Neutral lipid metabolism of the rotifer *Brachionus plicatilis* during periods of starvation

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Abstract

The rotifer *Brachionus plicatilis* is a halophilic invertebrate often used for research and feeding young fish. One concern in this work is to provide young fish with energy-rich lipids. Past research suggests that neutral lipids serve a primarily structural role in rotifers, while other research suggests that neutral lipids are broken down for energy during starvation of rotifers, but these ideas are not mutually exclusive. In this research, Nile Red staining technique was used to quantify neutral lipids during starvation coupled with liquid chromatography mass spectrometry (LCMS) to identify specific lipids present during periods of starvation. Results demonstrate that lipids are used for energy during periods of starvation up to the 48–hour point and that there is a change in fatty acid composition over longer periods of starvation. Thus, lipids appear to be an important biochemical in the storage of energy in rotifers.

Keywords: Energy storage, fatty acid, invertebrate, LCMS, lipid droplets, marine water

Phylum Rotifera is composed of around 2500 species ranging from 50 to 2000 μm in size (Wallace et al. 2015). Because of their short lifespans and ease of culture, species in this phylum are excellent research organisms. Rotifers are either halophilic or they live in freshwater; thus, rotifers can be used to feed fish or other commercially important species in both conditions (Wallace et al. 2015). Because of their size, nutritional makeup, and the fact that they store energy-rich lipids, rotifers are excellent energy sources for fish and are used to feed fish in research labs and fish farms (Lubzens 1987, Lee et al. 2006, Allen et al. 2016).

Over the last few decades, knowledge about rotifers has expanded rapidly, especially in the fields of aging, research, toxicology, and phylogeny. Unfortunately, there is still relatively little known about their physiology and lipid biochemistry (Fernández–Reirez and Labarta 1996, Wallace et al. 2015). Nevertheless, the halophilic rotifer, *Brachionus plicatilis,* and its freshwater counterpart, *Brachionus calyciflorus,* have been studied intensively.

According to Szyper (1989), fish need omega-3 fatty acids in their diet. One way to determine whether or not fish are getting these fatty acids is liquid chromatography mass spectrometry (LCMS). Knittelfelder et al. (2014) demonstrated that positive polarity electrospray ionization (ESI) can be used to qualitatively analyze neutral lipids in microorganisms, while others have used different methods of analysis, such as gas chromatography (Smith and Knight 1971). To my knowledge, there are no published studies that use LCMS to qualitatively elucidate the fatty acid composition of the rotifer *B. plicatilis*. Therefore, it is unknown whether fish are receiving the omega-3 fatty acids that they need from the rotifers in their diet. Because *B. plicatilis* is a popular rotifer for research and aquaculture, it is reasonable to investigate its lipidome (lipid makeup) and its use of lipids during starvation, along with whether omega-3 fatty acids are derived from rotifers or another organism in the food chain.

Recently, much work has been dedicated to lipid studies, which now includes investigations of the lipid contents of invertebrates (Barber and Blake 1981, Boechat and Adrian 2005, Subramanian et al. 2011). Rotifers store lipids in spherical droplets within their guts, so how they use these lipids is of particular interest (Wallace et al. 2015). In 1992, Frolov and Pankov determined the dry weights of lipids in rotifers. They showed that rotifers follow the starvation strategy of most animals by using lipids and then probably switching to proteins for energy. In 1994, Rainuzzo and colleagues enriched rotifers with specific fatty acids and discovered that the fatty acid profile of the rotifers matched that of the enrichment. Using gas chromatography, Fernández–Reirez and Labarta (1996) found that the level of neutral lipids in rotifers depended on their metabolic rate. Later, Olsen and Øie (1997) discovered that rotifer lipid content is constant during periods of abundant food availability and quality. Past research by Wilde (2016) suggested that the lipid droplet count of rotifers decreased during starvation, while lipid droplet volume remained the same throughout starvation.

