginis* (Marshall et al., 2014). These acTAGs differ from the more common long-chain triacylglycerols (lcTAGs) in that they have an acetyl group rather than a fatty moiety at the third position on the glycerol backbone. It appears that these acTAGs are produced by acetylation of the existing lcTAG pool:

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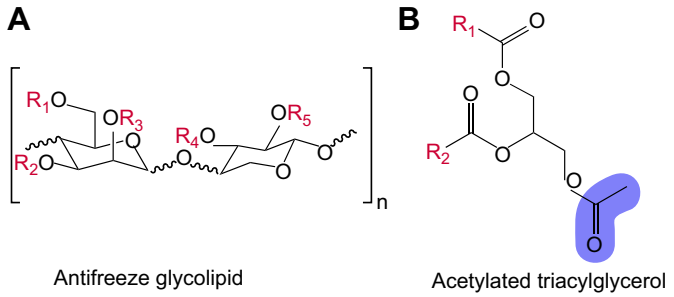

Fig. 3. General structures of two lipids with potential cryoprotective activity. (A) Antifreeze glycolipids have been described from several overwintering insects, frogs and plants, and consist of a repeating mannose-xylose disaccharide (where *n*=5 to 70), with fatty moieties either electrostatically or covalently bonded at each of the R groups (where R can also include no fatty moiety). Structure based on Walters et al. (2009, 2011, 2013). (B) Acetylated triacylglycerols have been described from the freeze-tolerant pre-pupae of the goldenrod gall fly *Eurosta solidaginis* (Marshall et al., 2014), and have the usual assortment of fatty moieties at positions R_1 and R_2 (see Fig. 1B); however, they have an acetyl group esterified instead of a third fatty moiety (highlighted in blue).

there is stoichiometric balance between acTAGs, lcTAGs and non-esterified fatty acids, and acTAGs appear to be rapidly converted back into lcTAGs in a stoichiometric ratio in the spring (Marshall et al., 2014). acTAGs contain one fewer fatty moiety than lcTAGs and, therefore, have lower energy density (Durrett et al., 2010). However, acetylation lowers the melting point of acTAGs to –17°C (relative to 5°C for the nearest equivalent lcTAG), which allows them to remain liquid at subzero temperatures. Given that acTAGs are found in FBC lipid droplets, rather than the membrane, this may explain observations of lipid coalescence in isolated frozen FBCs (Salt, 1959; Lee et al., 1993). Given that acTAGs are slightly polar and interact with water (Marshall et al., 2014), they may play a role in the cryoprotection of FBCs, which survive intracellular freezing (Salt, 1959). An alternative form of cryoprotection could be via the predicted ability of acTAGs to form micelles in solution (Goto et al., 1992). Finally, we speculate that acetylation may enable storage lipids to remain liquid with a reduced peroxidation risk associated with unsaturation. However, no other insect (or indeed any other animal) accumulates large quantities of acTAGs and, therefore, it is unclear to what extent the synthesis of acTAGs is responsible for (or is driven by) the unique aspects of the life history and biology of *E. solidaginis*.

The lipid component of overwintering insects has seldom been separated into neutral lipid classes, partly owing to technical reasons; in particular, non-esterified fatty acids are not usually isolated and quantified separately from DAGs and TAGs. However, in the study mentioned above (Marshall et al., 2014), the synthesis of acTAGs by *E. solidaginis* was accompanied by a 1:1 increase in the concentration of non-esterified fatty acids (NEFAs). It is possible that the increase in the concentration of NEFAs is simply a side-effect of the production of acTAGs, and we do not know whether these NEFAs are bound to lipophorins or are FFAs. It is possible that FFAs could be used as fuel [e.g. by providing ketone bodies for brain metabolism, as they do in hibernating mammals (Rauch and Behrisch, 1981)]. However, FFAs can have functional roles, particularly in innate immunity, where they are associated with both immune signalling and direct bactericidal effects (Hwang, 2000; Desbois and Smith, 2010). Because these bactericidal effects are not enzyme-mediated, we speculate that they may be less temperature-sensitive than other components of the insect immune system and, therefore, may play an important role in the immunity of overwintering insects [see Sinclair et al. (2013a) for discussion]. Furthermore, *E. solidaginis* appears to have increased humoral immunity in mid-winter (Ferguson and Sinclair, 2017) – coinciding with the time point when the concentration of NEFAs is highest (Marshall et al., 2014). The abundance and identity of FFAs in other overwintering insects is, to our knowledge, only poorly understood. Thus, an opportunity exists to explore the functional role of FFAs in overwintering insects.

Conclusions

Although our understanding is still incomplete, lipid metabolism is clearly an important component of insect overwintering. In particular, we lack knowledge about the extent to which lipids are the primary fuel for overwintering insects, how lipid use is regulated, and whether and how insects have overcome some of the substantial barriers that might prevent lipid use, especially at subzero temperatures. The identification of functional roles for some lipids in insects also highlights the potential for these molecules (which are abundant and ubiquitous) to play important roles that we do not yet understand.

At a broader scale, our understanding of the mobilisation and consumption of lipids in insects is largely derived from studies of starving or flying insects. Although there are parallels between these processes and metabolism over winter, we argue that fuel selection and energy use in overwintering insects is not well-enough understood to conclude that the starvation literature can be directly applied to overwintering. To the extent that they have been investigated, overwintering insects appear to be using the same lipolytic machinery as better-understood species, although studies of the biochemistry of lipid consumption over winter are largely confined to a few studies of two gall-forming species by Storey and colleagues (e.g. Storey and Storey, 1986; Joanisse and Storey, 1996) and signalling pathway studies of diapausing mosquitoes (Denlinger and Armbruster, 2014). There is a significant opportunity to expand this small repertoire, and to leverage the substantial emerging picture of the cellular and endocrine pathways regulating lipid metabolism in *D. melanogaster* to thoroughly investigate the mechanistic basis of lipid use in overwintering insects. Finally, we hope that we have provided some useful hypotheses to stimulate future investigations of fuel selection and lipid metabolism in insects when exposed to low temperatures. Given the importance of overwintering to the population persistence and distribution of many insect species, unravelling the processes underlying lipid usage over winter may be the key to better understanding the mechanistic drivers of these patterns.

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Competing interests

The authors declare no competing or financial interests.

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