Many parasitoids lack adult fat accumulation, despite fatty acid synthesis: A discussion of concepts and considerations for future research

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Abstract

Fat reserves, specifically the accumulation of triacylglycerols, are a major energy source and play a key role for life histories. Fat accumulation is a conserved metabolic pattern across most insects, yet in most parasitoid species adults do not gain fat mass, even when nutrients are readily available and provided *ad libitum*. This extraordinary physiological phenotype has evolved repeatedly in phylogenetically dispersed parasitoid species. This poses a conundrum because it could lead to significant constraints on energy allocation toward key adult functions such as survival and reproduction. Recent work on the underlying genetic and biochemical mechanisms has spurred a debate on fat accumulation versus fat production, because of incongruent interpretation of results obtained using different methodologies. This debate is in part due to semantics, highlighting the need for a synthetic perspective on fat accumulation that reconciles previous debates and provides new insights and terminology. In this paper, we first clarify the distinct metabolic pathways involved in the complex process of lipogenesis and propose updated, unambiguous terminology for future research in the field, including “fatty acid synthesis” and “lack of adult fat accumulation”. Second, we discuss the benefits and drawbacks of the main methods available to measure fatty acid synthesis and adult fat accumulation. Most importantly, gravimetric/colorimetric and isotope tracking methods provide complementary information, provided that they are applied with appropriate controls and interpreted correctly. Third, we compiled a comprehensive list of fat accumulation studies performed during the last 25 years. Last, we present avenues for future research that combine chemistry, ecology, and evolution into an integrative approach, which we think is needed to understand the dynamics of fat accumulation in parasitoids.

Key words

Hymenoptera; Parasitic wasp; Lipids; Lipogenesis; Life histories; Nutrient metabolism

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1. Introduction

Lack of fat accumulation in adult insect parasitoids has been considered a major constraint shaping life histories. More than 25 years ago, the first observation showed that adult parasitoids do not increase their fat reserves when provided carbohydrates *ad libitum* (Ellers, 1996), for which the term "lack of lipogenesis" was coined. The dependence of adult parasitoids on host fat carried over from larval feeding has been cited as a prime example of repeated evolution (van der Kooi and Schwander, 2014; Keymer and Gutjahr, 2018). The lack of adult fat accumulation has further impacted strategies for the use of parasitoids as biocontrol agents. For example, better knowledge about inter-strain differences in larval fat accumulation on different hosts, as well as the ability of adults to accumulate fat when provided poor larval hosts may help biocontrol companies or end users to select the best strains given the hosts available in their areas (de Freitas Bueno et al., 2012). Knowledge on fat accumulation could further help to understand what dietary requirements should be provided during mass rearing of both larvae and adults to produce high-performing adult parasitoids for biological control programs (Liu et al., 2015; Weber et al., 2020, p. 20). Most of the conceptual work on "lack of lipogenesis" has focused on a lack of fat accumulation in adults as a physiological trait that may have cascading effects on parasitoid life histories.

A series of studies that have tracked the fates of metabolic substrates within adult parasitoids using stable isotopes have challenged the concept of "lack of lipogenesis" in parasitoids (Multerer et al., 2022). Although adults of most parasitoid species lack fat accumulation, there is evidence for fatty acid synthesis in limited quantities in several species

(Giron and Casas, 2003; Visser et al., 2017, 2021; Prager et al., 2019; Ruther et al., 2021) (Table 1). Yet, the fact that adult parasitoids can synthesize limited quantities of fatty acids does not nullify previous results on the lack of fat accumulation or the conceptual ramifications of those results for parasitoid life histories.

The current controversy regarding the “lack of lipogenesis” in adult parasitoids seems to be fueled predominantly by semantic confusion due to unclear terminology used by earlier studies (Visser and Ellers, 2008; Visser et al., 2010; Ruther et al., 2021; Multerer et al., 2022). Here, we aim to resolve the apparent conflict by clarifying the distinction between lipogenesis, adult fat accumulation, and fatty acid synthesis by providing clear definitions. Subsequently, we highlight differences in lipid metabolism between non-parasitoid and parasitoid insects. We then emphasize methodological considerations when measuring fatty acid synthesis and fat accumulation in adult parasitoids. We revisit the main question as to why parasitoids lack fat accumulation while giving due attention to the broader physiological, ecological, and evolutionary contexts for this observation, in addition to the more narrow biochemical perspective of lipid metabolism.

Table 1: List of hymenopteran parasitoid species for which studies have looked at three different measures in adults: fat accumulation, gene transcription of genes involved in fatty acid synthesis, and/or fatty acid synthesis in adults. "Yes" refers to an increase in accumulated fat, gene transcripts or fatty acids; "no" refers to similar or lower amounts. The methodology used is indicated in superscript: g = gravimetry, c = colorimetry, i = isotope tracking, t = gene transcription study. We report the number of species tested and the number of studies in bold at the end of the table.

Species	**Fat accumulation**	**Gene transcription**	**Fatty acid synthesis**	**Reference**
Acrolyta nens	yes^{g}	-	-	(Visser et al., 2010)
Anisopteromalus calandrae	-	-	yes^{i}	(Ruther et al., 2021)
Apanteles aristoteliae	no^{c}	-	-	(Lightle et al., 2010)
Aphelinus abdominalis	no^{g}	-	-	(Visser et al., 2010)
Aphidius ervi	no^{g}	-	-	(Visser et al., 2010)
Aphidius rhopalosiphi	no^{g}	-	-	(Visser et al., 2010; Le Lann et al., 2012)
Aphidius picipes (=avenae)	no^{g}	-	-	(Visser et al., 2010; Le Lann et al., 2012)
Aphidius platensis	no^{c}	-	-	(Souza et al., 2018)
Asobara tabida	no^{g}	-	-	(Ellers, 1996; Visser et al., 2010; Le Lann et al., 2014)
Asobara tabida	-	-	yes^{i}	(Ruther et al., 2021)
Baryscapus tineivorus	-	-	yes^{i}	(Ruther et al., 2021)
Cephalonomia tarsalis	-	-	yes^{i}	(Ruther et al., 2021)
Cotesia glomerata	no^{g}	-	-	(Visser et al., 2010)
Cotesia rubecula	no^{g}	-	-	(Visser et al., 2010)
Diadegma insulare	no^{c}	-	-	(Lee et al., 2004)
Dibrachys cavus	-	-	yes^{i}	(Ruther et al., 2021)
Diglyphus isaea	no^{c}	-	-	(Zhang et al., 2011)
Eupelmus vuilletti	no^{c}	-	-	(Casas et al., 2015; Muller et al., 2017)
Eupelmus vuilletti	-	-	no^{i}	(Giron and Casas, 2003)
Exeristes comstockii	-	-	yes^{i}	(Thompson and Barlow, 1972)
Gelis agilis	yes^{g}	-	-	(Visser et al., 2010)
Gelis agilis	-	-	yes^{i}	(Visser et al., 2017)
Gelis areator	-	-	yes^{i}	(Visser et al., 2017)
Goniozus nephantidis	no^{g}	-	-	(Visser et al., 2010)
Goniozus legneri	no^{g}	-	-	(Visser et al., 2010)
Habrobracon hebetor	no^{c}	-	-	(Gündüz et al., 2010; Işitan et al., 2011)