Although it is logical to assume that lipids are used for energy during periods of starvation, research has not demonstrated the extent to which this occurs in rotifers. Carman et al. (1991) showed that neutral lipid storage products are indicators of the nutritional status of invertebrates. Frolov and Pankov (1992) reported that lipids are used for energy during periods of starvation, supporting this idea. On the other hand, Guisande and Serrano (1989) posited that lipids serve a primarily structural role in rotifers. Lipids are likely to serve both roles simultaneously, but the details of lipid catalysis have yet to be elucidated. This research consisted of two components: (1) to compare the relative amounts of neutral lipids in *B. plicatilis* before and after periods of starvation and (2) to identify specific fatty acids in *B. plicatilis* using LCMS. To complete aim number one, the investigation done by Wilde in 2016 was replicated

with a larger sample size. The first hypothesis was that the lipid droplet count would decrease during starvation, but that lipid droplet volume would show no significant change during starvation based on the studies of Wilde (2016). Consistent with research that shows that neutral lipids are broken down during starvation, the second hypothesis was that there would be a change in neutral lipid profile of rotifers after periods of starvation (aim 2).

Methods

Culturing of B. plicatilis

Brachionus plicatilis and their foods (*Nannochloropsis* sp. and Roti-Rich®) were obtained from Florida Aqua Farms® (Dade City, FL). Rotifers hatched by incubating up to 5,000 diapausing embryos for up to three days in 15 parts per thousand (ppt) Instant Ocean® (IO) dissolved in distilled water. Food was added after 24-hr to the hatching embryos, but rotifers were not mature until seven days post-hatch. At that point the rotifers were ready for experimentation. Rotifer and algal cultures were maintained according to Allen et al. (2016) (Fig. 1). The procedure was as follows. To obtain a high population level, rotifers were fed approximately 100 mL *Nannochloropsis* sp. per liter of rotifer culture and two drops of the artificial food supplement Roti-Rich® once daily. *Nannochloropsis* sp. was cultured in 18 ppt IO. To maintain the salt concentration of rotifer cultures, about 100 mL of the algal culture was diluted with distilled water to reduce the salt concentration from 18 to 15 ppt IO before being added to the rotifer cultures. Depending on population density, rotifer cultures were harvested every two to three days by draining up to ½ of the culture and replenishing it with 15 ppt IO. This ensured that the cultures did not become too densely populated, which would lead to either starvation or induction of sexuality.

Collection of B. plicatilis

Once ready for harvesting, up to half of a rotifer culture (~500 mL) was collected in a beaker and filtered through a series of sieves of decreasing porosity (900–180 μm) to remove debris. In this way, up to ~1000 individuals were obtained in a short period of time. After harvesting, rotifers were backwashed into a beaker with 15 ppt fresh IO, where they were held for 30 min. to clear their guts. At this point the rotifers were prepared for either LCMS study or neutral lipid droplet analysis.

Lipid volume analysis

Lipids form droplets in the guts of marine zooplankton (Lee et al. 2006). The labs of both Greenspan et al. (1985) and Carman et al. (1991) found that Nile Red almost exclusively stains neutral lipids, which can then be visualized under 100–400x magnification. Therefore, after clearing the guts and starving the rotifers, they were filtered once again, backwashed into a small Petri dish with Nile Red and 15 ppt IO, and incubated for 60 min. Rotifers were then placed three to a slide and imaged using a Zeiss® microscope equipped with a Spot® (Insight FireWire) camera. The images were printed and lipid droplets were counted and measured using a Zidas® (Carl Zeiss Inc.) digitizing tablet. To determine if a difference existed in the recorded values, ANOVA and Tukey–Kramer statistical tests were performed using Excel.

Lipid profile analysis

To analyze rotifers for their lipid content, they were processed as described above, but rather than staining, they were placed in a 2:1 methanol:water solution immediately after starvation (Maity et al. 2012). The rotifers were sonicated in an ice bath using 1 sec pulses for 6 min. After sonication, the volume was doubled with chloroform, vortexed, and placed on ice. The sample was centrifuged and the chloroform layer was removed and its volume reduced using

a rotavap. The sample was then suspended in 2:1:1 water:methanol:acetonitrile solution and examined with an Agilent® 6320 LCMS. Using the proprietary computer program Agilent MassHunter, specific neutral lipids were identified.

