NEW FAT FUELS FROZEN FLIES

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Goldenrod gall flies are one of Canada's toughest animals, and are able to survive freezing of their body fluids. Since the flies can't eat all winter, we wanted to know what happens to their fat stores following these freezing events. We found a surprise—gall flies have an unusual kind of fat that stays liquid at low temperature and seems to help them survive the winter.

INTRODUCTION

During winter, animals that spend the winter in Canada and other temperate environments deploy many different strategies for surviving. Some find a warm spot like an insulated burrow for shelter, while others just tough it out. One of the most cold-tolerant animals in Canada is the goldenrod gall fly (*Eurosta solidaginis*). Goldenrod gall flies lay their eggs in early summer in growing goldenrod plants (Figure 1). Once a larva hatches, its chewing induces the goldenrod plant to make a gall around it, protecting it through the summer as it eats. In the fall, the goldenrod plant dies with the larva still inside. The larva spends the winter inside the gall, and in the spring it pupates, metamorphoses into a fly, then emerges and mates.

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Goldenrod gall fly larvae spend the winter above the snow in the gall, freezing solid every time the temperature falls below -9 °C. During the winter, their metabolic rate is suppressed, they do not eat, can survive hypoxia (lack of oxygen), and tolerate being frozen solid.

Many ectothermic (cold-blooded) animals that live in polar and temperate climates can survive freezing; these include the woolly bear caterpillar, the wood frog, and the painted turtle. Generally, these animals survive freezing by preventing ice from forming inside their cells. They use ice nucleators (which 'seed' ice formation in specific locations) to initiate ice formation in the hemolymph (insect blood). Because only water is incorporated into ice, all of the other molecules in the hemolymph (including cryoprotectants – the sugars and other molecules that the animal accumulated to protect against cold) are left in the unfrozen component, which becomes more and more concentrated. This high concentration of sugars outside of the cell draws water from inside the cell to outside cells through osmosis. This means that the remaining water in the cells has too much concentrated solute to freeze – and the cells, although shrunken, have avoided freezing.

Overwintering goldenrod gall fly larvae, however, are an exception to this general process. These larvae can withstand ice formation inside at least one cell type – the fat body cells. Fat body cells are used by insects to store carbohydrates and fats, synthesize cryoprotectants, and they function almost like the insect equivalent of the liver. The goldenrod gall fly's fat body cells can even nucleate ice. Reginald Salt, a Canadian scientist working in Lethbridge, Alberta, first described the ability of these cells to survive intracellular freezing (ice formation inside the cell) in 1959. He also noted that even when the cells were frozen, there were unfrozen globules of fat in the cells.

Since gall flies experience very cold conditions in their galls above the snow through the winter, we were interested in what happens to their fat stores over the winter. While that's still an interesting question, along the way we uncovered a biochemical mystery—an unusual form of fat in the gall flies. We identified

 DOI: 10.13034 / JSST-2015-009

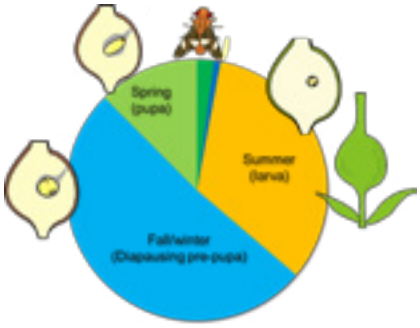

Figure 1.

The life cycle of the goldenrod gall fly. Drawings supplied by Dr. Hiroko Udaka.

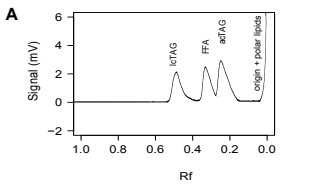

Figure 2.

Characterization of the major neutral lipid classes found in Goldenrod gall flies. (a) Chromatogram from TLC-FID of the neutral lipid composition of goldenrod gall flies showing: lcTAG (long-chain triaclyglycerols), FFA (free fatty acids), and acTAG (acetylated triaclyglycerols) after separation in (70:30:05) benzene: chloroform: formic acid (v/v/v). The origin includes any remaining polar lipids that will not migrate in the solvent system. (b) General structure of lcTAGs: glycerol backbone with three fatty acids attached by ester bonds. (c) General structure of acTAGs: glycerol backbone with two fatty acids attached by ester bonds, plus an acetyl group on the third position of the glycerol.