Habrobracon hebetor	-	-	yes[i]	(Ruther et al., 2021)
Lariophagus distinguendus	-	-	yes[i]	(Ruther et al., 2021)
Leptopilina boulardi	no[g]	-	-	(Visser et al., 2010)
Leptopilina boulardi	yes[g+c] + no[g+c]	-	-	(Moiroux et al., 2010, 2012)
Leptopilina heterotoma	no[g]	-	-	(Eijs et al., 1998; Visser et al., 2018)
Leptopilina heterotoma	yes[g]	-	-	(Visser et al., 2010)
Leptopilina heterotoma	yes[g] + no[g]	-	-	(Le Lann et al., 2014)
Leptopilina heterotoma	yes[g] + no[g]	-	yes[i] + no[i]	(Visser et al., 2021)
Leptopilina heterotoma	-	-	yes[i]	(Ruther et al., 2021)
Lysibia nana	yes[g]	-	-	(Visser et al., 2010)
Macrocentrus grandii	no[g]	-	-	(Olson et al., 2000)
Meteorus pulchricornis	no[g]	-	-	(Sheng et al., 2019)
Meteorus pulchricornis	no[g]	yes[t]	-	(Wang et al., 2020)
Microplitis mediator	no[g]	-	-	(Luo et al., 2010)
Muscidifurax raptorellus	-	-	yes[i]	(Ruther et al., 2021)
Muscidifurax uniraptor	-	-	yes[i]	(Ruther et al., 2021)
Nasonia vitripennis	no[c]	-	-	(Rivero and West, 2002)
Nasonia vitripennis	no[g]	no[t]	no[i]	(Visser et al., 2012)
Nasonia vitripennis	-	no[t]	-	(Lammers et al., 2019)
Nasonia vitripennis	-	-	yes[i]	(Prager et al., 2019; Multerer et al., 2022)
Nasonia vitripennis	no[i]	-	yes[i]	(Ruther et al., 2021)
Nasonia giraulti	-	-	yes[i]	(Prager et al., 2019)
Nasonia longicornis	-	-	yes[i]	(Prager et al., 2019)
Neochrysocharis formosa	no[c]	-	-	(Wang et al., 2014)
Orthopelma mediator	no[g]	-	-	(Visser et al., 2010)
Pachycrepoideus vindemmiae	no[g]	-	-	(Visser et al., 2010)
Pimpla turionellae	no[c]	-	-	(Ortel, 1991)
Pteromalus bedeguaris	no[g]	-	-	(Visser et al., 2013)
Pteromalus puparum	yes[g]	-	-	(Visser et al., 2010)
Spalangia erythromera	no[g]	-	-	(Visser et al., 2010)
Spalangia cameroni	no[c]	-	-	(Taylor et al., 2022)
Tachinaephagus zealandicus	no[g]	-	-	(Lammers et al., 2020)
Tachinaephagus zealandicus	-	-	yes[i]	(Ruther et al., 2021)
Trichogramma evanescens	-	-	yes[i]	(Ruther et al., 2021)
Trichopria drosophilae	no[g]	-	-	(Visser et al., 2010)
Urolepis rufipes	-	-	yes[i]	(Ruther et al., 2021)
Venturia canescens	no[c]	-	-	(Casas et al., 2003; Gomes et al., 2021)
No. of species/No. of studies	**35/29**	**2/3**	**20/8**	

2. Solving the semantics on lipid metabolism

For over a decade, the term “lack of lipogenesis” has been used in research to describe the extraordinary observation that insect parasitoids typically do not accumulate fat stores as adults (Visser and Ellers, 2008). Fat, in the form of triacylglycerols, is generally the main energetic reserve for insects (Box 1)(Arrese and Soulages, 2010). Yet in contrast with other insects, adults of most parasitoids do not accumulate substantial fat reserves when provided carbohydrates *ad libitum* (Visser et al., 2010). This lack of adult fat mass gain has so far been demonstrated in more than 29 parasitoid species (Table 1). However, our prior use of the term “lack of lipogenesis” has admittedly been a poor choice because lipogenesis is an overarching term that covers two major biochemical processes: first, the conversion of glucose or other substrates into fatty acids, and second the synthesis of triacylglycerols (Kersten, 2001) (Box 1). Lipogenesis is often referred to in the context of the accumulation of triacylglycerols or fat stores, further providing potential confusion. Earlier studies by some of us and others have neglected to distinguish between these components, treating lipogenesis as a ‘black box’ with glucose as input and stored triacylglycerols as output. As more sophisticated molecular and chemical analyses have become commonplace, the focus of discussions has shifted from an organismal-level evolutionary perspective to obtaining mechanistic insights in the underlying pathways of lipid metabolism in parasitoids (Kraaijeveld et al., 2019; Lammers et al., 2019; Ruther et al., 2021). This shift in perspectives within the literature necessitates an update to provide precise terminology for separate and specific processes related to fat metabolism and accumulation in parasitoids.