Results

Lipid volume

Staining with Nile Red allowed for easy observation of the neutral lipid droplets within the rotifers (Fig 2). Changes in average lipid droplet count and volume after starvation are recorded in Table 1 and illustrated in Figs. 3 and 4. With the exception of a Tukey–Kramer *p*–value of 0.246 between 48 and 72 hr, both mean lipid droplet volume and count decreased significantly after every 24–hr period of starvation, as well as total lipid content per rotifer (0–24, $p<0.001$; 24–48, $p<0.001$). During starvation, total lipid content per rotifer decreased by 83.5% between zero and 24 hr, 86.6% between 24 and 48 hr, and by 71.3% between 48 and 72 hr (Table 1).

Lipid profile

The number of present fatty acids and the specific fatty acids present changed throughout starvation up to 72 hr, although most fatty acids exhibited no change in presence or absence throughout the starvation trials (Tables 2 and 3). Some fatty acids became undetectable as the periods of starvation lengthened, while others became detectable. Docosahexaenoic acid (DHA) was never detected throughout the starvation trials. On the other hand, Eicosapentaenoic acid (EPA), a related omega–3 fatty acid, was detected at the 48–hr mark, but at no other point in the starvation trials. Arachidonic acid was detected at zero and 48 hr, but not at 24 and 72 hr. Eicosatrienoic and Eicosapentaenoic acids were not detected at zero, 24, and 72 hr, but did show

up at the 48-hr mark. At zero hr of starvation, seven fatty acids were detected. That number decreased to six at 24 hr, increased to eight fatty acids at 48 hr, and decreased to just three at 72 hr of starvation.

Discussion

Biochemical analyses are widespread across many phyla. However, relatively little research has been undertaken to study the role of lipids as energy storage molecules in rotifers. Rotifers store lipids in spherical droplets within their guts (Wallace et al. 2015). Unpublished data from Wilde (2016) suggests that lipid droplet volume does not change during periods of starvation, but that lipid droplet count decreases. This research expanded her work to include total lipid content per rotifer before and after periods of starvation, thereby providing more direct results to elucidate the role of lipids in rotifers during starvation. Based on previous research, the hypothesis that lipid droplet count decreases during starvation, lipid droplet volume remains the same during starvation, and total lipid content per rotifer decreases during starvation. This would be indicative of neutral lipids as storage molecules in rotifers. Prior research on lipid storage molecules suggests that fatty acids are catalyzed for energy during starvation (Gilbert 2004). Thus, it was hypothesized that there would be a change in the fatty acid profile of rotifers over a period of starvation.

The fatty acid profile of the diet of rotifers is closely related to the fatty acid profile of the rotifers (Rainuzzo et al. 1994). Although the diet of the *B. plicatilis* culture was closely controlled, diet is variable in natural ecosystems. Results of these lipid analyses apply more to commercial fish farming than wild ecosystems where their diet is more variable. Interestingly, two important omega–3 fatty acids, DHA and EPA, were not detected consistently throughout

the starvation studies. EPA was detected at the 48–hr mark of starvation in *B. plicatilis*, but at no other point in the study. According to Szyper (1989), most fish require high amounts of omega–3 fatty acids in their diet. The fact that these two important omega–3 fatty acids were undetectable adds a level of uncertainty to the quality and effectiveness of lab-grown *B. plicatilis* as a food source for fish. On the other hand, if fish are found to be malnourished on a diet of lab–grown rotifers, the rotifers can be enriched with certain fatty acids until they are a sufficient food source for fish.

In regards to using rotifers to feed fish, these rotifers did not demonstrate a well–rounded fatty acid profile, although there are some explanations for this. (1) Certain fatty acids may not elute from the column and are therefore never subjected to the mass spectrometer. (2) The methods for lipid extraction have yet to be assessed for efficacy in the LCMS that was used. (3) The extraction method that was followed (Maity et al. 2012) called for approximately 60 individuals/mL of the methanol:water solution, which could be too few rotifers and too little fatty acid to be detected by the LCMS. Also, it was difficult to get peaks from some long-chain fatty acid standards on the LCMS, indicating that some natural long–chain fatty acids in the rotifers may go undetected. However, DHA standard was detected on LCMS, indicating that it and EPA would likely elute from the column and be detected by the mass spectrometer were it at detectable levels in the extracted fatty acid solution.

