

9-2-2016

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Recommended Citation

Edwards, Kristin; Rice, Gary; and Sanderson, S. Laurie, Separating oil from water: suspension-feeding goldfish ingest liquid vegetable oil (2016). *Canadian Journal of Fisheries and Aquatic Sciences*, 74(4), 524-532.
<https://doi.org/10.1139/cjfas-2016-0197>

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

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Draft

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

13 **Introduction**

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

28 Goldfish (*Carassius auratus*, Cyprinidae) are omnivorous benthic feeders (Sibbing and Witte
29 2005) that also use crossflow filtration during facultative suspension feeding (Sanderson et al.
30 2001). In aquaria, goldfish often suspension feed at the surface on small neutral and low-density
31 food particles (Burggren 1982). In manmade outdoor ponds, goldfish can use continuous
32 suspension feeding at the surface, drawing the surface layer of water through their oral cavities
33 and out past the opercula repeatedly (personal observation).

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

36 the surface as a potential food source. The aquatic surface microlayer at the water-air interface, a
37 few microns to a millimeter thick, accumulates microorganisms and organic nutrients including
38 surfactants such as fatty acids and other lipids (Wotton and Preston 2005; Drudge and Warren
39 2014; Seliskar and Gallagher 2014). In lakes and ponds, the surface microlayer can become
40 enriched with bacteria, ciliates, flagellates, amoeba, and phytoplankton (Södergren 1979, 1993;
41 Parker and Hatcher 1974; Maki and Hermansson 1994), and has been shown to attract larvae of
42 insects such as blackflies and mosquitoes (Wotton 1982; Wotton et al. 1997). Surface
43 microlayers rich in organic nutrients have also been well studied in marine environments
44 (Cunliffe et al. 2013; Elliott et al. 2014; Zhou et al. 2014) and can be important habitats for larval
45 fish (Wurl and Obbard 2004).

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

52 In this study, we assess quantitatively whether untrained goldfish (1) feed voluntarily on
53 liquid oil at the surface of the water and (2) can ingest measurable amounts of liquid oil. We
54 performed fatty acid analysis on goldfish gut contents after feeding experiments using canola oil,
55 a component of commercial fish feeds (Tacon et al. 2011). Previous studies have developed
56 methodologies for using fatty acid analysis of gut contents and tissues to determine diets and
57 food webs for marine and freshwater organisms (Carreón-Palau et al. 2013; Couturier et al.
58 2013a). We also conducted feeding experiments with a combination of liquid oil and Tetramin™

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

64

65 **Materials and methods**

66

67 **Feeding experiments**

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

76 For all experiments, goldfish were transferred individually into 38 L aquaria equipped with a
77 bubble-up filter (Second Nature Whisper Size 2). Each fish was allowed to acclimate for 3–5 d,
78 during which the fish was fed twice daily at the surface on finely ground Tetramin™ flakes (0.1–
79 0.5 mm diameter). For 36 h prior to the experiment, fish were not fed and plastic grating (1.5 cm
80 x 1.5 cm x 1.0 cm) was inserted on the bottom of the aquarium to reduce feeding on sunken food
81 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

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

106

107 *Canola oil + Tetramin™ feeding experiments*

108 In the canola oil + Tetramin™ treatment ($n = 5$ fish), 0.3 mL of canola oil from the same
109 container of oil used in the above experiments was added with a 1 mL syringe and was spread by
110 spatula, and the bubble-up filter was turned off. Next, 15.0 mg of finely ground Tetramin™
111 flakes (0.1–0.5 mm diameter), measured on a Fisher Scientific XA-100 analytical balance, was
112 sprinkled directly from the weighing pan evenly across the water surface. The aquarium lid was
113 put back into place and the experimenters stepped away from the aquarium. The fish was
114 allowed to feed on the canola oil and Tetramin™ for 20.0 min, timed from the first feeding.
115 During this period, the time spent feeding at the surface was recorded using a stopwatch and the
116 fish was videotaped at 30 fps on MiniDV cassettes using a Sony Handycam (DCR-HC36) for
117 behavioral analysis. After 20.0 min, the fish was caught in a hand net that was pulled through the
118 surface layer of oil and Tetramin™.