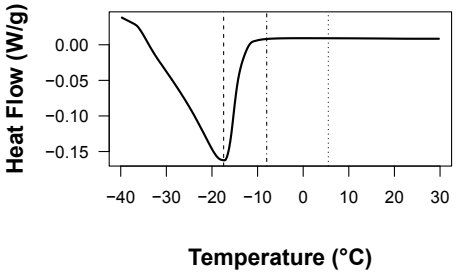

Figure 3.

Seasonal changes in acTAG concentration of goldenrod gall flies. The neutral lipid pool of goldenrod gall flies shifts dramatically from late summer until early spring (2011-2012) in relation to life stage (indicated by arrows: a, onset of freeze tolerance; b, pupation; and c, eclosion).

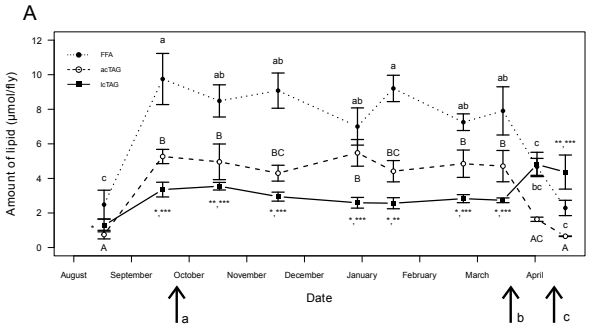

Figure 4.

Heat flow during melting of acTAGs extracted from goldenrod gall flies. Dashed line indicates melting point of acTAGs. Dot-dash line indicates whole-body supercooling point of goldenrod gall flies. Dotted line indicates melting point of triolein (lcTAG with 18:1 fatty acids esterified).

the unusual fats, and did some additional experiments to shed light on why the gallfly might be producing them.

TYPES OF FATS

Every organism has lots of different kinds of fats that serve different important roles in the body. Some fats make up lipid membranes, some are used as the building blocks of hormones, while others store chemical energy. Each kind of fat has a different structure that fits the function it will be performing in the body. These different structures give each kind of fat a different polarity, which makes some fats better or worse at dissolving in chemicals like chloroform. We first extracted all the fats out of the fly using chloroform, then used a technique called thin-layer chromatography coupled to flame ionization detection (TLC-FID) to separate the different kinds of fats. Thin-layer chromatography (the "TLC") separates each kind of fat by its polarity—how well it dissolves in chloroform determines the position of the fat after it has been soaked along a thin silica-covered rod; fats that dissolve best in chloroform moving furthest from the point where the fat extract was placed on the rod. A detector then moves along the rod, burning off the fats to measure how much material is at each point (that's the flame ionization detection part of the name). While this type of procedure is particularly good at measuring how much of each type of fat there is in an animal, it doesn't identify the structures of each one. Instead, it can help identify what fats might be present by comparing the distance that

fats with a known structure travel down the rod to the distance unknown fats travel. When we looked at the fats that were in the gall fly, we found that almost one third of all the fat didn't end up matching polarity with any known fat (Figure 2A).

At this point, we turned to colleagues who were specialists in identifying unknown chemicals. First, a graduate student at the University of Toronto named Áron Roxin developed a method for purifying the unknown fat from all the others in the gall fly. Then a postdoctoral fellow at the University of Western Ontario named Eric Chen took that purified fat and used a technique called proton nuclear magnetic resonance (H1 NMR) that identified the position and numbers of all the protons in the unknown fat. Finally a postdoctoral fellow at the University of Western Ontario named Raymond Thomas used gas chromatography coupled with mass spectrometry (GC-MS) and mass spectrometry-mass spectrometry (MS-MS). In GC-MS, acid is first added to break any ester bonds in the unknown, then methanol is added to form methyl esters where the original esters occurred. This step increases the polarity of unknown compounds, which helps them become volatized so they can travel down a column at different speeds based on their polarity. Finally at the end the MS bombards the unknown with electrons, breaking the chemical into fragments that can be identified by their mass to charge ratios. In MS-MS, this occurs twice—the unknown chemical is first bombarded once to break the molecule into large pieces, then each of those pieces is bombarded to break it into even smaller pieces. By knowing the number and size of each piece of molecule, the original can be reassembled.

