Ingestion of Low Density Food Particles by Suspension-Feeding Goldfish

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Ingestion of Low Density Food Particles by Suspension-Feeding Goldfish

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from The College of William and Mary

by

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Accepted for Honors

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Introduction

Goldfish (*Carassius auratus*) are known to be omnivorous, benthic, facultative suspension feeders (Staab et al. 2012, Callan and Sanderson 2003). In the wild goldfish generally avoid the surface due to the threat from predators, which is heightened by their bright coloration (Burggren 1982). However, in captivity goldfish can be trained to feed from the surface on neutral and low-density feeds (Burggren 1982). In manmade outdoor ponds, goldfish use continuous suspension feeding at the surface, drawing the surface layer into their oral cavities repeatedly (personal observation). We suggest that this surface feeding behavior could allow goldfish and related species to take advantage of the freshwater surface microlayer as a potential food source. The surface microlayer is enriched with hydrophobic compounds such as lipids that are released from photosynthetic plankton, particularly during light inhibition near the surface. Bacteria, small algae, and flagellates also live in the surface microlayer, potentially drawing nutrients from the released macromolecules (Sodergren 1979), and some species of phytoplankton have been shown to become enriched in the microlayer of lakes and ponds (Parker and Hatcher 1974). This microlayer has also been shown to attract various insect larvae, such as blackflies and mosquitoes (Wotton 1982, Wotton et al. 1997).

In this study, we posed the question of whether goldfish can feed on oils on the surface and successfully ingest them. We did this by performing fatty acid analysis on gut contents after feeding experiments on canola oil, a source of oils in commercial fish feeds. Previous studies have established a precedent for using fatty acid analysis of gut contents and tissues as a way to determine the diet of various marine and freshwater organisms (Pozernick and Wiegand 1997, Phillips 2001, Aras 2009, Couturier 2013). We also conducted feeding experiments with oil and Tetramin flakes to test if the introduction of a familiar food would lead to higher oil consumption. Once it became clear that goldfish were in fact ingesting canola oil, we also defined and quantified three associated behaviors: surface feeding, spitting, and processing,
in order to see if any of these were correlated with either increased oil consumption or feeding on any particular food type or combination thereof.

Dietary requirements of most fish species are not well defined since they tend to vary based on age, season, and species, and most of what is known is due to the need of aquaculturists to formulate flesh-maximizing diets. However, in a study conducted by Sánchez-Vázquez et al. (1998), goldfish were shown to select a diet (g/kg bodyweight/day) consisting of approximately 22% protein, 32% fat, and 46% carbohydrate from among three different macronutrient-enriched food types. It should be noted that this study also revealed a positive correlation between fat consumption and carbohydrate consumption, which could be metabolically significant since fish are not as reliant on carbohydrates as other vertebrates (Leaver 2008). They were also shown to adjust this diet based on what they had eaten previously, suggesting that they are able to select for a balanced diet. Lipids are important in the diets of all organisms, for use in the structure of cell membranes as well as energy production and storage (Leaver 2008). Goldfish are able to produce some polyunsaturated fatty acids (Pozernick and Wiegand 1997), but their main natural source of fatty acids is likely from phytoplankton and zooplankton. Since goldfish are facultative feeders, this source will vary depending on habitat and available niches (Specziàr et al. 1997).

During times of hypoxia and anoxia, goldfish have the capability of “air gulping” or aquatic surface respiration (ASR) which is distinct from the well-studied air breathing done by some other fish species (He et al. 2015). Air-gulping consists of a goldfish protruding its upper jaw above the surface and repeatedly depressing its lower jaw in a way that moves a small amount of water in the buccal cavity back and forth before it passes through the gill filaments and exits posteriorly from the operculum. This motion causes mixing with air trapped in the goldfish’s buccal cavity and allows the fish to respire without a major morphological adaptation. This was shown to take place even in goldfish specifically kept from feeding at the surface, suggesting it is not a learned behavior (Burrgren 1982). This behavior
is nearly identical to the way captive goldfish feed from the surface, as observed by the experimenters and noted by Burggren, and suggests a possible connection between the adaptation for respiration during hypoxia and the ability to modify that behavior to suspension feed on the surface layer.