119 In the control for the oil + Tetramin™ treatment ($n = 5$ fish), the filter was turned off first, and
120 then 15.0 mg of Tetramin™ was sprinkled evenly across the surface. The filter was turned off
121 before Tetramin™ was added because the action created by the air bubbles rising to the surface
122 caused the flakes to sink. Canola oil was not added to the aquarium and the fish were allowed
123 free access to the surface. The aquarium lid was put back into place and the experimenters
124 stepped away from the aquarium. The fish was allowed to feed on the Tetramin™ for 20.0 min,
125 timed from the first feeding. During this period, the time spent feeding at the surface was
126 recorded using a stopwatch and the fish was videotaped at 30 fps on MiniDV cassettes using a

127 Sony Handycam (DCR-HC36) for behavioral analyses. After 20.0 min, the fish was caught in a
128 hand net that was pulled through the surface layer of Tetramin™.

129

130 **Preparation of gut samples and lipid extraction**

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

141 The gut segment was then cut longitudinally using microdissection scissors while held with
142 forceps inside the centrifuge tube, to transform the gut to an open sheet with contents exposed.
143 The scissors and forceps were rinsed with 750 μ L of heptane (Fisher Scientific, 99.7%) into the
144 centrifuge tube using a Pipetman micropipette. The sample was then vortexed for 30 s with a
145 Fisher Scientific Vortex Genie 2. The empty gut wall was removed from the centrifuge tube and
146 the forceps used were rinsed into the tube with 250 μ L of heptane. This 1.0 mL sample was
147 centrifuged at 5000 rpm for 5 min with a Fisher Scientific Micro 7 microcentrifuge. A 500 μ L
148 subsample was micropipetted from the surface of this gut sample and transferred directly into a
149 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 $^{\circ}\text{C}$) 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 Centrifuge 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 $^{\circ}\text{C}$, 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 $\text{mL}\cdot\text{min}^{-1}$ and helium was used as the carrier gas. The inlet temperature was 280 $^{\circ}\text{C}$ with a
169 split injection set at 100:1. The initial oven temperature was 150 $^{\circ}\text{C}$, which was increased at a
170 rate of 5 $^{\circ}\text{C}\cdot\text{min}^{-1}$ until the final temperature of 260 $^{\circ}\text{C}$ was reached. The total run time was 22
171 min.

172 Identification of methylated fatty acids from the gut extracts was verified using a NIST mass
173 spectral library which compares mass fragmentation and ion intensity patterns of known
174 compounds within the database to mass spectra from unknown samples. The methylated fatty
175 acids were consistently identified with 95-99% confidence in all cases when a sufficient quantity
176 of compound was detected from the extracts. As the first step in calculating the mass of canola
177 oil in the 2.5 cm sections of gut from the feeding experiments, we quantified the area of the oleic
178 acid (18:1*n*-9) peak of each FAME injection sample, which had a retention time of 13.0 min as
179 determined from preparation of FAMEs using known concentrations of canola oil. Oleic acid is
180 the major fatty acid component of canola oil (approximately 63% by mass; Syed 2012), which
181 when converted into a methyl ester becomes methyl oleate. A known standard of methyl oleate
182 (99%, Aldrich) was diluted to a concentration of 1 mg·mL⁻¹ in heptane by dissolving 100 mg
183 into 10 mL of heptane (Fisher Scientific, 99.7%) and then dissolving a 1 mL subsample into
184 another 10 mL of heptane. The methyl oleate standard was analyzed each day of experiments
185 using the same GC-MS procedure as above, and the area of this standard peak was compared to
186 the area of the 18:1*n*-9 peak from each FAME injection sample that was analyzed with the GC-
187 MS on that day. Peak areas were quantified using the AutoIntegrate function of MSD
188 ChemStation software (Agilent Technologies) or a Manual Integration function for peaks with
189 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
190 FAME injection samples were compared with the known concentration of the methyl oleate
191 standard to determine the solution concentration. The fatty acid composition of canola oil, based
192 on 63% oleic acid composition (Syed 2012), and the dilution factors used to prepare the gut
193 sample were then used to calculate the mass of canola oil in the original 2.5 cm gut segment.
194