Finally with all the evidence we were able to identify the molecule as acetylated triacylglycerols (acTAGs). These are fats that look a lot like long-chain triacylglycerols (lcTAGs, Figure 2B), which are the common storage lipid in animals, except that instead of having three fatty acids per molecule, our molecules only had two fatty acids along with an acetyl group (Figure 2C). This was rather odd—while these fats had been found in very small quantities in the burning bush plant, as well as mammals like the Japanese deer and domestic cow, there was no report of an animal making large quantities of it. Fatty acids are the molecules in lcTAGs that store chemical energy, and it was surprising that an animal that had to survive all winter without eating would store so much energy in a molecule with only two fatty acids rather than three. So next we needed to understand why gall flies might be making acTAGs.

WHY DO GOLDENROD GALL FLIES MAKE acTAGs?

We first tried to answer this question by looking at other very cold tolerant insects that live near the goldenrod gall fly, including freeze tolerant caterpillars, wasps, and beetles. Using the TLC-FID, we found that no other species produced acTAGs, and that the plant the gall fly eats doesn't either. Then we looked at how much acTAG goldenrod gall flies had at different times of year. We found in the spring and summer flies didn't produce very much, but in the fall and winter goldenrod gall flies suddenly made lots (Figure 3). So we concluded that acTAGs must have something to do with surviving the winter, which made us think about the gall fly's extreme freeze tolerance.

We decided to stress flies out by freezing them many times, like they would experience in a cold winter. We froze some flies for 120 hours at -20 ºC, while we froze others ten times for 12 hours each at -20 °C. All of them survived (which is impressive!), and we found the flies that had frozen repeatedly made more acTAGs than the flies that only froze once, or not at all. This led us to believe that something about the way that acTAGs handled freezing might be important.

To understand the properties of acTAGs better we used a differential scanning calorimetry (DSC) machine, which measures heat flow while heating up a chemical. When a compound melts, it absorbs heat energy to fuel the change in state between solid and liquid. By measuring heat flow during heating, you can identify the temperature at which absorption occurs, which tells you the melting point of the substance. We found the melting point of acTAGs was very low for a fat; -17 ºC (Figure 4). That means that even when the whole gall fly freezes at -9 °C, the acTAGs in its body are still liquid. Most animal fats have melting points well above 0 °C.

DOI: 10.13034 / JSST-2015-009

Finally, we thought that maybe if the fats remained liquid, they could help the gall fly by interacting with water differently than most fats, reducing the damage caused by ice crystal formation. To test this idea, we mixed up some acTAGs with water, then prepared a mix of lcTAGs with water in the same proportion. We froze these solutions, then warmed them up slowly again. When we recorded the temperature at which the solutions melted, we found that acTAGs lowered the melting point of the water by 1.2 ºC while the lcTAGs only lowered the melting point by 0.02 °C. This meant that acTAGs were interacting with water in a way that may help the fly survive freezing.

WRAP-UP

Sometimes scientists are lucky enough to stumble across something new. In our case, we found that goldenrod gall flies make and consume a fat that no other animal on the planet uses. These acetylated triacylglycerols seem to be important for surviving the winter, and manage to stay liquid at very low temperatures, even when the whole fly is frozen. While we don't know yet if these fats are the reason why gall flies are so cold tolerant, we think these fats explain Reginald Salt's observations in 1959 of the liquid drops of fat that remain when the whole cell freezes.

A fat that stays liquid at very low temperatures might be useful for humans too. Biodiesels, which are fuels made from fats that living organisms make, are more sustainable than fossil fuels (which are in limited supply). But biodiesels usually either don't stay liquid at low temperatures, or need a lot of expensive chemical alteration to help them out. Perhaps these acTAGs might be a good alternative. Additionally, since acTAGs have fewer fatty acids per molecule than lcTAGs, they have fewer calories per molecule, which might be helpful for people trying to lose weight. After finding a new fat in flies that seems to help them survive freezing, there are still lots of questions to answer. Next we want to know how gall flies synthesize acTAGs – investigating the genes responsible would be an exciting first step.