We propose that practitioners in the field use the more accurate terms “fatty acid synthesis” and “fat accumulation” to distinguish between the two major processes of past focus (Box 1). The term fat accumulation has indeed been used in a wide range of taxa to describe the process of bulk storage of triacylglycerols (e.g., Pullin, 1987; Teixeira et al., 2003; Schmid et al., 2005; Guo et al., 2008; Sinclair and Marshall, 2018; Didion et al., 2021). Fat or triacylglycerols can be synthesized from dietary carbohydrates, fatty acids, or proteins (Arrese and Soulages, 2010). These triacylglycerols are stored within finely regulated sub-cellular structures called lipid storage droplets. Within the insect fat body, more than 90% of the contents of lipid storage droplets is composed of triacylglycerols (Arrese and Soulages, 2010).

By contrast, the term “fatty acid synthesis” more narrowly defines and denotes the conversion of acetyl-CoA and malonyl-CoA into fatty acids (Box 1; Figure 1). While fatty acids can be incorporated into triacylglycerols, they can also be used for many other physiological processes. These include, but are not limited to, the production of *i)* cuticular lipids, *ii)* pheromones, and *iii)* diglycerides, which can be used as cell-signaling molecules or converted into phospholipids (Stanley-Samuelson et al., 1988; Howard and Blomquist, 2005; Arrese and Soulages, 2010; Stanley and Kim, 2019; Toprak et al., 2020). Given the wide range of potential functional fates for fatty acids synthesized by insects, a clear distinction between fatty acid synthesis and the accumulation of stored fat is crucial for understanding the biological significance and life history consequences of adult parasitoid fat reserves.

Box 1 Definitions

Lipid: A class of molecules usually composed of carbon chains (or carbon rings, e.g., sterols) that can either be amphipathic, possessing both polar and non-polar groups (e.g., phospholipids), or purely hydrophobic, containing only non-polar, neutral groups (e.g., triacylglycerols). Lipids can be classified into simple (e.g., fatty acids, acylglycerols) and complex (e.g., phospholipids, sterols)(Garrett and Grisham, 1999; Bailey Jr and Bailey, 2000).

(True/neutral) fat reserve/stored fat: Common name for triacylglycerols.

Triacylglycerol (TAG)(also known as triglyceride (TG), triacylglyceride): Molecule consisting of three fatty acids and a glycerol molecule (i.e., a triester) and the form in which lipids are stored in the insect fat body (Bailey Jr and Bailey, 2000, p. 200; Arrese and Soulages, 2010; Williams et al., 2011). Triacylglycerol is the correct chemical name, but triglycerides and triacylgycerides are also commonly used interchangeably.

Fat synthesis: Esterification of fatty acids to glycerol, a process forming triacylglycerol.

Fat accumulation: Bulk storage of fat in the insect fat body.

Fatty acid: Long-chain carboxylic acid (10 – 24 carbon atoms). Palmitate (palmitic acid; C16:0), stearate (stearic acid; C18:0), and oleate (oleic acid, C18:1) are generally the most common fatty acids in insects (Bailey Jr and Bailey, 2000; Chapman, 2013).

Fatty acid synthesis: The conversion of acetyl-CoA and malonyl-CoA (derived from sugars, carbohydrates or proteins) into fatty acids. The saturated fatty acid palmitate (C16:0) is the first to be produced by the fatty acid synthesis pathway through the action of fatty acid synthase (FAS). Other enzymes (e.g., desaturases, elongases) then add double bonds and additional carbon units to the chain to produce more complex fatty acids, e.g., polyunsaturated fatty acids (PUFAs), e.g., linoleic acid (C18:2) containing multiple double bonds (Garrett and Grisham, 1999).

Lipogenesis: Entails the entire process from the conversion of glucose or other substrates into fatty acids to the synthesis of triacylglycerols (Kersten, 2001).