195 **Calculations of mass of oil ingested**

196 Equation (1) below uses the ratio of the known concentration in $\text{mg}\cdot\text{mL}^{-1}$ 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 18:1n-
199 9 peak, C = concentration of 18:1n-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 $1 \text{ mg}\cdot\text{mL}^{-1}$ (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) $C_s = \frac{1 \text{ mg}}{\text{mL}}$

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

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

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

216

217 **Behavioral analyses**

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

223

224 **Statistical analysis**

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

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

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

250

251 **Experienced goldfish feeding on oil**

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

260

261 **Results**

262

263 **Mass of canola oil in the gut**

264 While there was high variability among fish, canola oil was present in the guts of the majority
265 of the canola oil treatment fish and in two of five fish from the canola oil + Tetramin™ treatment
266 (Table 1). Overall, nine of 15 fish that fed at the surface in the presence of oil had detectable oil
267 in their guts. In contrast, none of the 15 control fish samples showed a peak at the 18:1*n*-9
268 retention time, indicating that the oil in the experimental samples resulted from ingestion during
269 feeding in the presence of oil and that contamination of gut samples with oil did not occur. The
270 guts of the fish in the canola oil treatment group had a significantly higher mass of oil than the
271 guts in the control group, which contained no detectable oil (two-sample Fisher Pitman
272 permutation test, $p = 0.005$, $n_i = 10$). The mass of oil in the guts of the canola oil + Tetramin™
273 group was not significantly different than the zero mass of oil in the control group ($p = 0.22$, $n_i =$
274 5). No correlation was found between the time spent feeding at the surface and the mass of oil in
275 the gut segments from fish in the canola oil treatment (Pearson's product-moment correlation, r^2
276 = 0.24, $p = 0.19$, $n = 10$).

277

278 **Detection limit**

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

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

289 The detection limit, when a peak that is detectable above background noise at the expected
290 retention time for 18:1*n*-9 is so low that it cannot be identified as 18:1*n*-9 by the GC-MS
291 software, was determined to occur between 0.3 and 0.6 mg of canola oil in the 2.5 cm gut
292 segment. This was established by performing a serial dilution with five known volumes of canola
293 oil (0.16 μL , 0.31 μL , 0.63 μL , 1.25 μL , 2.5 μL) dissolved in 500 μL of heptane and then treated
294 with the same FAME preparation process and data analysis procedure as the experimental
295 samples. The known volumes of canola oil were converted from μL to mg using $0.92 \text{ g}\cdot\text{mL}^{-1}$ as
296 the density of canola oil (Rousseau 2004). All of the FAMES from this serial dilution resulted in
297 a detectable peak at the expected retention time, but the peak at the lowest oil volume of 0.16 μL
298 could not be identified by the mass spectral compound identification software. This indicates that
299 the GC-MS would have detected a peak for 18:1*n*-9 in the FAME sample prepared from any 2.5
300 cm gut segment that contained ≥ 0.3 mg of canola oil (equivalent to 0.16 μL of oil in the 500 μL
301 gut subsample).

302

303 **Behavioral analyses**

304 Three main behaviors associated with feeding were defined: feeding bouts, spitting bouts, and
305 processing bouts (Table 2). Because a series of repeated motions usually composed a bout, we
306 counted bouts rather than singular motions. The bouts tended to follow a sequence, beginning
307 with a feeding bout and followed by a spitting bout, a processing bout, neither, or both.
308 Occasionally a fish performed two bouts of the same behavior in a sequence, but these never
309 occurred consecutively, with the exception of feeding bouts. For example, a spitting bout would

310 be followed by a processing bout or a feeding bout before another spitting bout took place, but a
311 feeding bout could be followed immediately by another feeding bout.