Methods

Feeding Experiments

Comet Goldfish, a conventional pond variety, were maintained in the laboratory in a 284 L aquarium at 24 °C. Goldfish were trained to feed at the water surface on Tetramin™ flakes 0.1 – 1.0 cm in diameter, but were not exposed to canola oil prior to the experiments.

Goldfish were transferred individually into 38 L tanks equipped with a bubble-up filter (Second Nature Whisper Size 2). Each fish was kept in this tank to acclimatize for 3-5 days, during which they were fed twice daily at the surface on finely ground Tetramin™ flakes 0.1 – 0.5 mm in diameter. Fish were not fed for 36 hours prior to the experiment and plastic grating (1.5 cm x 1.5 cm) was added to the bottom of the tank to prevent the fish from feeding on any sunken food particles or feces. The bottom of the tank was also cleaned by siphoning twice a day prior to the experiment in order to eliminate any confounding food particles or feces.

There were different set-up protocols for different food types in order to maximize the spread of the food across the surface and to minimize disturbance to the fish.

Canola oil feeding experiments: In the oil treatment, 2.0 mL of pure canola oil (Crisco®) was added with a 5 mL syringe as evenly as possible on the water surface. The oil was partly distributed by the surface motion created by the bubble-up filter and was also spread with a spatula before the filter was turned off. In the control treatment, the bubble-up filter was turned off before oil was added and the filter was removed from the tank. This was done to make room for additional pieces of plastic grating (described above) that were used to sequester the fish away from the surface. The grating was inserted from above.
so that one edge rested along the longer tank bottom edge and the opposite one rested against the tank glass of the opposite side. Three pieces of grating were used in total, with one overlapping the other two. Once the grating was in place, canola oil was added by the same method as described above.

**Canola oil and Tetramin™ feeding experiments:** In the oil and Tetramin treatment, 0.3 mL of oil were added first with a 1 mL syringe, spread by spatula, and then the filter was turned off. Following this, 15.0 mg of finely ground Tetramin, measured on a Fisher Scientific XA-100 analytical balance, was sprinkled evenly across the surface directly from the weighing pan. In the control for the canola oil and Tetramin™ feeding experiments the filter was turned off first, and then the 15.0 mg of Tetramin was sprinkled evenly across the surface. The filter needed to be turned off before Tetramin could be added since the action created by the air bubbles rising to the surface caused the flakes to sink. The spatula also could not be used on the flakes since it made them more likely to sink.

Once the food was added, the tank lid was put back into place and the experimenters stepped away from the tank. The fish was allowed to feed for 20.0 minutes, timed from its first feeding. During this period, the time spent feeding at the surface was recorded using a stopwatch and the fish was videotaped at 30 fps on MiniDV cassettes using a Sony Handycam (DCR – HC36) for behavioral analysis later on. In the case of the oil controls, when no food was added to the tank, the fish were not videotaped and 20.0 minutes were allowed to pass after the grating was added.

**Fatty Acid Extraction and FAME Production**

Upon being removed from the tank using a handnet, goldfish were transferred into a paper towel to remove any surface oil. Fish were euthanized by cervical transection and pithing, while being held lightly to avoid redistributing the gut contents. They were then wiped down again before dissection to prevent transfer of any surface oil into the body cavity. The first 2.5 cm of the gut immediately posterior to the esophageal sphincter were removed. The length of the gut to be removed was measured and forceps
clamped on either end just behind where the foremost cut and in front of where the hindmost cut would be made. This segment was transferred into a 1.5 mL centrifuge tube. The total length, fork length, and standard length of each fish was recorded. To expose gut contents, the gut was cut longitudinally using microdissection scissors while held inside the centrifuge tube so that the gut was an open sheet with contents exposed. The scissors and forceps used were rinsed with 750 µL of heptane (Fisher Scientific, 99.7%) into the centrifuge tube using a Pipetman micropipette. The sample was then vortexed for 30 sec with a Fisher Scientific Vortex Genie 2. The empty gut wall was removed from the centrifuge tube and the forceps used were rinsed into the tube with 250 µL of heptane. This 1.00 mL sample was centrifuged at 5000 rpm for 5 minutes with a Fisher Scientific Micro 7 microcentrifuge. 500 µL were then micropipetted from the surface of this sample and transferred directly into a 15 mL centrifuge tube.