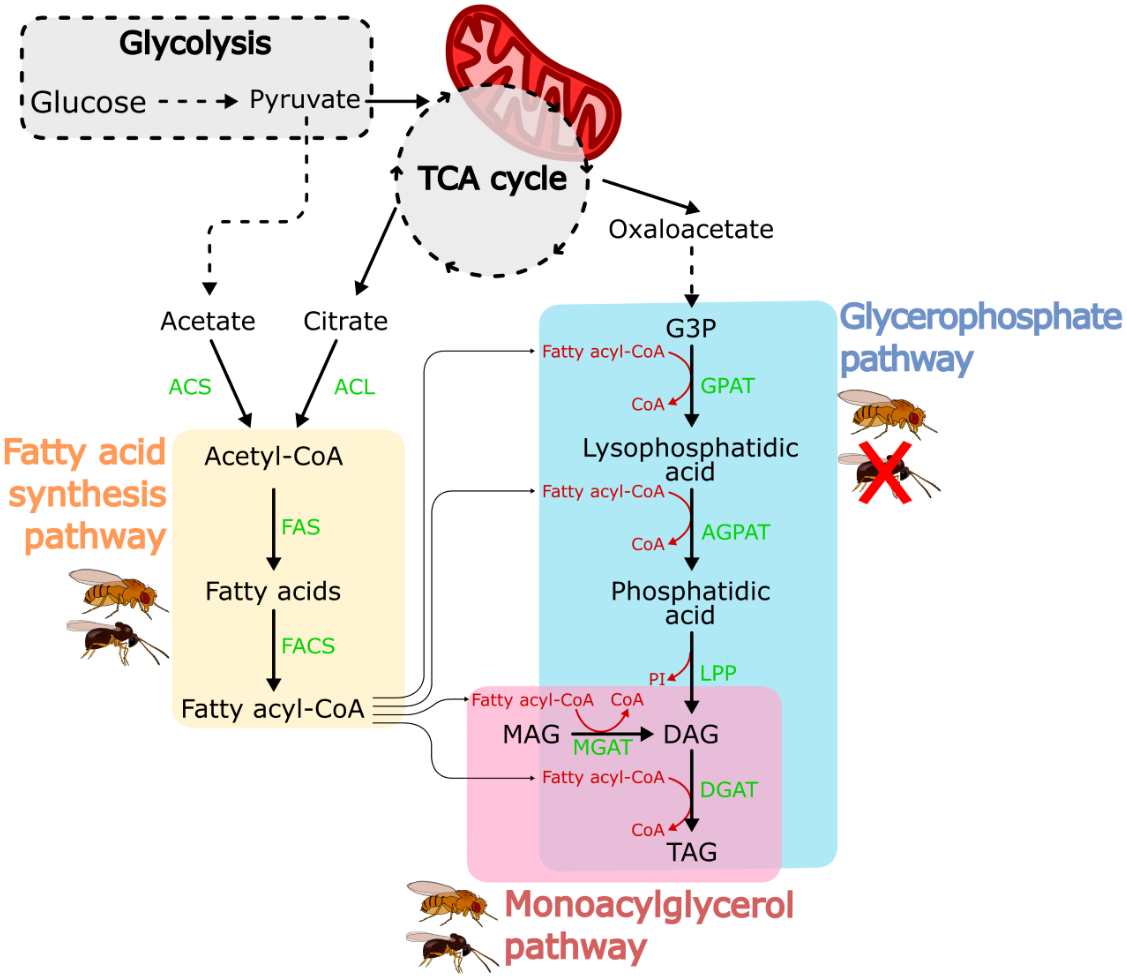

Figure 1. Lipid synthesis in adult parasitoid and non-parasitoid insects. Ingested sugars are converted to pyruvate through glycolysis, after which pyruvate is either converted to acetate or used in the tricarboxylic acid (TCA) cycle to produce substrates for triacylglycerol (TAG) synthesis (citrate and glycerol-3-phosphate, i.e., G3P). Acetate and citrate are converted to acetyl-CoA that enters the fatty acid synthesis pathway (left), and oxaloacetate is used to produce G3P that enters the glycerophosphate pathway (right). Triacylglycerols are also synthesized through the monoacylglycerol pathway. All three pathways are functional and active in insects in general. In contrast, in some parasitoid species larvae do not seem to synthesize triacylglycerols *de novo* through the glycerophosphate pathway, leading to a lack of triacylglycerol accumulation (Barlow and Jones, 1981; Jones et al., 1982). The majority of adult parasitoids also lack triacylglycerol accumulation (see Table 1). Depicted metabolic pathways are simplified with enzymes highlighted in green, and enzymatic reactions shown in red. Abbreviations: ACL, ATP-citrate lyase; ACS, Acetyl-coA synthetase; FAS, Fatty acid synthase; FACS, Fatty acyl-coA synthetase; GPAT, glycerophosphate acyltransferase; AGPAT, acylglycerophosphate acyltransferase; LPP, lipid phosphate phosphohydrolase; DGAT, diacylglycerol acyltransferase; MGAT, monoacylglycerol acyltransferase; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; TCA cycle: tricarboxylic acid cycle.

3. Fatty acid synthesis and fat accumulation in parasitoids versus other insects

In insects, the consumption of sugars and other carbohydrates that form a major part of their diet leads to the synthesis of fatty acids that can subsequently be used for the synthesis of triacylglycerols (Arrese and Soulages, 2010). Insects generally do indeed increase fat reserves when feeding on an excess of sugar-rich sources (Service, 1987; Chapman, 2013). Two direct precursors of triacylglycerols are diacylglycerols and monoacylglycerols (Figure 1). The synthesis of tri- and diacylglycerols from monoacylglycerols is referred to as the monoacylglycerol synthesis pathway. The monoacylglycerol pathway can readily use lipid molecules produced during catabolism, such as degraded phospholipids or deacylated triacylglycerols through the action of lipases. Triacylglycerols can also be synthesized *de novo* through the glycerophosphate pathway (also called the Kennedy pathway; Figure 1). In insects, both pathways can be used to synthesize triacylglycerols (Beenakkers et al., 1985; Soulages et al., 2015).

For adult parasitoids, most species do not accumulate triacylglycerols in adulthood (Visser et al., 2010)(Table 1; Figure 2). Using bulk fat quantification methods, this lack of adult fat accumulation is represented by stable or decreasing fat content compared between individuals at emergence and after feeding on sugars (Figure 2A; middle, right panel). The unusual nature of this pattern becomes immediately clear when comparing fat accumulation in adult parasitoids with other insects that have a more typical adult fat accumulation phenotype, where stored fat quantities significantly increase in feeding adults, such as in *Drosophila melanogaster* (Figure 2A, left panel). Given this pattern of depletion of fat stores in adult parasitoids rather than accumulation, one might predict that adult parasitoids synthesize no fatty acids *de novo*. However, the lack of triacylglycerol accumulation in adults may also be due to low fatty acid synthesis, metabolism of synthesized fatty acids for purposes other than triacylglycerol accumulation, or a combination of both (Giron and Casas, 2003; Visser et al., 2012, 2017; Prager et al., 2019; Ruther et al., 2021; Multerer et al., 2022).

Several studies using sensitive isotopic tracking methods have shown that some adult parasitoid species can synthesize small quantities of fatty acids (Giron and Casas, 2003; Visser et al., 2017, 2021; Prager et al., 2019; Ruther et al., 2021; Multerer et al., 2022) . For example, recently Ruther et al. (2021) used a sensitive quantitative method for stable isotope tracing of carbon from glucose into fatty acids followed by mass spectrometry to show that some parasitoid species, including *N. vitripennis,* synthesized fatty acids from glucose. In a follow-up study, Multerer et al. (2022) showed that newly synthesized fatty acids could be incorporated into matured eggs. However, despite the demonstration of limited amounts of fatty acid synthesis, no adult fat accumulation was noted in *N. vitripennis* (Ruther et al., 2021), even though the metabolic pathways and associated genes are present and functional (Werren et al., 2010; Visser et al., 2012; Kraaijeveld et al., 2019; Lammers et al., 2019). Furthermore, when compared to stable isotope incorporation from glucose to fatty acids in *D. melanogaster*, the degree of fatty acid synthesis was much lower in the parasitoids *E. vuilletti* and *N. vitripennis*, lacking adult fat accumulation, compared to *D. melanogaster*, a species that readily accumulates fat stores in adulthood (Figure 2B, C). We, therefore, think it is critical to place rates of fatty acid synthesis in adult parasitoids into context with rates of fatty acid synthesis in insects that show the more typical pattern of adult fat accumulation.