312 The number of bouts was counted for each 20-min video (Table 3), and a MANOVA was
313 performed to determine whether counts of each type of behavior (feeding bouts, spitting bouts,
314 processing bouts) differed significantly among food types (canola oil only, canola oil +
315 Tetramin™, Tetramin™ only). The MANOVA gave results as follows – Pillai-Bartlett: $p = 0.09$,
316 Roy: $p = 0.006$, Hotelling-Lawley: $p = 0.03$, Wilks: $p = 0.05$. The Pillai test is considered to be
317 the most conservative and robust, with the Roy giving a lower bound of the p -value (Quinn and
318 Keough 2002). The post-hoc ANOVAS showed feeding bouts as the only dependent variable to
319 have significant differences between independent variable groups ($p = 0.04$), indicating that the
320 number of feeding bouts differed significantly among food types: canola oil only, canola oil +
321 Tetramin™, and Tetramin™ only. Because of the high correlation between feeding time and
322 number of feeding bouts, feeding time was not included in the MANOVA. A separate one-way
323 ANOVA was performed on the feeding time data that also gave a significant result ($p < 0.001$)
324 and post-hoc Tukey-Kramer tests revealed significant differences between all treatment groups.

325 Although by definition the number of feeding bouts affects the number of spitting and
326 processing bouts, the number of spitting bouts and the number of processing bouts did not differ
327 significantly among food types (post-hoc ANOVAs, $p = 1.00$). This suggests a relationship not
328 visible in the previous MANOVA. Therefore, a one-way ANOVA was performed on the ratio of
329 processing bouts to feeding bouts, following a reciprocal transformation ($Y' = 1/Y$). The ratio of
330 processing bouts to feeding bouts was significantly different among food types ($p = 0.002$).
331 Tukey-Kramer tests showed significant differences between the canola treatment and the canola
332 + Tetramin™ treatment ($p = 0.001$) and the Tetramin™ treatment and the canola + Tetramin™

333 treatment ($p = 0.02$), but not between the canola treatment and the Tetramin™ treatment ($p =$
334 0.29) (Figure 1). The ratio of processing bouts to feeding bouts in the canola oil + Tetramin™
335 treatment was significantly lower than this ratio in the treatments that used only one food type.
336 When a reciprocal transformation and one-way ANOVA were applied to the ratio of spitting
337 bouts to feeding bouts, there was no significant difference among food types ($p = 0.06$).

338

339 **Experienced goldfish feeding on oil**

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

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

351

352

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).

375 While some goldfish swallowed a relatively large amount of oil, this alone does not indicate
376 whether oil ingestion was purposeful or incidental. However, despite the fact that all of the fish
377 with access to oil at the surface were observed to feed at the surface during the experiments,
378 some did not have a detectable level of oil in the gut. If ingestion had been incidental, we would
379 expect a more consistent pattern of oil ingestion correlated with time spent feeding or the number
380 of feeding bouts. This pattern would be expected particularly in the canola + Tetramin™
381 treatment group, where fish in the presence of oil were actively ingesting Tetramin™ particles
382 from the surface that were later visible in the gut during dissection. Three of the five fish in this
383 group did not ingest oil despite ingesting Tetramin™, suggesting that the other two fish may
384 have ingested oil using an unknown selection mechanism rather than incidental ingestion.

385

386 **Potential mechanisms of oil ingestion**

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

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

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

416 Particle selection in goldfish is aided by action of the palatal organ, a ridged, protrusible,
417 highly chemosensory pad of tissue on the roof of the anterior pharynx. Muscular projections of
418 the palatal organ in cyprinids can pin larger solid food particles against the floor of the oral
419 cavity while inorganic material is expelled by spitting (Sibbing et al. 1986; Callan and Sanderson
420 2003; Finger 2008). The palatal organ could also assist in differentiating between oil and