Consistent with changes in fatty acid composition through the starvation periods shown in Tables 2 and 3, it appears that *B. plicatilis* does indeed use fatty acids during periods of starvation. This is consistent with the findings of Frolov and Pankov (1992), who suggested that *B. plicatilis* switches between lipids and proteins for energy throughout starvation. These results indicate no significant change in neutral lipid content after the 48–hr mark, suggesting that the

switch from neutral lipids to proteins occurs some time around 48 hr after the beginning of starvation. Further research should expand these findings to determine when exactly this change happens, which could provide more information about how long rotifers remain nutritious for fish.

Tables 2 and 3 showed that fatty acids do change in their composition throughout starvation, with some becoming detectable and some becoming undetectable at certain points in starvation. This confirms the hypothesis that fatty acid composition changes throughout starvation. While the basic mechanism of fatty acid breakdown for energy is well understood, it would be interesting to investigate the specific pathways of fatty acid consumption to determine why some fatty acids become detectable and others do not.

There are multiple directions in which this research could be taken. One is to relate this research to natural ecosystems. This ranges from tracking fatty acids in wild-caught rotifers over time to catching wild rotifers, starving them, and tracking the changes in fatty acids. One particularly interesting investigation would be to simply quantify the deaths of rotifers throughout starvation periods to determine how long an average rotifer can live without food. It would also be enlightening to repeat this research on *Brachionus calyciflorus*, a close relative to *B. plicatilis*. On the topic of related species, a review article comparing the lipidome of *B. plicatilis* to that of related invertebrates is necessary. Lipidomes have been reported by Boechat & Adrian (2005) for *Balanion planctonicum* (Prostomatea), *Urotricha farcta* (Prostomatea), and *Cryptomonas phaseolus* (Cryptophyta), and for *Chromodoris michaeli* (Gastropoda) by Zhukova (2014), among many others.

The behavior of fatty acid standards in the LCMS should be further investigated to determine the best method(s) to employ to identify lipids in our facility. These new methods

should be used to repeat the present research with more specific lengths of starvation periods.

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Table 1. Change in average lipid droplet count and volume over different lengths of starvation in the rotifer *Brachionus plicatilis.*

Time (hours)	Count	Volume (µL)	Total lipid volume (µL)	Percent change in total lipid volume from previous
0	64.23	17.49	1123.70	—
24	24.67	7.52	185.57	–83.5
48	6.97	3.56	24.77	–86.6
72	5.20	1.37	7.10	–71.3

Table 2. The presence or absence of selected fatty acids throughout different periods of starvation, up to 72 hours in the rotifer *Brachionus plicatilis*.

Fatty Acid	0 hours	24 hours	48 hours	72 hours
Linoleic acid	Present	Present	Present	Not Present
Alpha-Linoleic acid	Present	Present	Not Present	Not Present
Nonadecenoic acid	Present	Present	Not Present	Not Present
Eicosadienoic acid	Not Present	Not Present	Not Present	Present
Eicosatrienoic acid	Not Present	Not Present	Present	Not Present
Eicosapentaenoic acid	Not Present	Not Present	Present	Not Present
Docosahexaenoic acid	Not Present	Not Present	Not Present	Not Present
Arachidonic acid	Present	Not Present	Present	Not Present
Octadecatetraenoic acid	Present	Present	Present	Not Present

Table 3. The presence or absence of all fatty acids throughout different periods of starvation up to 72 hours in the rotifer *Brachionus plicatilis*.