So far, the exact physiological mechanism underlying the lack of adult fat accumulation has remained elusive. For parasitoid larvae, early work revealed that compared to typical insect triacylglycerol synthesis only few triacylglycerols were synthesized *de novo* through the glycerophosphate pathway. The majority of triacylglycerols were produced through the monoacylglycerol pathway (i.e., from lipid molecules produced during catabolism of host tissues, see Figure 1)(Barlow and Jones, 1981; Jones et al., 1982). For larvae of the parasitoids *Exeristes roborator* and *Itoplectis conquisitor*, 75% and 97% of triacylglycerols are formed from diacylglycerols through the action of the monoacylglycerol pathway, respectively. Substantial inhibition of the *de novo* triacylglycerol synthesis pathway could be due to a lack of need in larvae feeding on lipid-rich host tissues (Barlow and Jones, 1981; Jones et al., 1982). This could also explain the lack of triacylglycerol accumulation observed in adults. Further investigation of the relative activities of the monoacylglycerol and glycerophosphate pathways using specific isotope tracking experiments in both parasitoid larvae and adults are needed to understand how each of these biochemical pathways are associated with adult lack of fat accumulation as a trait.

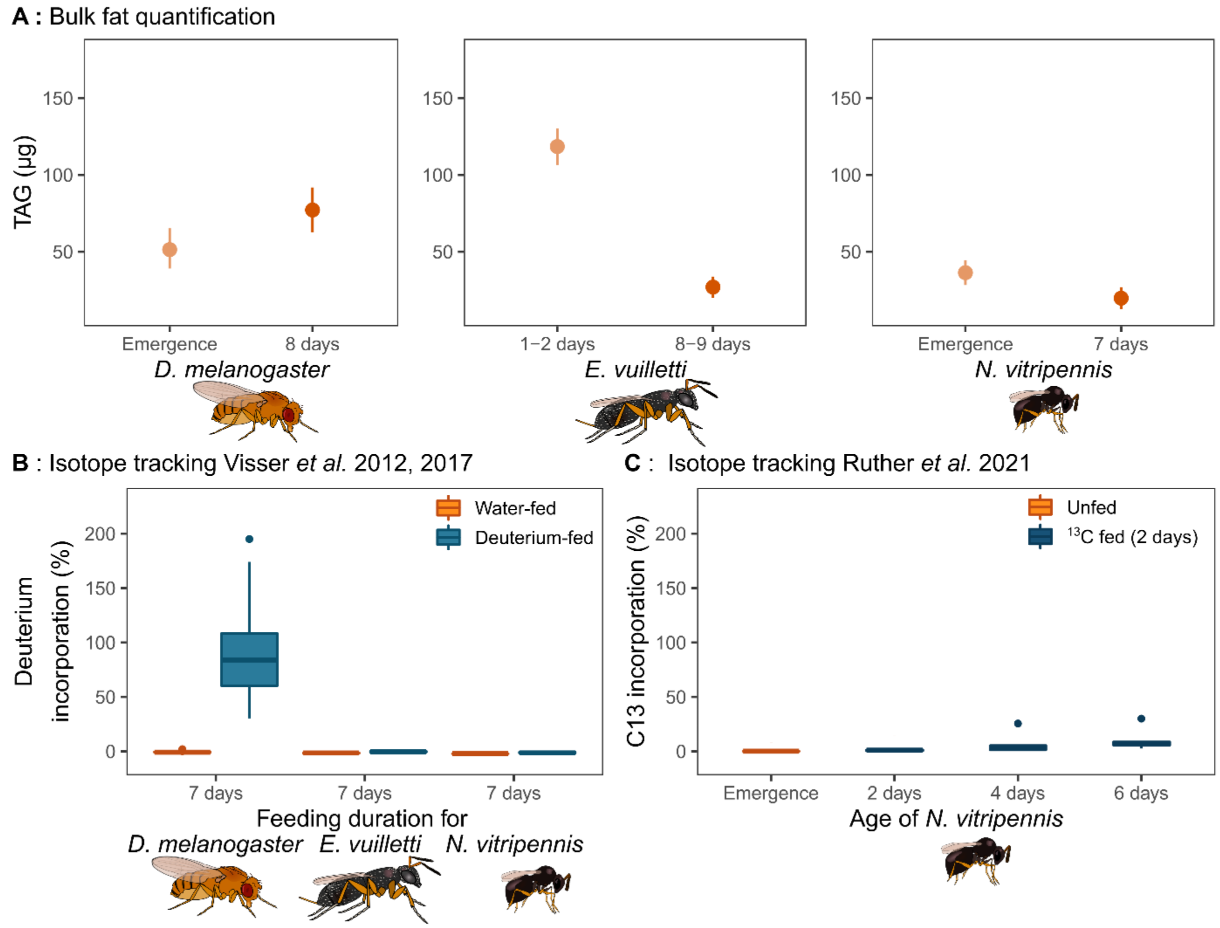

Figure 2. Fat accumulation (TAG) and fatty acid synthesis (% isotope incorporation) in the non-parasitoid *D. melanogaster* and two parasitoids. (A) Mean triacylglycerol quantities (in µg with 95% confidence intervals) in female *D. melanogaster* (redrawn from Shirazi et al. 2014), and in the parasitoids *Eupelmus vuilletti* (redrawn from Muller et al. 2017) and *N. vitripennis* (based on data from Visser et al. 2012) at emergence and after 7 to 9 days of feeding on sugars. (B) Incorporation of the stable isotope deuterium (%) into the fatty acid palmitic acid (C16:0) in *D. melanogaster*, *E. vuilletti* and *N. vitripennis* females (based on data from (Visser et al., 2012, 2017). In these experiments, females were either fed a sugar/honey solution with or without deuterated water. Percent deuterium incorporation was calculated for molecules with 1, 2, or 3 incorporated deuterons (m+1 to m+3) that were subsequently summed to capture the total amount of newly synthesized C16:0 fatty acids. (C) ^{13}C-incorporation (%) into palmitic acid (C16:0) in *N. vitripennis* females at emergence, and in females that had access to hosts for 0, 2, or 4 days followed by 2 days of feeding on fully ^{13}C-labelled α-D-glucose (Ruther et al., 2021).

