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Separating oil from water: suspension-feeding goldfish ingest liquid vegetable oil

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Abstract: We show that goldfish (*Carassius auratus*) voluntarily ingest liquid canola oil at the surface of the water and can swallow significant quantities of oil. The ability of fish to separate floating oil from water has not been tested previously, and the mechanisms used to retain oil in the form of suspended droplets, globules, or a surface film are unknown. Chromatograms of fatty acid methyl esters (FAMEs) prepared from gut samples confirmed that goldfish were able to obtain a substantial proportion of their daily lipid intake from canola oil at the surface of laboratory aquaria. Quantification of goldfish suspension-feeding, processing, and spitting behavior suggested that upper jaw protrusion with a closed mouth during processing was important for the handling of different food types, including oil. Crossflow filtration and the generation of vortices could be involved in oil retention by goldfish, as these processes are used industrially to separate oil from water. These results have implications for the uptake of hydrophobic pollutants and dietary lipids at the surface by suspension-feeding fishes.

Introduction

Suspension-feeding fishes with economic and ecological importance, including carp, menhaden, and many tilapia, can filter particles as small as 5 microns from enormous volumes of water (Beveridge et al. 1991; Friedland et al. 2006; Smith and Sanderson 2013). Rather than using mechanical dead-end sieving during which water is forced to travel perpendicularly through the filter, most suspension-feeding fishes that have been studied use crossflow filtration, during which the water to be filtered is moved tangentially across filtering structures inside the oral cavity (Sanderson et al. 2001; Callan and Sanderson 2003; Motta et al. 2010). Although industrial crossflow filtration is a major technology for separating oils from wastewater (Masoudnia et al. 2013; Tashvigh et al. 2015), the possibility that suspension-feeding fish may be able to ingest lipids by separating liquid oil from water inside their oral cavities has not been investigated. In addition, principles of vortical cross-step filtration (Sanderson et al. 2016) could enable fish to generate vortices inside their oral cavities, potentially concentrating oil, surfactant-coated air bubbles, and other positively buoyant materials with a density ($g \cdot cm^{-3}$) less than that of water.

Goldfish (*Carassius auratus*, Cyprinidae) are omnivorous benthic feeders (Sibbing and Witte 2005) that also use crossflow filtration during facultative suspension feeding (Sanderson et al. 2001). In aquaria, goldfish often suspension feed at the surface on small neutral and low-density food particles (Burggren 1982). In manmade outdoor ponds, goldfish can use continuous suspension feeding at the surface, drawing the surface layer of water through their oral cavities and out past the opercula repeatedly (personal observation).

Based on our observations of this suspension-feeding behavior at the surface in goldfish and other fish species, we designed experiments to determine whether goldfish can use liquid oil at

the surface as a potential food source. The aquatic surface microlayer at the water-air interface, a few microns to a millimeter thick, accumulates microorganisms and organic nutrients including surfactants such as fatty acids and other lipids (Wotton and Preston 2005; Drudge and Warren 2014; Seliskar and Gallagher 2014). In lakes and ponds, the surface microlayer can become enriched with bacteria, ciliates, flagellates, amoeba, and phytoplankton (Södergren 1979, 1993; Parker and Hatcher 1974; Maki and Hermansson 1994), and has been shown to attract larvae of insects such as blackflies and mosquitoes (Wotton 1982; Wotton et al. 1997). Surface microlayers rich in organic nutrients have also been well studied in marine environments (Cunliffe et al. 2013; Elliott et al. 2014; Zhou et al. 2014) and can be important habitats for larval fish (Wurl and Obbard 2004).

Lipids are important in the diets of all animals, for use in the structure of cell membranes as well as energy provision and storage (Leaver et al. 2008). Pozernick and Wiegand (1997) reported that juvenile goldfish are capable of producing important polyunsaturated fatty acids using fatty acid precursors from the canola oil in their pellet food. The main sources of fatty acids in wild goldfish are likely to be from their natural diet of detritus, diatoms, and zooplankton (Specziár et al. 1997; Specziár and Rezsu 2009).

In this study, we assess quantitatively whether untrained goldfish (1) feed voluntarily on liquid oil at the surface of the water and (2) can ingest measurable amounts of liquid oil. We performed fatty acid analysis on goldfish gut contents after feeding experiments using canola oil, a component of commercial fish feeds (Tacon et al. 2011). Previous studies have developed methodologies for using fatty acid analysis of gut contents and tissues to determine diets and food webs for marine and freshwater organisms (Carreón-Palau et al. 2013; Couturier et al. 2013*a*). We also conducted feeding experiments with a combination of liquid oil and Tetramin™

flakes to test whether the introduction of a familiar food at the surface would lead to higher oil consumption. After establishing that the goldfish were ingesting canola oil, we defined and quantified three feeding behaviors (surface feeding, spitting, and processing), to determine whether the occurrence of these behaviors was correlated with food type (oil and/or Tetramin™) and with oil consumption.

Materials and methods

Feeding experiments

Juvenile comet goldfish (5.2 – 7.3 cm standard length, SL; approximately 9 g body weight), a conventional pond variety, were obtained through the aquarium trade and maintained in the laboratory in a 284 L aquarium at 24 °C. The fish were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011), and the research protocol was approved by the Institutional Animal Care and Use Committee of the College of William & Mary (IACUC-2015-02-03-10023-slsand). Goldfish were fed daily with Tetramin™ flakes (1–10 mm diameter) that were introduced at the water surface, but the fish were not exposed to canola oil prior to the experiments.