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

423 An alternative mechanism for separating oil from water is that by protruding the upper jaw
424 above the surface during a feeding bout, goldfish might engulf the entire surface layer and pump
425 this layer posteriorly along the palatal organ towards the esophagus as a continuous thin film.
426 This oil ingestion mechanism might be possible due to the goldfish's angled body position
427 relative to the water-air interface during surface feeding, which could place regions of the palatal
428 organ and the esophagus level with the surface of the water. Engulfment and intra-oral transport
429 of an intact surface film could involve a more passive consumption of oil than the creation of
430 intra-oral oil emulsions. In this case, ingestion of oil might actually be reduced by repetitive
431 lower jaw movements during feeding that disrupt the floating film of oil inside the oral cavity. If
432 the number of repetitive jaw movements within each feeding bout differed among individuals,
433 this could explain how some fish swallowed substantially larger masses of oil. However, we did
434 not quantify the number of repetitive jaw movements within each feeding bout.

435

436 **Behavioral analyses**

437 The significant relationship between food type and the ratio of processing bouts to feeding
438 bouts (Figure 1) indicates that processing could be important for handling different food types.
439 The canola + Tetramin™ group had the lowest ratio of processing to feeding, even lower than
440 Tetramin™ alone. Processing has been described previously in the closely related common carp
441 (*Cyprinus carpio*) as a mechanism for sorting and repositioning food in the oral cavity before
442 swallowing (Sibbing et al. 1986). Handling multiple food types simultaneously would seem to
443 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.

490

491 **Role of lipids in fish nutrition**

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

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

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

513 diet. The $\text{g} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1}$ 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. 2013a, 2013b;
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).

536 **Acknowledgements**

537 M.D. LaMar provided guidance on statistical analyses and R.M. Chambers, J.E. Graves, and
538 P.D. Heideman commented on the manuscript. Thanks also to P.D. Heideman and C.J. Abelt for
539 advice, C.M. LaValley for filming of goldfish feeding, and J. Molloy for assistance. K.M.E.
540 received a W&M Honors Fellowship through the Roy R. Charles Center for Academic
541 Excellence and conducted this research in partial fulfillment of the requirements for the degree of
542 Bachelor of Science with Honors in Biology from the College of William & Mary.

543

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Draft

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 \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
45.8 \pm 84.9	0 \pm 0	23.8 \pm 38.3	0 \pm 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
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

* 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.

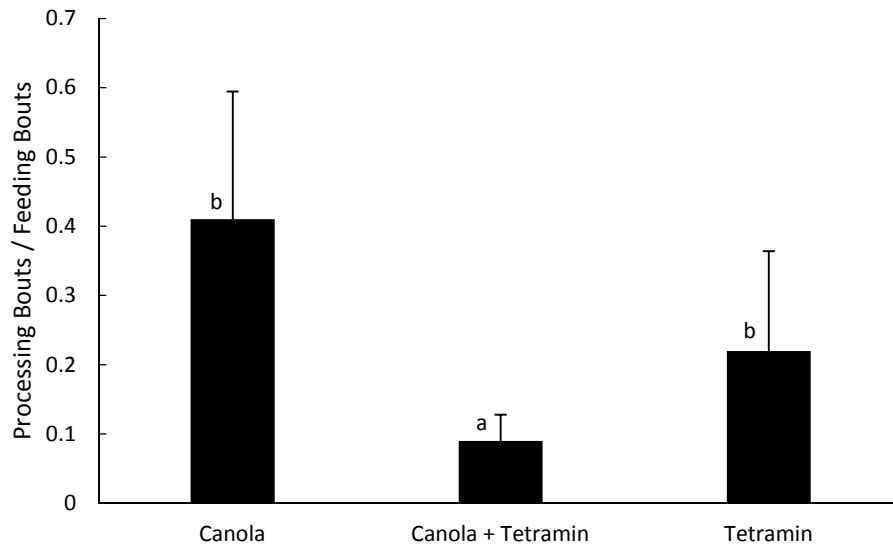


Figure 1. Average ratios of processing bouts to feeding bouts with 95% confidence intervals. Treatments labeled with different letters are significantly different.

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).

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