4. Considerations when measuring fat accumulation and fatty acid synthesis in parasitoids

Methods for quantifying fat accumulation

A variety of methods can be used to measure fat accumulation in insects when adults have access to a dietary source of sugars and/or proteins, but the different methods do not necessarily provide the same information about the phenotype. Here, we address the main methods used, as well as their limits and advantages, for extracting and quantifying fat. These methods include gravimetry (based on weight, as in Visser et al., 2010, 2012, 2018, 2021), or colorimetry (conversion of chromophores measured by light absorbance, as in Muller et al., 2017), as well as enzymatic assays and chromatography/mass spectrometry methods. We summarize these methods as well as their pros and cons in Table 2.

Typically, experiments testing adult fat accumulation compare recently emerged individuals to individuals that had access to food (Figure 2A). A significant increase in fat quantity is considered indicative for fat accumulation. A lack of significant fat accumulation, on the other hand, cannot be taken as proof that no fatty acids are being synthesized. This is because the net increase in fat reserves depends on the rates at which lipids are metabolized (Giron and Casas, 2003; Visser et al., 2010, 2012), which could happen when lipid use (i.e., catabolism) exceeds lipid synthesis (i.e., anabolism). In other words, adult parasitoids could simply be producing and turning over fatty acids and triacylglycerols very quickly, so they do not accumulate stored fat.

Table 2: Overview of different methods for estimating adult fat accumulation in parasitoids (and other insects), including their pros and cons. Based on Williams et al. (2011) where gravimetric and colorimetric methods were compared to results of TLC followed by GC-FID.

Method			
	Description	**Pros**	**Cons**
Gravimetry (Ether extraction)	Extracts neutral lipids (mainly triacylglycerols)	- A good predictor of triacylglycerol content - No need for high-tech equipment	- Not suitable for differentiating subtle variation in lipid content - No distinction between classes of neutral lipids
Gravimetry (Folch extraction = chloroform:methanol) (Folch et al., 1957)	Non-polar neutral lipids extracted with chloroform. Polar lipids extracted with methanol	- A repeatable method for total lipid extraction - No need for high-tech equipment	- Overestimation of total lipid content, as polar lipids (and other compounds) are also extracted. Not suitable when the insect contains large quantities of polar non-water solutes (e.g., glycerol in overwintering insects). - No distinction between classes of neutral lipids
Colorimetry (generally Folch extraction, vanillin reagent) (van Handel, 1985)	Sulphuric acid hydrolyzes triacylglycerols releasing fatty acids that, in turn, react with sulphuric acid to form chromogens. The latter then react with vanillin leading to a	- Accurate and repeatable when using a mix of fatty acids in standard curve	- Vanillin reacts with monounsaturated fatty acids; hence this method is not suitable for comparisons where fatty acid unsaturation levels are expected to vary between treatments (e.g.,

	colour change, measurable with spectrophotometry (compared to a standard curve)		between seasons, differential consumption during migration) - Underestimation of total triacylglycerol content
Enzymatic assay (chloroform extraction)	Based on hydrolysis of triacylglycerols to glycerol and free fatty acids by a lipase of bacterial origin	- Commercially available	- Measures only glycerol - Underestimates total lipid content, less repeatability compared to other methods
Quantitative thin Layer Chromatography – Flame Ionisation Detector (GC-FID)	Separation of different lipid types (mono, di, triacylglycerols, cholesterol esters etc…)	- High accuracy and repeatability in estimating lipid quantities	- Costly
Gas Chromatography (e.g., GC-MS, GC-MS-MS, LC-MS, etc...)	Estimation of triacylglycerol content based on separation of derivatized fatty acids on a column and estimate of peak area of separated fatty acids	- C16 and C18 often represent majority of the triacylglycerol content (e.g., >95% in Wood, Harlow, and Lambremont 1969)	- Requires derivatization to fatty acids Potentially underestimates triacylglycerol content if not all fatty acids are considered - Costly
Liquid Chromatography (e.g., LC, HPLC, HPLC-MS, etc…)	Estimation of triacylglycerol content based on separation of lipids in a solvent on a column	- High accuracy and repeatability in estimating lipid quantities when compared to known standards on the same system	- Costly

Methods for quantifying fatty acid synthesis

Isotope tracking

Isotope labelling methods (e.g., radioactive isotopes such as ^{14}C and tritium - ^{3}H, or stable isotopes like deuterium-^{2}H or ^{13}C) allow tracing the metabolic conversion of sugars or other substrates into fatty acids (e.g., Giron and Casas, 2003; Visser et al., 2012, 2017, 2021; Muller et al., 2017; Prager et al., 2019; Ruther et al., 2021; Multerer et al., 2022). There are several methodological considerations regarding the isotope-tracking method to avoid misinterpretation of results that we feel is important to discuss here.

Appropriate controls

To assess if fatty acid synthesis is occurring in different parasitoid species, and understanding the magnitude of difference from a typical insect, incorporation levels of tracers into fatty acids need to be compared with a positive control: a non-parasitoid insect (e.g., *D. melanogaster, Apis mellifera*; Figure 2)(Visser et al., 2012, 2017). Isotope-tracing studies further require appropriate negative controls. Incorporation rates should be compared between wasps that are fed at comparable levels with and without the isotope. If one compares, for example, isotope-fed individuals with starved individuals, two different and potentially confounding factors are varied at the same time: *i)* isotope labelling (yes or no) and *ii)* feeding status (fed vs starved)(Prager et al., 2019; Ruther et al., 2021; Multerer et al., 2022). This is because starvation is well-known to affect many aspects of intermediary metabolism, including fatty acid synthesis and lipid metabolism in general (Chapman, 2013).

The choice of isotopes

In isotope-labeling studies, the choice of isotope matters. For example, there is some controversy regarding the use of ^{13}C labeling techniques for measuring fatty acid synthesis, because discrimination against ^{13}C incorporation into the lipid fraction increases with dietary ^{13}C concentration (Wessels and Hahn, 2010). The advantage of using the equally stable hydrogen isotope, deuterium, is that it is the cheapest and easiest method to measure fatty acid synthesis from acetate, glucose, or other precursors (i.e., proteins)(Murphy, 2006). In parasitoids, the tracing of deuterium was indeed successfully used for tracking synthesis of fatty acids through the incorporation of hydrogen from heavy (deuterated) water (Visser et al., 2021). An alternative to using deuterated water is to use deuterated glucose (Wallace et al., 2018) or deuterated acetate (Votruba et al., 2001), which have so far not been applied in parasitoid studies. Furthermore, ^{13}C and ^{2}H can be combined to parameterize metabolic reactions more fully.