For all experiments, goldfish were transferred individually into 38 L aquaria equipped with a bubble-up filter (Second Nature Whisper Size 2). Each fish was allowed to acclimate for 3–5 d, during which the fish was fed twice daily at the surface on finely ground Tetramin™ flakes (0.1–0.5 mm diameter). For 36 h prior to the experiment, fish were not fed and plastic grating (1.5 cm x 1.5 cm x 1.0 cm) was inserted on the bottom of the aquarium to reduce feeding on sunken food particles or feces. The bottom of the aquarium was cleaned by siphoning twice each day.

82

83 ***Canola oil feeding experiments***

84 In the oil treatment (n = 10 fish), 2.0 mL of liquid canola oil (Crisco®) was added with a 5
85 mL syringe as evenly as feasible on the water surface, and the oil was spread with a spatula. The
86 bubble-up filter was then turned off, the aquarium lid was put back into place, and the
87 experimenters stepped away from the aquarium. The fish was allowed to feed on the canola oil
88 for 20.0 min, timed from the first feeding. During this period, the time spent feeding at the
89 surface was recorded using a stopwatch and the fish was videotaped at 30 fps on MiniDV
90 cassettes using a Sony Handycam (DCR-HC36) for subsequent behavioral analyses. After 20.0
91 min, the fish was caught in a hand net that was pulled through the surface layer of oil.

92 In the control for the oil treatment (n = 10 fish), the bubble-up filter was turned off and
93 removed from the aquarium before oil was added. This provided space for additional pieces of
94 plastic grating (described above) that were used to sequester the fish away from the surface. The
95 grating was inserted from the top of the aquarium at an angle such that one edge rested along the
96 bottom length of the aquarium and the opposite edge of the grating rested against the aquarium
97 glass directly beneath the surface. The angled grating allowed water to move freely in the
98 aquarium. Approximately one-half of the aquarium volume was accessible to the fish swimming
99 beneath the grating, but the fish could not reach the surface. After the grating was in place, 2.0
100 mL of canola oil was added and spread by the method described above. As these control fish did
101 not have access to the surface and did not exhibit feeding behavior, they were not videotaped and
102 20.0 min were allowed to pass after the grating was added. The grating was then removed and
103 the fish was caught in a hand net that was pulled through the surface layer of oil. Thus, this

control for the oil treatment enabled quantification of potential contamination in gut contents from goldfish that had been exposed to surface oil but had been unable to feed on the oil.

Canola oil + Tetramin™ feeding experiments

In the canola oil + Tetramin™ treatment (n = 5 fish), 0.3 mL of canola oil from the same container of oil used in the above experiments was added with a 1 mL syringe and was spread by spatula, and the bubble-up filter was turned off. Next, 15.0 mg of finely ground Tetramin™ flakes (0.1–0.5 mm diameter), measured on a Fisher Scientific XA-100 analytical balance, was sprinkled directly from the weighing pan evenly across the water surface. The aquarium lid was put back into place and the experimenters stepped away from the aquarium. The fish was allowed to feed on the canola oil and Tetramin™ for 20.0 min, timed from the 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. After 20.0 min, the fish was caught in a hand net that was pulled through the surface layer of oil and Tetramin™.

In the control for the oil + Tetramin™ treatment (n = 5 fish), the filter was turned off first, and then 15.0 mg of Tetramin™ was sprinkled evenly across the surface. The filter was turned off before Tetramin™ was added because the action created by the air bubbles rising to the surface caused the flakes to sink. Canola oil was not added to the aquarium and the fish were allowed free access to the surface. The aquarium lid was put back into place and the experimenters stepped away from the aquarium. The fish was allowed to feed on the Tetramin™ for 20.0 min, timed from the 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 analyses. After 20.0 min, the fish was caught in a hand net that was pulled through the surface layer of Tetramin™.

Preparation of gut samples and lipid extraction

After removal from the aquarium using a hand net, goldfish were transferred into a paper towel to absorb any oil from the body surface. Fish were euthanized immediately using cervical transection followed by pithing, while being held lightly to avoid redistributing the gut contents. Fish were then blotted with paper towel before dissection to avoid transfer of any residual surface oil into the body cavity. While still connected, the anterior portion of the gut was straightened and laid flaccidly across the exposed body cavity. The first 2.5 cm of the gut immediately posterior to the esophageal sphincter was measured, forceps were clamped at each end of this section, and the section was removed using microdissection scissors. This gut segment was transferred directly into a 1.5 mL centrifuge tube. The total length, fork length, and standard length of each fish were recorded.

The gut segment was then cut longitudinally using microdissection scissors while held with forceps inside the centrifuge tube, to transform the gut to an open sheet with contents exposed. The scissors and forceps 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 s 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 1.0 mL sample was centrifuged at 5000 rpm for 5 min with a Fisher Scientific Micro 7 microcentrifuge. A 500 µL subsample was micropipetted from the surface of this gut sample and transferred directly into a 15 mL centrifuge tube.

150

151 **Fatty Acid Methyl Ester (FAME) preparation**

152 Fatty acid methyl ester (FAME) preparation was carried out using the protocol described by
153 Zhang et al. (2014). 1.0 mL each of diethyl ether, petroleum ether, and 0.4 M KOH in methanol
154 were added to 500 µL of the gut subsample in that order. This mixture was vortexed for 30 s and
155 left at room temperature (21 °C) for 2.5 h. 2.0 mL of deionized water was added and the mixture
156 was centrifuged at 3400 rpm for 2 min with a Fisher Scientific Centrific Model 228.