Fatty Acid Methyl Ester (FAME) production was carried out using the protocol described by Zhang et al. (2014). 1.0 mL each of diethyl ether, petroleum ether, and 0.4 M KOH in MeOH were added to the sample in that order. The KOH solution was prepared in a low-humidity environment to ensure the KOH did not react with water in the air; this was done by simply placing a watch glass over the top of the beaker as the KOH dissolved. The sample was then vortexed for 30 seconds and left at room temperature (21 °C) for 2.5 hours. 2.0 mL of DI water was added and then the sample was centrifuged at 3400 rpm for 2 minutes with a Fisher Scientific Centrifuge Model 228. 100 µL was then extracted from the top (organic) layer of this sample and added to 400 µL of diethyl ether in a 1.5 mL glass sample vial (Thermo Scientific). FAME samples were stored at -5 °C and evaporated diethyl ether was replaced using a Pasteur pipette before analysis. Before samples were stored, the meniscus was noted on the sample vial so that evaporation could be observed. If the sample was stored long enough for evaporation to occur, diethyl ether was added one drop (approx. 20-25 µL, or 4-5% of the total sample volume) at a time until the meniscus was level with the previous mark.
**GC/MS Analysis**

FAME extracts in diethyl ether were analyzed on an Agilent 6890N gas chromatograph interfaced to an Agilent 5973 mass spectrometer. A fused silica Rxi-1ms nonpolar column (30 m, 25 mm ID, 0.25 µm film, Restek) was used. The flow rate was 1.1 mL/min and Helium was used as the carrier gas. The inlet temperature was 280°C and the inlet pressure was 15.00 psi. The initial oven temperature was 150°C, which was increased at a rate of 5°C/min until it reached a final temperature of 260°C. The total run time was 22 minutes. The splitting ratio was set at 100:1.

The mass of canola oil in the gut samples from the feeding experiments was calculated by quantifying the area of the oleic acid (C18:1n-9) peak (retention time 13.0 min, determined from preparation of FAMES from known concentrations of pure canola oil). Oleic acid is the major fatty acid component (approx. 63% by mass (Syed 2012)) of canola oil, which when converted into a methyl ester becomes methyl oleate. A known standard of methyl oleate (99%, Aldrich) was diluted to a concentration of 1 mg/mL in heptane by dissolving 100 mg into 10 mL of heptane (Fisher Scientific, 99.7%) and then taking a 1 mL subsample and dissolving it into another 10 mL of heptane. This methyl oleate solution was then analyzed using the same GC procedure as above and the area of this standard peak was compared to the area of the C18:1n-9 peak of the sample. This was done each day before samples were put through the GC/MS so as to account for any equipment-related fluctuations between uses. Peak areas were quantified using the AutoIntegrate function of MSD ChemStation (E. 02. 02. 1431, Agilent Technologies).

In some samples, the software was unable to distinguish between peaks with low signals, in which case the Manual Integration function was used to define the base width of the peak of concern. The relative area of the two peaks was combined with the known methyl oleate concentration, the fatty acid composition of canola oil, and the previous dilutions to calculate a mass of canola oil in the original gut sample.
Calculations

The calculations are shown below. Equation (1) uses the ratio of the known concentration in mg/mL of the standardized methyl oleate to the peak area of the standard in order to calculate the concentration of oleic acid in the gut sample injection. This calculation is shown simplified in equation (3), which is possible since the known concentration of the standard is 1 mg/mL (equation (2)). In order to convert concentration of oleic acid in the gut sample injection to a mass of canola oil in the gut, the gut sample injection concentration is divided by 0.63, since oleic acid only makes up 63% of canola oil (Syed 2012). This value can then be divided by the 1/5 dilution factor of the 100 µL extraction placed in diethyl ether before GC/MS analysis, which gives us the canola oil concentration in the gut sample organic layer. This concentration is then multiplied by 2.5 mL, the volume of the organic layer (including ethers, heptane, and FAMES) at the end of the FAME preparation process, giving the mass of canola oil in the gut sample organic layer. This mass can then be divided by the dilution factor of ½ from the halving of the sample following the first centrifugation. This is represented in equation (4), which gives the mass of canola oil contained in the original 2.5 cm gut sample. By substituting equation (3) into equation (4), all of the above steps can be calculated at once as shown in equation (5).