Metabolic flux

To date, only few studies have investigated how isotope incorporation into fatty acids relates to triacylglycerol accumulation (Visser et al., 2012, 2021). The question is whether isotope incorporation into fatty acids can reveal any information about the fat accumulation phenotype. To do this, isotopes need to be traced into accumulated fat stores and we need to understand the turnover (i.e., breakdown) of stored triacylglycerols. We suggest that future research may benefit from explicitly comparing short-term, pulse-chase isotopic labeling and longer-during labeling experiments, or even alternating labels to more carefully distinguish patterns of metabolic flux in parasitoids. Moreover, typical stable isotope studies only focus on a subset of fatty acids and broader monitoring of the fates of isotopic labels into a range of fatty acids and fatty acid-derived compounds may provide additional perspectives (but see Wood, Harlow, and Lambremont 1969 showing that in a lepidopteran 95% of the fatty acids used in triacylglycerols are C16 and C18 fatty acids).

In conclusion, there is no singular suitable method to study fatty acid synthesis and the lack of fat accumulation in parasitoids. Both types of methodologies (gravimetry/colorimetry and isotope tracking) offer valuable and complementary information, provided that they are applied and interpreted appropriately. Ideally, these two methodologies should be used concomitantly to understand how they relate to each other and whether estimated rates of fatty acid synthesis could be interpreted as a good estimate of total triacylglycerol accumulation or turnover.

5. Current research and perspectives

More than 25 years after the first discovery of lack of adult fat accumulation in a parasitoid species (Ellers, 1996), the question still stands - why do most adult parasitoids lack fat accumulation despite possessing functioning biochemical machinery for fat synthesis? The original hypothesis was that adult parasitoids carry over substantial fat quantities from their larval hosts, rendering fat accumulation during the adult stage unnecessary (Visser and Ellers, 2008; Visser et al., 2010). It was thought that the high fat stores at adult emergence are sufficient for lifetime investment in eggs and survival (Ellers, 1996). We now know that the fat synthesis and accumulation phenotype is more complex (Ruther et al., 2021; Visser et al., 2021). Several wasp species can synthesize fatty acids, but only few species accumulate fat as adults (Visser et al., 2010) whereas others do not (Ruther et al., 2021), and this can be plastic in some species (Visser et al., 2021). Whether fatty acid synthesis thus far observed in parasitoids is biologically relevant for adult fat reserves versus other functions like membrane maintenance, cuticular lipids, or signaling remains to be tested. In the next few paragraphs, we emphasize important avenues for future research on fat accumulation in parasitoids.

Evolutionary patterns of fat accumulation phenotypes

Triacylglycerol accumulation in adult stores can be absent or present (i.e., constitutively expressed) or be plastic depending on the environment. For example, adults of the amber wasp *Leptopilina heterotoma* generally synthesize fatty acids and accumulate triacylglycerols in significant quantities when larval development occurs on lean hosts and carbohydrates are provided *ad libitum* (Visser et al., 2021; Quicray et al., 2023)(but see (Eijs et al., 1998) where no fat was accumulated despite low teneral fat content). In contrast, when *L. heterotoma* larvae develop on fat hosts, no fatty acid synthesis and triacylglycerol accumulation takes place in adults (Visser et al., 2021). A threshold for switching fat synthesis and accumulation on or off may be dependent on the genetic background and local environmental conditions (i.e., host quality in terms of fat content through space and time, temperature). We now need to determine if plasticity in fatty acid synthesis and fat accumulation traits is more common throughout the parasitic Hymenoptera, using both isotope labelling and bulk fat estimation methods (Table 1). Indeed, for some species the evolution of a complete lack of fat synthesis and accumulation can be expected. For example, *A. tabida* and *E. vuilletti* have strong behavioral preferences for fat-rich host stages that may provide sufficient fat in the larval diet to support the needs of adults.

At the other extreme, constitutive fat synthesis and accumulation may be necessary for species that cannot rely on fat-rich hosts, for example generalists such as *Gelis* species, that emerge with only 4-8% body fat (Visser et al., 2017; Visser, Le Lann, et al., 2010). Additional synthesis and accumulation of fat reserves is then required during adult life (Visser et al., 2010, 2017).

Life history consequences of distinct fat accumulation phenotypes

To study the impact of plastic fat synthesis and accumulation on life histories, *L. heterotoma* is an excellent model because distinct fat synthesis and accumulation phenotypes can easily be generated in the laboratory using hosts that vary in fat content (Visser et al., 2021; Enriquez et al., 2022). This plasticity in initial fat loads (fat versus lean) and propensity to accumulate fat in adulthood provides opportunities to phenotypically engineer a gradient of fat storage and accumulation phenotypes that both encompasses and exceeds natural variation in these physiological traits. This gradient can then be used to determine potential consequences on adult life history traits, such as fecundity, egg size and composition, dispersal ability, longevity, etc. Our predictions are that if fat loads at adult emergence are low, the ability to synthesize and accumulate fat from adult feeding will improve some of the life history traits listed above. However, when parasitoid larvae are developing on lean hosts, adult fat accumulation may not yield life history benefits as large as adults emerging with high fat loads (as a consequence of developing on high-fat hosts). In other words, we expect that parasitoids will benefit from a capital-breeding life history with regard to fat compared to an income-breeding life history, perhaps due to the greater costs of adult fat synthesis compared to simply acquiring fat when feeding on the host as a larva.

The role of the microbiota in fatty acid synthesis

Another unresolved concept to be tested is whether the parasitoid microbiome may play any role in synthesizing fatty acids or other aspects of lipid metabolism in adult parasitoids. It is possible that the fatty acids that have been observed to be synthesized within adult parasitoid wasps may not actually be the products of the wasp cells, but instead could be produced by the wasp microbiota. The (gut) microbiota could contribute to the production of some fatty acids that are subsequently absorbed by the adult wasp digestive tract. Bacteria generally use type II dissociate fatty acid synthesis and possess all the necessary genes for fatty acid synthesis (Rock and Jackowski, 2002). Nevertheless, it currently remains unclear what role the parasitoid microbiota may play in adult parasitoid lipid physiology. Much progress could be made in this area by first simply testing for fat synthesis and accumulation in axenic parasitoids that are completely free of bacteria, then in gnotobiotic parasitoids experimentally colonized with bacterial strains that possess or lack lipid synthetic ability.