157 A 100 µL subsample was micropipetted from the top (organic) layer of this mixture and added
158 to 400 µL of diethyl ether in a 1.5 mL glass sample vial (Thermo Scientific). When these
159 FAMEs were stored at -5 °C, the meniscus was noted on the sample vial so that evaporation
160 could be detected. If diethyl ether evaporation occurred before analysis, diethyl ether was
161 replaced one drop at a time using a Pasteur pipette until the volume was reestablished at the
162 meniscus.

163

164 **Gas chromatography-mass spectrometry (GC-MS) analysis**

165 FAME samples in diethyl ether were injected into an Agilent 6890N gas chromatograph
166 interfaced to an Agilent 5973 mass spectrometer detector (MSD). A fused silica Rxi-1ms
167 nonpolar column was used (30 m, 25 mm ID, 0.25 µm film, Restek). The column flow rate was
168 1.1 $mL \cdot min^{-1}$ and helium was used as the carrier gas. The inlet temperature was 280 °C with a
169 split injection set at 100:1. The initial oven temperature was 150 °C, which was increased at a
170 rate of 5 $°C \cdot min^{-1}$ until the final temperature of 260 °C was reached. The total run time was 22
171 min.

Identification of methylated fatty acids from the gut extracts was verified using a NIST mass spectral library which compares mass fragmentation and ion intensity patterns of known compounds within the database to mass spectra from unknown samples. The methylated fatty acids were consistently identified with 95-99% confidence in all cases when a sufficient quantity of compound was detected from the extracts. As the first step in calculating the mass of canola oil in the 2.5 cm sections of gut from the feeding experiments, we quantified the area of the oleic acid (18:1*n*-9) peak of each FAME injection sample, which had a retention time of 13.0 min as determined from preparation of FAMEs using known concentrations of canola oil. Oleic acid is the major fatty acid component of canola oil (approximately 63% by mass; Syed 2012), 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 $mg \cdot mL^{-1}$ in heptane by dissolving 100 mg into 10 mL of heptane (Fisher Scientific, 99.7%) and then dissolving a 1 mL subsample into another 10 mL of heptane. The methyl oleate standard was analyzed each day of experiments using the same GC-MS procedure as above, and the area of this standard peak was compared to the area of the 18:1*n*-9 peak from each FAME injection sample that was analyzed with the GC-MS on that day. Peak areas were quantified using the AutoIntegrate function of MSD ChemStation software (Agilent Technologies) or a Manual Integration function for peaks with low signals to define the base width of the 18:1*n*-9 peak. The areas of the 18:1*n*-9 peak from the FAME injection samples were compared with the known concentration of the methyl oleate standard to determine the solution concentration. The fatty acid composition of canola oil, based on 63% oleic acid composition (Syed 2012), and the dilution factors used to prepare the gut sample were then used to calculate the mass of canola oil in the original 2.5 cm gut segment.

195 **Calculations of mass of oil ingested**

196 Equation (1) below uses the ratio of the known concentration in $mg \cdot mL^{-1}$ of the standard
197 methyl oleate solution to the peak area of the standard in order to calculate the concentration of
198 oleic acid in the FAME sample that had been injected into the GC-MS, where A = area of 18:1*n*–
199 9 peak, C = concentration of 18:1*n*–9, s = methyl oleate standard, and f = FAME sample. This
200 calculation is shown simplified in equation (3), which is possible since the concentration of the
201 standard was known to be 1 $mg \cdot mL^{-1}$ (equation (2)). Equation (4) shows the calculations
202 necessary to convert the concentration of oleic acid in the FAME sample to the mass of canola
203 oil in the 2.5 cm gut segment. The FAME sample concentration is multiplied by 2.5 mL, the
204 volume of the organic layer (including ethers and heptane) at the end of the initial FAME
205 preparation process. This value is then divided by 0.63 since canola oil is only 63% oleic acid
206 (Syed 2012). The FAME sample concentration in Equation 4 is also divided by 0.2 to account for
207 the 100 µL sample dilution to 500 µL with diethyl ether during FAME preparation and by 0.5 to
208 account for only one half of the original heptane gut extract being used for the FAME. By
209 substituting equation (3) into equation (4), all of the above steps were calculated at once as
210 shown in equation (5) to obtain the mg canola oil in the 2.5 cm gut sample.

211 (1) $\frac{A_s}{C_s} = \frac{A_f}{C_f}$

212 (2) $C_S = \frac{1\ mg}{mL}$

213 (3) $C_f = \left(\frac{A_f}{A_s}\right)\left(\frac{1\ mg}{mL}\right)$

214 (4) $\frac{C_f\ (2.5\ mL)}{(0.63)\ (0.5)\ (0.2)} =$ mass of canola oil in 2.5 cm gut segment (mg)

215 (5) $\frac{\left(\frac{A_f}{A_s}\right)\left(\frac{1\ mg}{mL}\right)(2.5\ mL)}{(0.63)\ (0.5)\ (0.2)} =$ mass of canola oil in 2.5 cm gut segment (mg)

216

Behavioral analyses

The videos taken during feeding experiments were viewed frame-by-frame on a Sony DVCam (DSR-11) using a remote control with a jog/shuttle (DSRM-20). Videos were analyzed for the presence of three main behaviors, which were defined after preliminary review of multiple videos: feeding bouts, spitting bouts, and processing bouts. Occurrences of each type of bout were counted for the duration of the 20 min experiments.