Fatty Acid	0 hours	24 hours	48 hours	72 hours
Alpha-linoleic acid	Present	Present	Not present	Not present
Arachidic acid	Not present	Not present	Not present	Not present
Arachidonic acid	Present	Not present	Present	Not present
Docosadienoic acid	Not present	Not present	Not present	Not present
Docosahexaenoic acid	Not present	Not present	Not present	Not present
Docosanoic acid	Not present	Not present	Not present	Not present
Docosatetraenoic acid	Not present	Not present	Not present	Not present
Docosatrienoic acid	Not present	Not present	Not present	Not present
Docosenoic acid	Not present	Not present	Not present	Not present
Eicosadienoic acid	Not present	Not present	Not present	Present
Eicosapentaenoic acid	Not present	Not present	Present	Not present
Eicosatrienoic acid	Not present	Not present	Present	Not present
Eicosenoic acid	Not present	Not present	Not present	Not present
Heneicosanoic acid	Not present	Not present	Not present	Not present
Heneicosadienoic acid	Not present	Not present	Not present	Not present
Heneicosenoic acid	Not present	Not present	Not present	Not present
Heptadecaenoic acid	Present	Present	Present	Present
Heptadocosadienoic acid	Not present	Not present	Not present	Not present
Hexacosadienoic acid	Not present	Not present	Not present	Not present
Lauric acid	Not present	Not present	Not present	Not present
Lignoceric acid	Not present	Not present	Not present	Not present
Linoleic acid	Present	Present	Present	Not present
Margaric acid	Not present	Not present	Not present	Not present
Myristic acid	Not present	Not present	Not present	Not present
Nonadecaenoic acid	Present	Present	Not present	Not present
Nonadecylic acid	Not present	Not present	Not present	Not present
Octadecatetraenoic acid	Present	Present	Present	Not present
Oleic acid	Not present	Not present	Not present	Not present
Palmitic acid	Not present	Not present	Not present	Not present
Palmitoleic acid	Present	Present	Present	Present
Pentacosadienoic acid	Not present	Not present	Not present	Not present
Pentadecaenoic acid	Not present	Not present	Not present	Not present
Stearic acid	Not present	Not present	Present	Not present
Tetracosadienoic acid	Not present	Not present	Not present	Not present
Tetracosahexaenoic acid	Not present	Not present	Not present	Not present
Tetracosapentaenoic acid	Not present	Not present	Not present	Not present
Tetracosatetraenoic acid	Not present	Not present	Not present	Not present