The physiological and genomic mechanisms of fat accumulation phenotypes

The evolution of the lack of adult fat accumulation was initially investigated only at the phenotypic level (Visser et al., 2010). Genes and enzymes involved in fatty acid synthesis and fat accumulation were later found to remain functional and intact in several parasitoid species (Visser et al., 2012, 2017, 2021; Kraaijeveld et al., 2019; Lammers et al., 2019; Prager et al., 2019; Ruther et al., 2021). This points towards the evolution of regulatory rather than structural genomic changes. There are, however, still substantial gaps in our knowledge of the physiological and genetic mechanisms leading to diverse fat synthesis and accumulation phenotypes in parasitoids. We still need to determine which biochemical and genetic pathways promote or deter accumulation of fat in adult parasitoids. Previous work in parasitoid larvae revealed that the *de novo* triacylglycerol pathway appears to be non-functional, but not the monoacylglycerol pathway (Barlow and Jones, 1981; Jones et al., 1982)(Figure 1). Only three genes are part of the *de novo* triacylglycerol pathway: glycerol-3-phosphate acyltransferase (*gpat*), 1-acylglycerol-3-phosphate O-acyltransferase 1 (*agpat*), and lipid phosphate phosphohydrolase (*lpp*). In *N. vitripennis*, qRT-PCR revealed that wasps feeding on sugar for 1 to 3 days significantly upregulated *agpat* (Visser et al., 2012). The only gene that did not respond to long-term feeding was *gpat*, which could be a prime candidate gene for future work exploring the biochemical basis of lack of fat accumulation. Testing for fat accumulation phenotypes using multiple parasitoid and non-parasitoid species could shed further light on the molecular basis. For species that do accumulate sufficient triacylglycerol stores as adults, we can manipulate expression of candidate genes using RNA interference (e.g., Guo et al. 2008) or create deletion mutants using tools like CRISPR-Cas9 (Sander and Joung, 2014) to recreate a lack or a low fat accumulation phenotypes.

The biological relevance of synthesized fatty acids

Another promising avenue for future research is to better understand the different biochemical purposes of the fatty acids that have been observed to be synthesized in adult parasitoids. The maintenance of the functional genetic and enzymatic machinery for fatty acid synthesis in species where substantial fat accumulation does not occur in adults raises the question of what synthesized fatty acids are used for, if not lipid storage. For instance, fatty acids can undergo different steps of desaturation and elongation from precursors for the production of pheromones used for sexual communication (Jurenka, 2004), the production of eicosanoids for immune function (Stanley and Kim, 2019), or as part of homeoviscous adaptation to maintain proper membrane function under stressful environmental conditions (Hazel, 1995), among other potential functions. In the parasitoid *N. vitripennis* that was found not to accumulate fat as an adult (Visser et al., 2012; Ruther et al., 2021), Multerer et al. (2022) found that synthesized fatty acids were present in substantial quantities in eggs. Thus it is possible that the ability to synthesize fatty acids in adulthood has a positive effect on parasitoid lifetime reproductive output. However, Multerer et al. (2022) compared fatty acid synthesis in adults that were fed ^{13}C-labeled carbohydrates with adults only provided water, thus covarying carbohydrate starvation with lack of fatty acid synthesis. To test the extent to which the ability to synthesize fatty acids contributes to adult parasitoid reproduction, carbohydrate feeding and fatty acid synthesis must be experimentally decoupled using tools like RNAi targeting fatty acid synthesis enzymes (as described above) or pharmacological inhibitors of fatty acid synthesis (Popham and Chippendale, 1996; Toth et al., 2005). Our understanding of why some parasitoid species display a greater ability for fatty acid synthesis than others might thus depend on the specific biological functions for which fatty acids and their derivatives are used by a certain species (Malcicka et al., 2018).

Lack of fat accumulation as a model for resisting obesity

Understanding lipid metabolism and accumulation in parasitoids may provide insights that could ultimately be useful to biomedical applications. Because excessive fat accumulation typically leads to obesity-related pathologies in insects, insects can be used as models for research in obesity and metabolic syndrome (Schilder and Marden, 2006; Musselman and Kühnlein, 2018; Walkowiak-Nowicka et al., 2021). In this vein of thinking, a useful question is to what extent do parasitoids that can become very fat (up to 40% of the dry weight) suffer negative fitness consequences? Under the premise that fat parasitoids will shut off fat accumulation completely while continuing to feed on sugars, and do not seem to suffer any negative consequences of being fat, we expect that they could serve as excellent model systems able to resist obesity-related pathologies. To test this, one could measure phenotypic traits characterizing typical pathologies associated with obesity in flies and humans by comparing leaner and very fat adult parasitoids. Experiments for determining obesity-related pathologies in *D. melanogaster* include: *i)* comparisons of sugar and carbohydrate metabolism to determine if a high-sugar diet can induce hyperglycemia and insulin resistance (Musselman et al., 2011), *ii)* heartbeat parameters (Fink et al., 2009) and electrical pacing to determine heart functioning and heart failure rate (Wessells and Bodmer, 2004; Wessells et al., 2004), *iii)* flight exercise and endurance to measure muscle function, as well as *iv)* food intake rates and consumption (Ja et al., 2007) to determine functioning of the central nervous system.

6. Conclusions

We urge that future work on lipid metabolism in parasitoids uses more precise definitions that clearly identify the processes/phenotypes under consideration. The unambiguous terminology proposed here aims to distinguish, both conceptually and experimentally, the differences between fatty acid synthesis and fat accumulation. We believe that this will unite researchers to understand the biological relevance of the lack of adult triacylglycerol accumulation in parasitoids and avoid misleading conclusions (e.g., that adult parasitoids in general can replenish ebbing fat resources through substantial fat accumulation (Kaczmarek and Boguś, 2021)). Combining biochemistry, physiology, ecology, and evolution into a truly interdisciplinary and integrative approach is needed to further our understanding of the dynamics of fat accumulation in parasitoids, and ultimately the downstream life-history consequences of accumulating stored nutrients, or not, from adult dietary intake.

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