Statistical analysis

Analyses were performed with the statistical software R (v.3.2.1), using tests appropriate for small sample sizes with high variance within treatments and non-normal distributions. For the comparison of mass of oil in the gut segment, a non-parametric permutation test was chosen because the data lacked a normal distribution and the treatment and control groups had different variances (Whitlock and Schluter 2015). Using the R package "coin" (Hothorn et al. 2008), two-sample Fisher Pitman permutation tests were used to compute an exact p-value for the mass of oil ingested during each of the two feeding experiments. In addition, a Pearson's product-moment correlation was done to determine if a relationship existed between the time spent feeding at the surface and the mass of oil in the gut segments from fish in the canola oil treatment.

The first five fish of the canola oil feeding experiments were not videotaped. Therefore, feeding time data and behavioral counts were not recorded for these first fish and they were not included in the behavioral analyses. A regression analysis showed that the feeding time data and the feeding bout behavioral counts were highly correlated ($r^2 = 0.85$). Therefore, feeding time data were excluded from the MANOVA described below.

Due to the large differences in variances and the non-normal shape of the data distribution, the behavioral data were transformed using a log transformation (Y' = ln(Y)). A series of F-tests was then performed to compare the variances of the counts for the different feeding behaviors, which gave non-significant results for all pairs, indicating that the transformed datasets did not have significant differences in variance. A MANOVA was performed on the transformed behavioral data with food type (canola oil only, canola oil + Tetramin™, Tetramin™ only) as the independent variable and type of behavior (feeding bouts, spitting bouts, processing bouts) as the dependent variable. This was followed by univariate post-hoc ANOVAs with Bonferroni adjustments for repeated tests. A separate one-way ANOVA was also performed on data for feeding time and was followed by post-hoc Tukey-Kramer tests.

Experienced goldfish feeding on oil

Following completion of all experiments, seven juvenile goldfish (approximately 7.0 – 7.5 cm SL) that had not been introduced previously to canola oil were maintained in a 284 L aquarium. Using a polyethylene cannula (1.14 mm I.D., 1.57 mm O.D., Intramedic PE-160) on a 5 mL syringe that was held manually in one corner of the aquarium, the experimenter released a total of 1 mL of canola oil into the aquarium over a period of approximately 15 min. Oil was released from the cannula either above the water surface or approximately 1 cm beneath the surface. This procedure was followed once each day for 4 – 5 d each week. Goldfish were fed their typical diet of Tetramin™ after each oil-feeding session as well as on days when oil was not fed to the fish.

Results

Mass of canola oil in the gut

While there was high variability among fish, canola oil was present in the guts of the majority of the canola oil treatment fish and in two of five fish from the canola oil + Tetramin™ treatment (Table 1). Overall, nine of 15 fish that fed at the surface in the presence of oil had detectable oil in their guts. In contrast, none of the 15 control fish samples showed a peak at the 18:1*n*-9 retention time, indicating that the oil in the experimental samples resulted from ingestion during feeding in the presence of oil and that contamination of gut samples with oil did not occur. The guts of the fish in the canola oil treatment group had a significantly higher mass of oil than the guts in the control group, which contained no detectable oil (two-sample Fisher Pitman permutation test, $p = 0.005$, $n_i = 10$). The mass of oil in the guts of the canola oil + Tetramin™ group was not significantly different than the zero mass of oil in the control group ($p = 0.22$, $n_i = 5$). No correlation was found between the time spent feeding at the surface and the mass of oil in the gut segments from fish in the canola oil treatment (Pearson's product-moment correlation, $r^2 = 0.24$, $p = 0.19$, $n = 10$).

Detection limit

One of the FAME samples analyzed from the canola oil treatment had a detectable peak at the expected retention time for 18:1*n*-9, but the peak area was too small to be identified or quantified by the GC-MS software. This sample was reanalyzed at 167% of the original concentration by dissolving the 100 µL FAME subsample in half the volume of diethyl ether (200 µL was added instead of 400µL) before GC-MS analysis. This provided a quantifiable peak that could be identified as 18:1*n*-9, resulting in a calculation of 0.3 mg of canola oil in the 2.5 cm gut segment. All calculations for mass of oil in the 2.5 cm gut segment were rounded to the nearest milligram.

Therefore, the value of 0.3 mg was rounded to zero, as indicated by an asterisk in Table 1. No other mass chromatograms had a peak that was not identifiable by the GC-MS software, so the above procedure was not performed on other samples.

The detection limit, when a peak that is detectable above background noise at the expected retention time for 18:1*n*-9 is so low that it cannot be identified as 18:1*n*-9 by the GC-MS software, was determined to occur between 0.3 and 0.6 mg of canola oil in the 2.5 cm gut segment. This was established by performing a serial dilution with five 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 analysis procedure as the experimental samples. The known volumes of canola oil were converted from µL to mg using 0.92 $g \cdot mL^{-1}$ as the density of canola oil (Rousseau 2004). All of the FAMEs from this serial dilution resulted in a detectable peak at the expected retention time, but the peak at the lowest oil volume of 0.16 µL could not be identified by the mass spectral compound identification software. This indicates that the GC-MS would have detected a peak for 18:1*n*-9 in the FAME sample prepared from any 2.5 cm gut segment that contained ≥ 0.3 mg of canola oil (equivalent to 0.16 µL of oil in the 500 µL gut subsample).