\[
\text{(1) } \frac{\text{[Standard C18:1n-9 peak area]}}{\text{[C18:1n-9 in standard]}} = \frac{\text{Gut Sample injection C18:1n-9 peak area}}{\text{[C18:1n-9 in gut sample injection]}}
\]

\[
\text{(2) } [\text{C18:1n - 9 in standard}] = 1 \text{ mg/mL}
\]

\[
\text{(3) } [\text{C18:1n - 9 in gut sample injection}] = \frac{\text{Gut Sample injection C18:1n-9 peak area}}{\text{[Standard C18:1n-9 peak area]}} \times 1 \text{ mg/mL}
\]

\[
\text{(4) } \frac{[\text{C18:1n-9 in gut sample injection]} \times 2.5 \text{ mL}}{0.63 \times 0.5 \times 0.2} = \text{mass of canola oil in 2.5 cm gut sample (mg)}
\]

\[
\text{(5) } \frac{(\text{Gut Sample injection C18:1n-9 peak area})}{\text{[Standard C18:1n-9 peak area]}} \times \frac{\text{mg}}{\text{mL}} \times 2.5 \text{ mL} = \text{mass of canola oil in 2.5 cm gut sample (mg)}
\]
Behavioral Analysis

The videos taken during feeding trials were analyzed for the presence of specific behaviors defined after preliminary review of multiple videos: feeding bouts, spitting bouts, and processing bouts. They were viewed on a Sony DVCam (DSR-11) and viewed at partial speed using a remote control with a jog/shuttle control (DSRM – 20).

Statistical Analysis

It should be noted that the statistical analysis of this data was limited due to the small sample sizes obtained coupled with high variance within treatments. This led to data that was not normally distributed and thus difficult to analyze robustly.

A two-sample Fisher Pitman permutation test was used to compute exact p-values for the two oil feeding experiments using the statistical software R (v.3.2.1) and the package “coin” (Hothorn et al. 2008). This non-parametric test was chosen because the oil content data lacked a normal distribution and transformation wasn’t possible due to the zero variance of the control groups. Since the treatment and control groups also had different variances, a permutation test was necessary (Whitlock and Schluter 2015).

The first five fish of the canola oil experiments were not filmed and feeding time and frequency data were not collected for the first fish, which were excluded from statistical analyses. Since the feeding time and frequency data were highly correlated as shown by regression analysis ($R^2 = 0.85$) and the planned test was a MANOVA, feeding time data were excluded from analysis as well. Due to the large differences in variances and the non-normal shape of the data distribution, the behavioral data was transformed using a log transformation ($Y' = \ln(Y)$). A series of F-tests were then performed to compare the variances of the frequencies for the different feeding behaviors, which gave nonsignificant results for all pairs, suggesting that the datasets did not have significant differences in variance. A MANOVA was
then performed on the behavioral data with food type as the independent variable and types of behaviors as the dependent variables. This was followed by univariate post-hoc ANOVAs with Bonferroni adjustments for repeated tests. A separate one-way ANOVA was also performed on the feeding time data and was followed by post-hoc Tukey-Kramer tests.

Results

Gut Oil Content

Canola oil was present in the guts of the majority of the Canola oil treatment fish and in two out of five of the canola oil + Tetramin treatment fish (Table 1). Gut oil content was highly variable between fish. However, none of the control fish samples in either category showed a peak at the C18:1n-9 retention time. Using a two-sample Fisher Pitman permutation test, the canola oil treatment group was shown to be significantly different from the control group with $p = 0.005$ ($n_i = 10$). The canola oil + Tetramin group was not shown to be significantly different from its control group with $p = 0.22$ ($n_i = 5$).

Detection Limit

One of the samples analyzed from the pure canola oil treatment group showed a peak at the expected retention time for C18:1n-9 but at too small of a concentration to be identified or quantified by the software. This sample was reanalyzed at twice the concentration by dissolving the FAMEs in half the volume of diethyl ether (200 µL was added instead of 400µL) before GC/MS analysis. This resulted in a quantifiable peak that could be identified as C18:1n-9, resulting in a final canola oil mass estimate of 0.3 mg in the gut. The final data point was rounded to the nearest milligram, so the value became zero, indicated in the data table by an asterisk. No other negative result chromatograms had a peak visible above the background noise, so this procedure was not performed on any other samples.