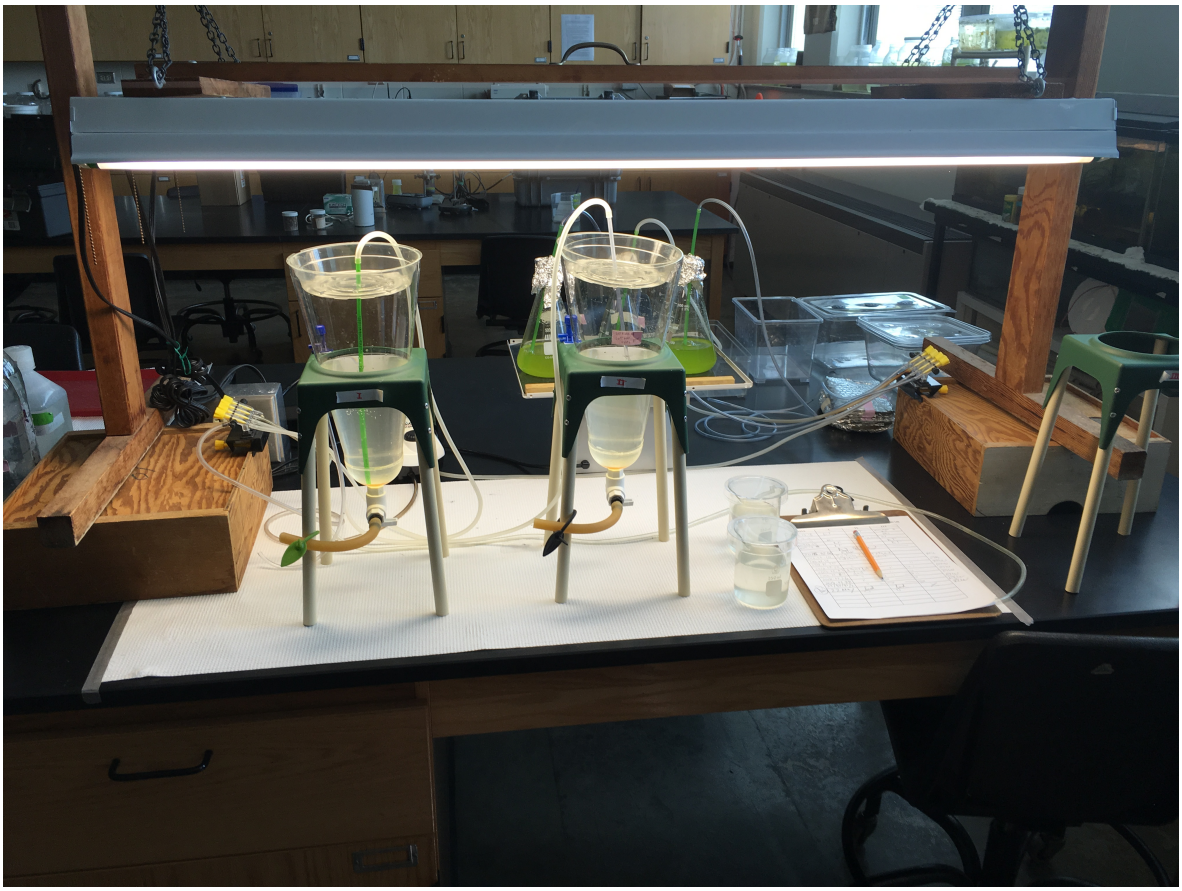

Figure 1. Cultures of *Brachionus plicatilis* in conical brine shrimp culture vessels. Pictured are two standing conical vessels hold rotifer cultures. Behind are several Erlenmeyer flasks that are culturing the algae *Nannochoropsis* sp. These cultures are housed at room temperature under fluorescent illumination as illustrated here.

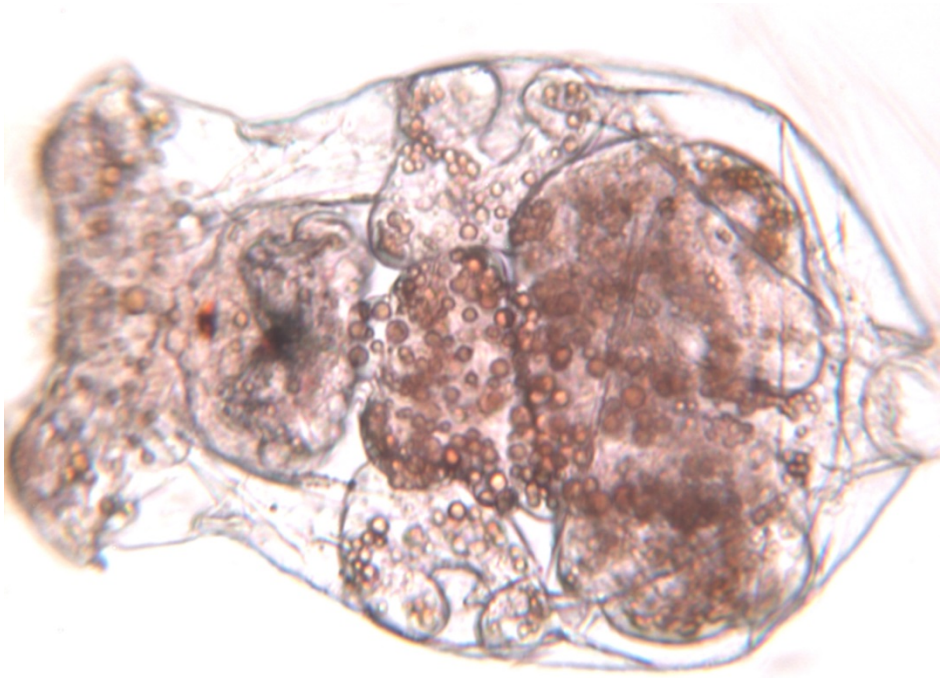

Figure 2. Rotifer *Brachionus plicatilis* under visible light microscopy after using Nile Red stain to make lipid droplets visible. Animal is approximately 350 μm in length.