Behavioral analyses

Three main behaviors associated with feeding were defined: feeding bouts, spitting bouts, and processing bouts (Table 2). Because a series of repeated motions usually composed a bout, we counted bouts rather than singular motions. The bouts tended to follow a sequence, beginning with a feeding bout and followed by a spitting bout, a processing bout, neither, or both. Occasionally a fish performed 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 a feeding bout before another spitting bout took place, but a feeding bout could be followed immediately by another feeding bout.

The number of bouts was counted for each 20-min video (Table 3), and a MANOVA was performed to determine whether counts of each type of behavior (feeding bouts, spitting bouts, processing bouts) differed significantly among food types (canola oil only, canola oil + Tetramin™, Tetramin™ only). The 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 (Quinn and Keough 2002). 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 among food types: 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 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.

Although by definition the number of feeding bouts affects the number of spitting and processing bouts, the number of spitting bouts and the number of processing bouts did not differ significantly among food types (post-hoc ANOVAs, $p = 1.00$). This suggests a relationship not visible in the previous MANOVA. Therefore, a one-way ANOVA was performed on the ratio of processing bouts to feeding bouts, following a reciprocal transformation ($Y' = 1/Y$). The ratio of processing bouts to feeding bouts was significantly different among food types ($p = 0.002$). Tukey-Kramer 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 ratio of processing bouts to feeding bouts in the canola oil + Tetramin™ treatment was significantly lower than this ratio in the treatments that used only one food type. When a reciprocal transformation and one-way ANOVA were applied to the ratio of spitting bouts to feeding bouts, there was no significant difference among food types (p = 0.06).

Experienced goldfish feeding on oil

Naive goldfish that had not been exposed previously to canola oil exhibited feeding bouts at the surface throughout the aquarium when canola oil was released from the cannula tip. In addition, one goldfish swam repeatedly to the underwater cannula tip and engulfed the globule of oil that was being extruded from the tip. Within two weeks after the first oil-feeding session, multiple goldfish exhibited feeding bouts directly beneath the cannula that was held just above the water surface as oil was released in drops from the tip. Goldfish also learned to engulf globules in a film of oil on the water surface that had been released from the cannula tip as the tip was being removed from the water (Video S1).

In manmade outdoor ponds, goldfish that had been introduced sporadically to liquid oil engulfed a thin layer of canola oil and interspersed oil globules at the surface, using continuous suspension feeding (personal observation, Video S2).

353 # Discussion

354

355 ## Liquid oil ingestion by goldfish

356 Untrained naive goldfish fed voluntarily on liquid canola oil at the surface of the water and
357 were able to retain and swallow liquid oil. All ten goldfish that had access to the surface during
358 the canola oil feeding experiments exhibited feeding behavior, and 70% of these fish had
359 detectable quantities of canola oil in the anterior 2.5 cm of their gut (Table 1). These fish
360 ingested between 0.01% and 14% of the 2.0 mL of oil present during the 20-min experiment. In
361 the canola oil + Tetramin™ feeding experiment, all five goldfish exhibited feeding behavior at
362 the surface and 40% of these fish ingested oil. The anterior 2.5 cm of the gut in these two fish
363 contained 11% and 32% of the 0.3 mL of oil present during the 20-min experiment. The gut oil
364 content quantified in these experiments is likely to have been underestimated because only the
365 anterior 2.5 cm of the gut was sampled. Oil was observed visually in some fish guts posterior to
366 the location where the gut segment was removed.

367 None of the fifteen control fish in the two experiments had GC-MS chromatogram peaks at
368 the expected retention time for 18:1*n*-9, suggesting that contamination with oil did not lead to
369 false positive results in the other treatment groups. The high variability of gut oil content among
370 fish could be due to small differences in fish personality (Mesquita et al. 2015; Pleizier et al.
371 2015), preference, or ability that led to differences in performance during the experiments.
372 Substantial inter- and intra-individual variability in oral flow speed, mucus production, and
373 particle retention in suspension-feeding fishes has been quantified by previous studies (Smith
374 and Sanderson 2008, 2013; Holley et al. 2015).

While some goldfish swallowed a relatively large amount of oil, this alone does not indicate whether oil ingestion was purposeful or incidental. However, despite the fact that all of the fish with access to oil at the surface were observed to feed at the surface during the experiments, some did not have a detectable level of oil in the gut. If ingestion had been incidental, we would expect a more consistent pattern of oil ingestion 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 in the presence of oil were actively ingesting Tetramin™ particles from the surface that were later visible in the gut during dissection. Three of the five fish in this group did not ingest oil despite ingesting Tetramin™, suggesting that the other two fish may have ingested oil using an unknown selection mechanism rather than incidental ingestion.

Potential mechanisms of oil ingestion

The ability of fish to separate oil from water has not been tested previously, and potential mechanisms that fish could use to separate oil from water have not been investigated. In our experiments, goldfish were observed to feed directly on the film of canola oil with larger interspersed oil globules that floated on the water surface, although smaller oil droplets and oil-coated air bubbles in suspension near the water surface may also have been available for ingestion. Many suspension-feeding fish species, including goldfish, use crossflow filtration to retain and swallow particles within the potential size range of suspended oil droplets to larger oil globules (approximately 30 µm – 5 mm; Sanderson et al. 2001; Smith and Sanderson 2013). During crossflow filtration, the gill rakers do not serve as dead-end mechanical sieves, and particles can be retained without contacting filtering elements. Particles are carried by flow patterns through the oral cavity to the esophagus (Sanderson et al. 2001; Sanderson et al. 2016).