The detection limit, when a peak that is visible above background noise at the expected retention time for C18:1n-9 is so low that it cannot be identified as C18:1n-9 by the GC/MS software, was determined
Table 1. Gut oil content after feeding experiments with means and standard deviations. 
* = peak was visible at C18:1n-9 retention time, but was neither quantifiable nor identifiable

to fall between 0.3 and 0.6 mg of canola oil in the original gut sample. This was established by
performing a serial dilution with 5 known volumes of canola oil (0.16 µL, 0.31 µL, 0.63µL, 1.25µL, 2.5 µL)
dissolved in 500 µL of heptane and then treated with the same FAME preparation process and data

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Oil in gut (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola Oil Feeding Experiments</td>
<td>Canola Oil (n=10)</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
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<tr>
<td></td>
<td></td>
<td>22</td>
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<td></td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td></td>
<td></td>
<td>114</td>
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<td></td>
<td></td>
<td>264</td>
</tr>
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<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>45.8 ± 84.9</td>
</tr>
<tr>
<td></td>
<td>Control (Oil present, but no access for feeding) (n=10)</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>Mean ± SD</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Canola Oil and Tetramin Feeding Experiments</td>
<td>Canola Oil and Tetramin (n=5)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>23.8 ± 38.3</td>
</tr>
<tr>
<td></td>
<td>Control (fed Tetramin only) (n=5)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>
analysis procedure as the experimental samples. The known volumes were converted from µL to mg using 0.92 g/mL as the density of canola oil (Rousseau 2004). All of the solutions showed a detectable peak at the expected retention time, but the lowest concentration (0.16 µL) could not be identified by the software. This indicates that any of the gut samples containing ≥ 0.3 mg should show a peak for C18:1n-9.

**Behavioral Quantification**

Observation of feeding goldfish and preliminary video analysis revealed three main behaviors associated with feeding, which were quantified by counting in all subsequent videos. We have determined these three main behaviors to be feeding bouts, spitting bouts, and processing bouts, as defined in Table 2. While the specific motions can be singular, they are more likely to be a series of repeated motions that make up a bout, thus the counting of bouts and not singular motions. These bouts also tend to follow a sequence, beginning with a feeding bout and followed by a spitting bout, processing bout, neither, or both. Occasionally a fish would perform two bouts of the same behavior in a sequence, but these never occurred consecutively, with the exception of feeding bouts. For example, a spitting bout would be followed by a processing bout or feeding bout before another spitting bout took place, but a feeding bout could be immediately followed by another feeding bout.

The behavioral data (Table 3) MANOVA gave results as follows – Pillai-Bartlett: p = 0.09, Roy: p = 0.006, Hotelling-Lawley: p = 0.03, Wilks: p = 0.05. The Pillai test is considered to be the most conservative and robust, with the Roy giving a lower bound of the p-value. The post-hoc ANOVAS showed feeding bouts as the only dependent variable to have significant differences between independent variable groups (p = 0.04), indicating that the number of feeding bouts differed significantly between the three treatments: canola oil only, canola oil + Tetramin, and Tetramin only. Because of the high correlation between feeding time and number of feeding bouts, feeding time was not included in the MANOVA. A separate
### Table 2. Descriptions of behavior bouts and the criteria used to distinguish them.