RESOURCES

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ACKNOWLEDGMENTS:

We thank C.M. Williams and M.A. Bernards for discussions, M.A. Bernards, J.E. Steele, and D.A. Wharton for valuable comments on the ms, C.G. Guglielmo and J.F. Staples for use of laboratory space and equipment, E.C. Boychuk with technical help with nanolitre osmometry, R. Guterman for technical help with differential scanning calorimetry, P. Pittiock of the Biological and Mass Spectrometry Laboratory (UWO) for technical assistance with ESI-MS/MS, and D. Hairsine of the Chemisitry Mass Spectrometry Laboratory for technical assistance with GC-MS. 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. Thanks to Erica Tennenhouse for encouraging us to write this 'from the source' article.

Q&A WITH DR. BRENT SINCLAIR

1. HOW WOULD YOU DESCRIBE YOUR SCIENTIFIC APPROACH?

I think I'm probably a natural historian at heart – I like to find out new things about animals, and explanations for why they

do what they do. The difference between me and a 'true' natural historian (whom I think of as being out in the woods watching birds and catching butterflies) is that I approach the world with a physiological toolbox. Thus, I try to engage in all aspects of the scientific method, including making observations and generating hypotheses, testing those hypotheses, and synthesising that information to gain new insights. It suits me to explore a range of different organisms, and to use lots of different techniques. I'm not an expert in any of them, which means I learn something new every day! In the context of this paper, we started by keeping our eyes open – Katie was supposed to be testing a completely different hypothesis! This led to a lot of careful hypothesis testing (each of our attempts to identify and characterise the acTAGs was essentially testing the hypothesis that it was x compound or had y properties), but the fun part was that we had the opportunity to find something completely new, which shed new light on the animal.

2. WHAT IS THE MOST FULFILLING ASPECT OF WORKING IN YOUR RESEARCH FIELD?

People. Comparative animal physiology is a wonderful, diverse, international community. It is collaborative, rather than competitive, which means that everyone is supportive of each other's efforts. It's a great community in which to be a student, and many of my closest and oldest friends are also my colleagues and collaborators.

3. WHAT ARE THE BIGGEST CHALLENGES FACED BY YOUR FIELD OF RESEARCH TODAY?

Translating our basic science into information relevant to the big issues faced by society. Comparative physiology can yield new molecules (like our study) and mechanisms, and lead to novel insights about agricultural pests, human disease or quality of life. Thermal biologists are also at the forefront of understanding and predicting the impacts of climate change, particularly on ectothermic animals. However, the lines of communication from those of us who do basic research in comparative physiology to those who work on applications are not always open, because we don't necessarily understand each other's approaches: I don't read reports on agricultural production methods, and they don't read papers on insect physiology.

4. IN WHAT DIRECTION DO YOU SEE YOUR FIELD MOVING?

There is a general shift at the moment towards gathering 'omics' datasets, but the problem is that these represent a snapshot of an animal's biology at a specific time, and while they generate hypotheses, they don't actually test them. If we are interested in how animals respond to stress and perturbations, we need to understand their physiology as a dynamic process. I expect that, over the next decade or so, we will see an increase in the number of studies that test the hypotheses generated by 'omics' techniques, and a shift towards measuring physiology in real time – even in insects.

5. WHAT ADVICE WOULD YOU GIVE TO HIGH SCHOOL AND UNDERGRADUATE STUDENTS INTERESTED IN ANIMAL PHYSIOLOGY?

Animal physiology is integrative. To do it successfully, you need to be able to use things you have learned from biology, physics, chemistry, mathematics. Thus, it is vitally important to approach classes with the intention of learning, not just memorising enough material to pass the exam. You need to be able to write – communication is essential as any kind of scientist. Finally, you need to be genuinely interested in animals, and how they work!

DOI: 10.13034 / JSST-2015-009