A similar mechanism could enable goldfish to retain and subsequently swallow oil droplets, larger oil globules, and/or surface films. This process could involve emulsion of the oil with the water inside the oral cavity, caused by the repetitive lower jaw movements that also allow water and air to mix during aquatic surface respiration (Burggren 1982), resulting in intraoral oil droplets or oil-coated air bubbles with the properties of a low-density particle rather than a surface film.

During hypoxia and anoxia, goldfish and some other fish species have the capability of "air gulping" or aquatic surface respiration (ASR), which is distinct from the well-studied air breathing in certain species (Burggren 1982; Chapman and McKenzie 2009; He et al. 2015). During ASR, goldfish protrude the upper jaw above the surface to engulf an air bubble and the underlying water at the air-water interface. From this position, goldfish repeatedly depress and raise the lower jaw, mixing the air and water within the oral cavity. This mixture is then passed between the gill filaments to exit posteriorly from the opercula, resulting in a significant elevation of blood oxygen content under hypoxic conditions compared to goldfish not using ASR (Burggren 1982). Engulfment of air and water during feeding at the surface is similar to the initial step in ASR (Burggren 1982), suggesting a possible connection between the adaptation of goldfish for ASR during hypoxia and the ability to modify that behavior for suspension feeding at the surface.

Particle selection in goldfish is aided by action of the palatal organ, a ridged, protrusible, highly chemosensory pad of tissue on the roof of the anterior pharynx. Muscular projections of the palatal organ in cyprinids can pin larger solid food particles against the floor of the oral cavity while inorganic material is expelled by spitting (Sibbing et al. 1986; Callan and Sanderson 2003; Finger 2008). The palatal organ could also assist in differentiating between oil and

Tetramin™, which could explain how some goldfish were able to ingest Tetramin™ without ingesting oil, discussed further below.

An alternative mechanism for separating oil from water 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 thin film. This oil ingestion mechanism might be possible due to the goldfish's angled body position relative to the water-air interface during surface feeding, which could place regions of the palatal organ and the esophagus level with the surface of the water. Engulfment and intra-oral transport of an intact surface film could involve a more passive consumption of oil than the creation of intra-oral oil emulsions. In this case, ingestion of oil might actually be reduced by repetitive lower jaw movements during feeding that disrupt the floating film of oil inside the oral cavity. If the number of repetitive jaw movements within each feeding bout differed among individuals, this could explain how some fish swallowed substantially larger masses of oil. However, we did not quantify the number of repetitive jaw movements within each feeding bout.

Behavioral analyses

The significant relationship between food type and the ratio of processing bouts to feeding bouts (Figure 1) indicates that processing could be important for handling different food types. The canola + Tetramin™ group had the lowest ratio of processing to feeding, even lower than Tetramin™ alone. Processing has been described previously in the closely related common carp (*Cyprinus carpio*) as a mechanism for sorting and repositioning food in the oral cavity before swallowing (Sibbing et al. 1986). Handling multiple food types simultaneously would seem to require more processing, yet the goldfish in the canola + Tetramin™ group had the lowest ratio

444 of processing bouts to feeding bouts of all treatment groups, and two of these five fish still
445 swallowed oil.
446 If the canola oil group exhibited relatively more processing bouts, this would suggest that oil
447 required processing before swallowing, but there was no significant difference between the oil
448 treatment and the Tetramin™ treatment (Figure 1). One explanation could be that increased
449 spitting in the canola + Tetramin™ treatment prevented fish from swallowing oil, but the ratio of
450 spitting to feeding was not significantly different among food types. Fish may have been able to
451 avoid the larger floating globules of oil visually, but in the canola + Tetramin™ experimental
452 setup, Tetramin was added on the top of the oil layer, so complete avoidance of oil globules
453 seems unlikely.
454 Processing bouts were characterized by repetitive partial upper jaw protrusion with a closed
455 mouth (Table 2). A similar closed mouth processing ("closed protrusion") was described as
456 essential for food handling in experiments conducted by Sibbing et al. (1986) with the common
457 carp, occurring infrequently throughout feeding but more often as food became “less manageable
458 or more soiled.” During suspension feeding by carp on small zooplankton, intraoral particle
459 selection was controlled by palatal organ activity and closed protrusion, which also served to
460 gather particles that had been retained for transport to the pharynx (Sibbing et al. 1986).
461 The upper jaw protrusion with a closed mouth that we observed in goldfish during processing
462 bouts is unique to cypriniforms due to the evolution of an elongated kinethmoid and modified
463 adductor muscles. These morphological novelties allow for a decoupling of the upper and lower
464 jaws not found in acanthomorphs (Gidmark et al. 2012; Hernandez and Staab 2015). This
465 decoupling enables cypriniforms to have more flexible and variable feeding movements
466 compared to acanthomorphs. Increased functional flexibility could allow cypriniforms to be