<table>
<thead>
<tr>
<th></th>
<th>Feeding Bout</th>
<th>Spitting Bout</th>
<th>Processing Bout</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water Column Location</strong></td>
<td>Surface</td>
<td>Anywhere</td>
<td>Anywhere, but generally in the midwater</td>
</tr>
<tr>
<td><strong>Upper/Lower Jaw Movement</strong></td>
<td>Upper jaw fully protruded to surface of the water and lower jaw fully depressed, with upper jaw at or above water surface</td>
<td>Upper jaw fully protruded and lower jaw fully depressed</td>
<td>Partial protrusion of upper jaw without depression of lower jaw</td>
</tr>
<tr>
<td><strong>Jaw Opening</strong></td>
<td>Alternates between fully open and fully closed throughout bout</td>
<td>Fully open, but sometimes preceded by a series of partial openings</td>
<td>Not open</td>
</tr>
<tr>
<td><strong>Anterior Expulsion from Oral Cavity</strong></td>
<td>None</td>
<td>Air bubbles, oil, or food particles</td>
<td>None</td>
</tr>
<tr>
<td><strong>Posterior Expulsion from Opercular Cavity</strong></td>
<td>Occasionally air bubbles</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Sequence</strong></td>
<td>Always begins the sequence</td>
<td>Follows feeding bout; follows or precedes processing bout</td>
<td>Follows feeding bout; follows or precedes spitting bout</td>
</tr>
<tr>
<td><strong>Repeated Motion</strong></td>
<td>Full protrusion of upper jaw at or above surface and then closing</td>
<td>Rapid opening and closing of jaws (not all repetitions need contain a full protrusion of the upper jaw and depression of the lower jaw so long as one is contained within the bout)</td>
<td>Partial protrusion of upper jaw</td>
</tr>
<tr>
<td><strong>End Indicator</strong></td>
<td>Upper jaw is brought below and deliberately away from the surface and jaw is closed</td>
<td>Either closing of the jaw or the expulsion of air, oil, or food from the oral cavity</td>
<td>Ceasing of motion or switch to different behavior</td>
</tr>
</tbody>
</table>
one-way ANOVA was performed on the feeding time data that also gave a significant result ($p < 0.001$) and post-hoc Tukey-Kramer tests revealed significant differences between all treatment groups.

The number of spitting and processing bouts did not differ significantly among groups ($p = 1.00$) which caught the experimenters’ attention since the number of spitting and processing bouts are by definition affected by the number of feeding bouts. This suggested a relationship not visible in the previous MANOVA. A one-way ANOVA was performed on the ratio of processing bouts to feeding bouts with $p = 0.002$. This dataset was transformed using a reciprocal transformation ($Y' = 1/Y$). Tukey-Kramer post-hoc

<table>
<thead>
<tr>
<th></th>
<th>Time Fed (sec)</th>
<th>Feeding Bouts</th>
<th>Spitting Bouts</th>
<th>Processing Bouts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Canola Oil Only</strong> <em>(n = 9)</em></td>
<td>70</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>32</td>
<td></td>
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<td>38</td>
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<td>49</td>
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<td>39</td>
<td>35</td>
<td>23</td>
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<td>67</td>
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<td>95</td>
<td>98</td>
<td>75</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>108</td>
<td>50</td>
<td>41</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>11</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>57.4 ± 31.1</td>
<td>44.7 ± 27.7</td>
<td>39.4 ± 23.8</td>
<td>20.0 ± 13.8</td>
</tr>
<tr>
<td><strong>Canola Oil + Tetramin</strong> <em>(n = 5)</em></td>
<td>400</td>
<td>199</td>
<td>71</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>307</td>
<td>203</td>
<td>115</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>287</td>
<td>182</td>
<td>67</td>
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<tr>
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<td>351</td>
<td>128</td>
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<td>21</td>
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<tr>
<td></td>
<td>288</td>
<td>159</td>
<td>52</td>
<td>11</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>326.6 ± 48.5</td>
<td>174.2 ± 31.1</td>
<td>73.0 ± 24.6</td>
<td>15.4 ± 4.7</td>
</tr>
<tr>
<td><strong>Tetramin Only</strong> <em>(n = 5)</em></td>
<td>197</td>
<td>90</td>
<td>54</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>246</td>
<td>147</td>
<td>37</td>
<td>22</td>
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<tr>
<td></td>
<td>98</td>
<td>32</td>
<td>18</td>
<td>16</td>
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<tr>
<td></td>
<td>161</td>
<td>58</td>
<td>34</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>116</td>
<td>99</td>
<td>68</td>
<td>15</td>
</tr>
<tr>
<td><strong>Mean ± SD</strong></td>
<td>163.6 ± 60.2</td>
<td>85.2 ± 43.6</td>
<td>42.2 ± 19.3</td>
<td>14.6 ± 5.0</td>
</tr>
</tbody>
</table>

**Table 3.** Behavioral data collected from video analysis with means and standard deviations. Bouts are measured in counts for the 20-minute experiment. Time Fed and Feeding Bouts were not quantified for the first fish and Spitting and Processing Bouts were not counted for the first five.
tests showed significant differences between the canola treatment and the canola + Tetramin treatment 
\( p = 0.001 \) and the Tetramin treatment and the canola + Tetramin treatment \( p = 0.02 \), but not 
between the canola treatment and the Tetramin treatment \( p = 0.29 \) (Figure 1). The same procedure 
was applied to the ratio of spitting bouts to feeding bouts with \( p = 0.06 \). No post-hoc tests were 
performed due to the non-significant result.