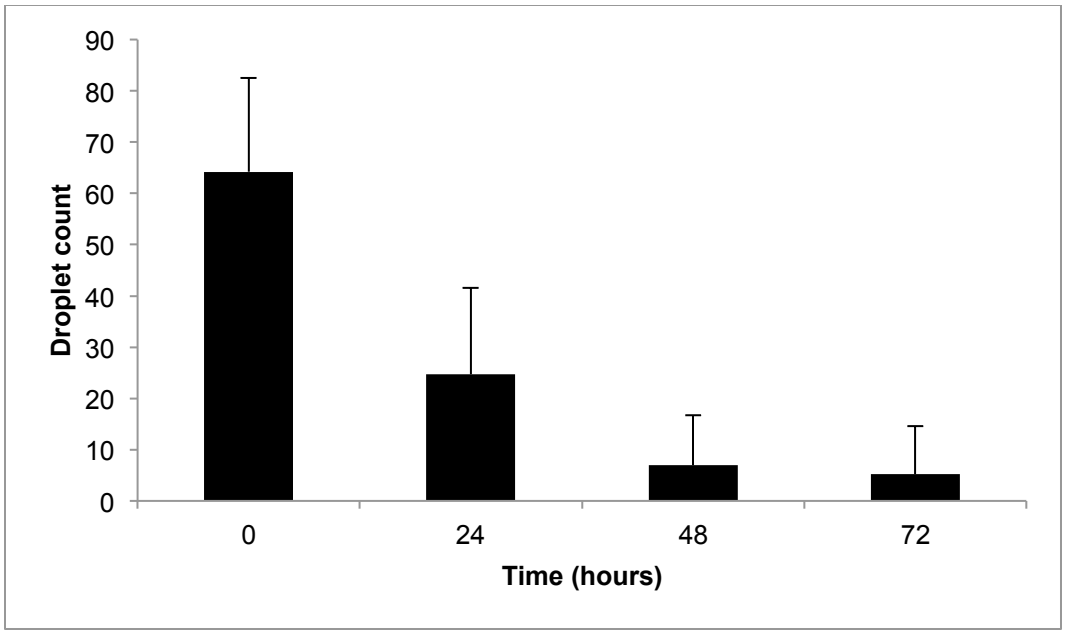

Figure 3. Mean droplet count vs. time. Lipid droplet (spherical bodies) counts as a function of starvation time (±1 SD). There was a significant decrease in lipid droplet count between zero and 24 hr and 24 and 48 hr, but not between 48 and 72 hr.

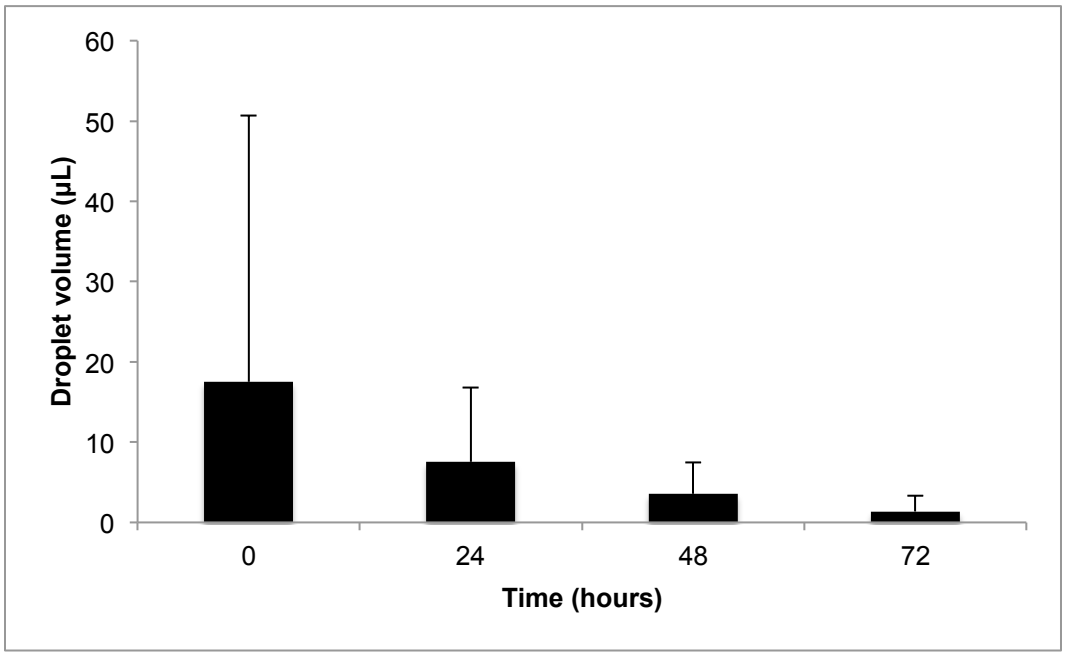

Figure 4. Mean droplet volume vs. time. Lipid droplet (spherical bodies) volumes as a function of starvation time (± 1 SD). There was a significant decrease in lipid droplet volume between zero and 24 hr and 24 and 48 hr, but not between 48 and 72 hr.