467 opportunistic in using a greater diversity of food types (Staab et al. 2012; Hernandez and Staab
468 2015), which, when coupled with cypriniform use of aquatic surface respiration (Fu et al. 2014;
469 He et al. 2015), makes them important future study species for potential feeding on surface films
470 as well as oil droplets and globules.
471
472 **Potential implications for uptake of hydrophobic pollutants**
473 Ingestion of liquid oil by fish in the form of suspended droplets, floating globules, or a surface
474 film could be a route for the uptake and transport of hydrophobic pollutants in the wild,
475 including polycyclic aromatic hydrocarbons (PAHs). The copepod *Calanus finmarchicus*, the
476 mussel *Mytilus edulis*, and the pelagic tunicate *Dolioletta gegenbauri* actively filter particles <
477 50 μm in diameter, which is the approximate size of the smallest fraction of petroleum oil
478 droplets that accumulate in the water column (Lee et al. 2012; Nordtug et al. 2015). Laboratory
479 and modeling studies indicate that bioaccumulation of polycyclic aromatic hydrocarbons (PAHs)
480 may occur due to active ingestion of petroleum oil droplets by these suspension-feeding
481 invertebrates (Viaene et al. 2014; Nordtug et al. 2015). The lower limit of particle size that can
482 be retained has not been reported for most suspension-feeding fish species, including goldfish.
483 However, suspended oil droplets < 50 μm in diameter are well within the size range of
484 polystyrene particles ingested incidentally by suspension-feeding tilapia species (Cichlidae) that
485 use crossflow filtration (Smith and Sanderson 2013). Since particle retention in these tilapia and
486 in goldfish is not dependent on mucus entrapment or mechanical dead-end sieving (Sanderson et
487 al. 2001; Smith and Sanderson 2013), investigation is needed to assess the potential exposure of
488 such fish species to hydrophobic pollutants through the ingestion of suspended oil droplets,
489 surfactant-coated air bubbles (Walls et al. 2014), or surface films.

Role of lipids in fish nutrition

Due to their importance in determining the growth rate of fish, lipids are an important area of focus in developing the optimal diet for aquaculture (Leaver et al. 2008). Unlike many terrestrial vertebrates, fish use lipids, fatty acids, and proteins as major macronutrients rather than carbohydrates (Leaver et al. 2008). Many studies have investigated the effects of varying fish dietary lipid levels and sources. There is an optimal level of lipid consumption in fish that interacts closely with protein utilization (Leaver et al. 2008; Bonvini et al. 2015; González-Félix et al. 2015). Wang et al. (2015) varied lipid levels in the diets of fish that they identified as a subspecies, *Carassius auratus gibelio*, and concluded that the optimal lipid level for juvenile growth was 11.6% of the diet by dry mass.

A number of studies have evaluated using plant oil sources to replace fish oil in aquaculture feeds, with varying but promising results (Pozernick and Wiegand 1997; Duan et al. 2014; Sprague et al. 2015). Given that plant oils can be used as an effective lipid source in solid aquaculture feeds, further study is needed to determine whether fish in aquaculture settings or in the wild can ingest plant and animal lipids in the form of suspended oil droplets or a surface film.

Dietary requirements of most fish species are not well defined because they tend to vary with 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 laboratory study conducted by Sánchez-Vázquez et al. (1998), adult goldfish selected a diet ($g \cdot kg$ body $weight^{-1} \cdot day^{-1}$) consisting of approximately 22% protein, 32% fat, and 46% carbohydrate on average by mass from among three different macronutrient-enriched food types. The goldfish adjusted their diet based on what they had consumed in the preceding days, suggesting that they were able to select for a balanced

513 diet. The g·kg body weight^{-1}·day^{-1} of oil (pollock visceral oil:soybean oil, 2:3) in the preferred
514 diet of adult goldfish reported by Sánchez-Vázquez et al. (1998) can be used to calculate a rough
515 estimate of the dietary importance of the oil ingested by goldfish during our study. Based on
516 these data, the seven goldfish that ingested a detectable amount of oil in the canola treatment of
517 our study swallowed approximately 30% of their daily lipid intake during the 20-min
518 experiment.

519 In conclusion, this ability of goldfish to ingest liquid oil in the form of suspended oil droplets,
520 floating oil globules, and/or a surface film could have important ecological and functional
521 morphological implications. Further study is needed of the mechanisms by which goldfish are
522 able to retain and swallow liquid oil, particularly in characterizing the location, movement, and
523 form of the oil within the oral cavity. Such research could determine whether the process is
524 purposeful or incidental and could aid in explaining the variation in oil ingestion among
525 individual goldfish in this study. Our results raise the question of whether other fish species can
526 ingest liquid oil by separating oil from water. Other cypriniforms that use aquatic surface
527 respiration are candidates for study. Ram suspension-feeding marine fishes such as menhaden
528 might use a crossflow or vortical cross-step filtration mechanism (Sanderson et al. 2001;
529 Sanderson et al. 2016) to retain suspended oil droplets or surfactant-coated air bubbles (Walls et
530 al. 2014), particularly juveniles that swim in shallow-water schools extending to the water-air
531 interface. In addition, further study is needed to determine whether ingestion of surface films or
532 surfactant-coated air bubbles might contribute to the unidentified source of fatty acids reported
533 recently in suspension-feeding manta rays and whale sharks (Couturier et al. 2013*a*, 2013*b*;
534 Rohner et al. 2013), which engulf water while positioning the upper jaw at or above the water
535 surface (Paig-Tran et al. 2013; Motta et al. 2010).