**Figure 1.** Average ratios of processing bouts to feeding bouts with 95% confidence intervals. Bars labelled with different letters are significantly different from one another.

**Discussion**

From this study we conclude that goldfish are able to retain and swallow liquid oil. 70% of fish \( n = 10 \) in 
the canola oil feeding experiment consumed oil, including the sample with 0.3 mg of oil that rounded to 
zero. Of the 2.0 mL of oil spread on the surface, they consumed between .01% and 14% of the oil 
present during the 20-minute experiment. 40% of fish \( n=5 \) in the canola oil + Tetramin feeding 
experiment consumed oil. These two fish consumed 11% and 32% of the 0.3 mL of oil present. The gut 

oil content quantified here is likely to have been slightly underestimated due to the fact that oil was not
retrieved from the intestine proper and in some specimens, oil was observed visually past the point where the gut sample was cut. As far as the experimenters are aware, liquid oil consumption by fish species has not been shown in any other study.

The high variability of oil content in the gut could be due to small differences in fish personality (Mesquita et al. 2015, Pleizier et al. 2015), preference, or skill that created large differences in their performance during experimental trials. None of the control fish in either experiment had peaks at the expected retention time for C18:1n-9, which suggests that any potential contamination did not lead to false positive results. This implies that the goldfish were able to swallow in some cases a relatively large amount of oil, although it says nothing about whether the ingestion was purposeful or incidental. Despite the fact that all of the fish, excluding the controls that were prevented from feeding at the surface, were observed feeding at the surface, not all of them were shown to have swallowed oil. If ingestion had been incidental, we would expect a more consistent pattern of oil consumption correlated with time spent feeding or the number of feeding bouts. This pattern would be expected particularly in the canola + Tetramin treatment group, where fish were actively ingesting food particles that were visible in the gut after dissection. Since the majority of this group did not ingest oil despite ingesting Tetramin, it is possible some selectivity is taking place by an unknown mechanism.

Goldfish have been shown to be crossflow suspension-feeders, meaning they do not use their gill rakers as dead-end mechanical sieves. In this mechanism of selection, particles do not need to come in contact with feeding structures in order to be retained, but rather are carried by flow patterns through the buccal cavity to the esophagus (Sanderson et al. 2001). The same or a similar mechanism could allow goldfish to retain and subsequently swallow oil globules or films. This process could require emulsion of the oil with the water inside the buccal cavity in order for the oil to have properties more like a low-density particle than a surface film, which could be made possible by the same repetitive jaw movement that allows water and air to mix during air-gulping (Burggren 1982). Particle selection in goldfish is aided
by action of the palatal organ, a ridged, protrusible, highly chemosensory organ found on the roof of the anterior pharynx. When the palatal organ senses food particles, fine muscular projections allow it to pin those particles against the floor of the buccal cavity while inorganic material is expelled by spitting (Callan and Sanderson 2003, Finger 2008). The palatal organ could also assist in differentiating between oil globules and Tetramin, which could explain how some goldfish were able to ingest Tetramin without ingesting oil, discussed further below.

An interesting alternative possibility is that by protruding the upper jaw above the surface during a feeding bout, goldfish might engulf the entire surface layer and pump this layer posteriorly along the palatal organ towards the esophagus as a continuous film. This might be possible due to the goldfish’s angled position relative to the surface during surface feeding, which would place the pharynx and esophagus even with the surface layer. In this case, ingestion of oil might actually be reduced by repeated feedings at the surface, which could explain how some fish swallowed significantly larger masses of oil if repetitions of feeding motions during feeding bouts, which we did not measure, differed as well. This has not been studied, but could be the subject of future research into mechanisms and could involve a more passive consumption of oil than if globules are consumed.