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Table 1. Mass of oil in gut segment (mg) for each experimental fish, calculated from GC-MS analysis of FAMEs.

Canola oil only		Canola oil + Tetramin	
Treatment	Control	Treatment	Control
52	0	0	0
4	0	88	0
22	0	0	0
0	0	31	0
0	0	0	0
0	0		
114	0		
264	0		
2	0		
0*	0		
Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
45.8 ± 84.9	0 ± 0	23.8 ± 38.3	0 ± 0

* Peak was visible at retention time for 18:1*n*-9, but was neither identifiable nor quantifiable using the GC-MS.

Table 2. Criteria used to distinguish goldfish behaviors during experiments.

Feature	Behavior		
	Feeding Bout	Spitting Bout	Processing Bout
Water Column Location	Surface	Anywhere	Anywhere, but generally in the midwater
Upper/Lower Jaw Movement	Upper jaw fully protruded at or above surface of the water, and lower jaw fully depressed	Upper jaw fully protruded and lower jaw fully depressed	Partial protrusion of upper jaw without depression of lower jaw
Jaw Opening	Alternates between fully open and fully closed throughout bout	Fully open, but sometimes preceded by a series of partial openings	Not open
Anterior Expulsion from Oral Cavity	None	Air bubbles, oil, or food particles	None
Posterior Expulsion from Opercular Cavity	Occasionally air bubbles	None	None
Sequence	Always begins the sequence	Follows feeding bout; follows or precedes processing bout	Follows feeding bout; follows or precedes spitting bout
Repeated Motion	Full protrusion of upper jaw at or above surface and then closing	Rapid opening and closing of jaws (not all repetitions need contain a full protrusion of the upper jaw and depression of the lower jaw, as long as one is contained within the bout)	Partial protrusion of upper jaw
End Indicator	Upper jaw is brought below and deliberately away from the surface and jaw is closed	Either closing of the jaw or the expulsion of air, oil, or food from the oral cavity	Cessation of motion or switch to different behavior

Table 3. Behavioral data from video analysis of each experimental fish; bouts measured in counts for each 20-min experiment.

	Time Fed (seconds)	Feeding Bouts	Spitting Bouts	Processing Bouts
Canola Oil Only*	70	31		
	44	32		
	38	25		
	49	40		
	39	35	23	6
	67	80	45	24
	95	98	75	40
	108	50	41	22
	7	11	13	8
Mean ± SD	57.4 ± 31.1	44.7 ± 27.7	39.4 ± 23.8	20.0 ± 13.8
Canola Oil + Tetramin	400	199	71	20
	307	203	115	13
	287	182	67	12
	351	128	60	21
	288	159	52	11
Mean ± SD	326.6 ± 48.5	174.2 ± 31.1	73.0 ± 24.6	15.4 ± 4.7
Tetramin Only	197	90	54	9
	246	147	37	22
	98	32	18	16
	161	58	34	11
	116	99	68	15
Mean ± SD	163.6 ± 60.2	85.2 ± 43.6	42.2 ± 19.3	14.6 ± 5.0

* Time Fed and Feeding Bouts were not quantified for the first fish and Spitting and Processing Bouts were not counted for the first five fish.

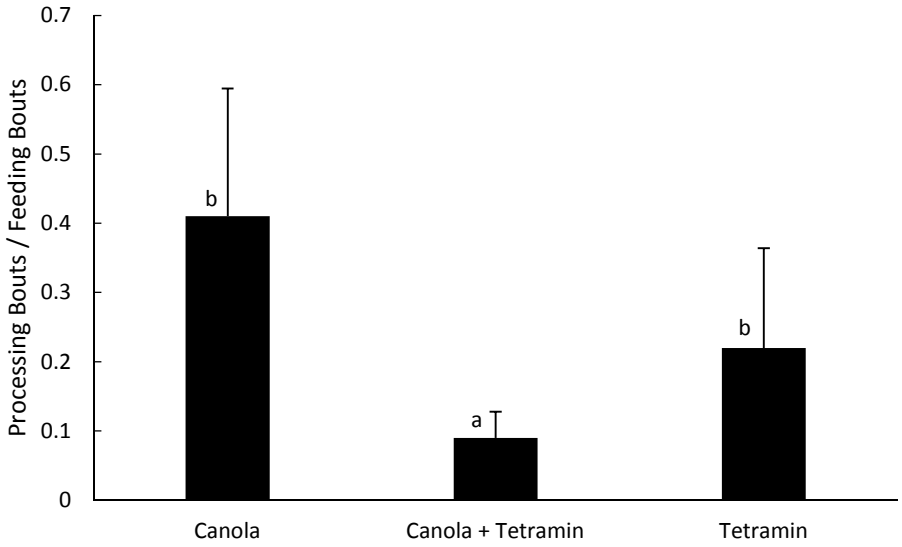

Figure 1. Average ratios of processing bouts to feeding bouts with 95% confidence intervals. Treatments labeled with different letters are significantly different.

Video S1. Following completion of experiments, juvenile goldfish in a laboratory aquarium learned to engulf globules in a film of canola oil on the water surface that had been released from the tip of a polyethylene cannula as the tip was removed from the water (240 frames·s^{-1}; video by C.M. LaValley).

Video S2. In outdoor ponds, suspension-feeding juvenile goldfish that had been introduced previously to liquid oil engulfed a thin layer of canola oil with interspersed oil globules at the surface (30 frames·s^{-1}).