The significant relationship between experimental treatment and the ratio of processing bouts to feeding bouts suggests that processing could be important for handling different food types. It is interesting to note that the canola + Tetramin group had the lowest ratio of processing to feeding, even lower than Tetramin alone. Processing has been identified and described previously in carps as a mechanism for repositioning food in the buccal cavity before swallowing (Sibbing et al. 1986). Handling multiple food types at once would seem to require more processing time, yet these fish took the least processing time of all treatment groups and three of five still managed to swallow no detectable oil. If the Tetramin group also had very little processing, this would suggest that something about oil required processing for it to be swallowed, but there was no significant difference between the Canola treatment
and the Tetramin treatment. One explanation could be that increased spitting in the canola + Tetramin
treatment prevented them from swallowing either oil or Tetramin, but there was no significant
difference found between the treatments for the ratio of spitting to feeding. It is possible fish were able
to avoid the larger globules of oil visually, but in the experimental setup, Tetramin was added on top of
the oil, so it seems unlikely these fish would be able to avoid it entirely.

Closed mouth processing was described as essential for food handling in experiments conducted by
Sibbing et al. (1986) with the common carp (Cyprinus carpio), occurring infrequently throughout feeding
but more often as food became “less manageable or more soiled.” These experiments also
differentiated between feeding styles on different types of food and showed repetitive “gulping”
behavior when feeding on small zooplankton that was distinct from the usual suspension feeding on
larger food types. During this type of feeding, particle selection was controlled by palatal activity and
closed mouth protrusion, which served to collect particles that had been retained for transport to the
pharynx. Sibbing et al.’s descriptions of feeding, spitting, and processing in the carp while feeding on
small particles are consistent with this study’s observations in goldfish, however lower jaw depression
during processing was not noted by the experimenters.

The closed mouth premaxillary protrusion necessary for this type of processing to take place was able to
evolve in cypriniforms due to an elongated kinethmoid, a suspended bone connected to the premaxillae
and maxillae by ligaments. The origin of this structure allowed for a decoupling of upper and lower jaw
extension not found in acanthomorphs. This uncoupling allows for cypriniforms to have more flexible
and variable feeding movements compared to acanthomorphs, many of which have a different
mechanism for premaxillary protrusion. Increased flexibility could allow cypriniforms to adapt to a wider
variety of food types (Staab et al. 2012, Hernandez and Staab 2015), which, when coupled with the
cypriniforms use of aquatic surface respiration (He et al. 2015, Fu et al. 2014), makes them important
future study species for potential feeding on surface films.
Lipids are an important area of focus in determining the optimal diets of aquaculture fish species due to their importance in determining the growth rate of fish (Leaver 2008). This has led to many studies investigating the varying effects of dietary lipid levels (Bonvini et al. 2015, González-Félix et al. 2015, Wang et al. 2015) and source (Pozernick and Wiegand 1997, Duan et al. 2014, Sprague et al. 2015). These conclude that there is an optimal level of lipid consumption in a fish diet that interacts closely with protein consumption. Wang et al. (2015) varied lipid levels in the diets of Prussian carp (Carassius gibelio), a close relative of goldfish once thought to be a subspecies, and concluded that 11.6% was the optimal lipid level for juvenile growth performance. Various studies have looked to replace fish oil in aquaculture feeds with vegetable oil sources, with varying but promising results (Pozernick and Wiegand 1997, Duan et al. 2014, Sprague et al. 2015). If vegetable oils can potentially be used as a lipid source in artificial feeds, then it is possible that fish in the wild could benefit from the consumption of such oils along with the free fatty acids from planktonic and animal sources found in the surface microlayer (Sodergren 1979). This is not limited to freshwater environments, as surface microlayers are well studied in marine environments and have been shown to be important habitats for larval fish (Kattner et al. 1983, Hardy et al. 1987, Wurl and Obbard 2004).

Further study is needed into the mechanism by which goldfish are able to retain and swallow liquid oil, particularly in characterizing the location, movement, and form of the oil within the buccal cavity. This could also shed light on whether the process is purposeful or incidental and could help to explain the large variation in the data collected in this study. Of morphological and ecological interest is the question of whether any other fish species are capable of ingesting liquid oil. Other cypriniforms that use aquatic surface respiration might also be capable of ingesting liquid oil, but there is also the question of whether they will exhibit the surface feeding behavior observed in captive goldfish. Other families of fish are capable of premaxillary protrusion, and studying whether this behavior is found in those families may shed light on the retention process for all species or could demonstrate an entirely
different oil retention mechanism. It would also be of interest to look at species that use ASR and whether they are more likely to feed on surface layers. The results of our study raise the question of whether other suspension-feeding fish species could retain liquid oil in the form of globules and/or a surface film.

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Works Cited


