Methods for tagging very small fish

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ABSTRACT

Identifying individuals over time and across contexts is essential in many scientific fields. There are a variety of well-established methods for uniquely marking individuals (e.g., visible implant elastomer, barcodes, paint). However, for some species, life history stages, and/or experiments, existing methods are not sufficient. Here, we describe two methods – a tattoo method and a piercing method – for tagging juveniles of the African cichlid fish, *Astatotilapia burtoni*, which are too small for the methods used with adults, or other known methods. The tattoo method is appropriate for the youngest, smallest *A. burtoni* juveniles and injects tattoo ink into the dorsal muscle. Different colors and injection locations can be used to distinguish among individuals over a period of weeks (up to 4 weeks, average 2.5-3 weeks under our conditions). Because fish this young and small are sensitive to handling and injection, we also include physiological data showing fish recover well from anesthetization and tagging. The piercing method is appropriate for fish of all sizes, including the youngest, smallest juveniles. It uses very thin fishing line threaded through the dorsal muscle and tied into a barbell or loop. Unique colors and patterns can be used to distinguish among individuals over a period of months. We also include open field exploration behavior data because a physical tag might impede normal movement in very small fish. We expect these effective and inexpensive methods to be useful for a variety of small species and will facilitate early-life, developmental, and longitudinal research.

KEY WORDS: *Astatotilapia burtoni*, development, fry, identification, juvenile, longitudinal, mark

INTRODUCTION

The ability to uniquely identify individuals is essential to many scientific endeavors. In some species, the natural, external patterns on the body are sufficiently distinctive for identification, such as humpback whales, *Megaptera novaeangliae* (Katona et al., 1979), the African cichlid fish, *Neolamprologus pulcher* (Kohda et al., 2015; Balzarini et al., 2017), the guppy, *Poecilia reticulata* (Kemp et al., 2009), and the bluebanded goby, *Lythrypnus dalli* (Reavis and Grober, 1999). For most species, however, it is necessary to mark individuals to reliably identify them over time and across contexts. The ideal tag allows for easy and unambiguous identification, lasts the duration of the experiment(s), and interferes minimally with the animal and experimental conditions (Malone et al., 1999). The method also must be legal and in compliance with the local regulations for the care and use of experimental animals. There are a variety of useful and well-established methods for marking individuals. For example, paint or dye is used in diverse species, from insects to mammals (e.g., Jones et al., 1996; Williamson et al., 2016); combinations of colored and metal bands are placed on bird legs (Frazier, 2015); fish fins can be uniquely clipped (e.g., Hammer and Lee Blankenship, 2001; Thompson et al., 2005); visible implant elastomer tags can be injected under the skin or scales of amphibians, reptiles, and fish (e.g.,(Malone et al., 1999; Thompson et al., 2005; Grant, 2008); unique identifiers (e.g., numbers, barcodes) can be glued to the animal (e.g., Formica et al., 2012); passive integrated transponders (PIT) can be implanted and detected by radio signal (e.g., Jørgensen et al., 2017; Kraus et al., 2017); surgical steel coded wire implant tags (e.g., Martin and Wainwright, 2013; Martin and Gould, 2020), and more (e.g., Hasler and Faber, 1941; Volk et al., 1999). Developments in automated tracking technology have also influenced identification methods, such as radio frequency identification (RFID) (e.g., Lewejohann et al., 2009;

68 Weissbrod et al., 2013) and computer-vision systems (e.g., bleach-marks on mouse fur, Ohayon
69 et al., 2013). The spatial and temporal scales involved, and the requirements and limitations of
70 the study species and experiment, together determine the most appropriate marking method.

71 Despite the variety of existing options, suitable marking solutions are still lacking for
72 some species, life history stages, and/or experiments. Here, we describe two tagging methods – a
73 tattoo method and a piercing method – we developed for juveniles of the African cichlid fish,
74 Burton's Mouthbrooder (*Astatotilapia burtoni*), a model system in social neuroscience
75 (Hofmann, 2003; Maruska and Fernald, 2018). Adults of this species are routinely tagged using
76 visible implant elastomer tags, a colorful liquid that dries as a pliable solid (Northwest Marine
77 Technology, Inc.), or a colored bead secured with a plastic tag through the dorsal muscle (as in
78 Trainor and Hofmann, 2006). However, the smallest, youngest juvenile *A. burtoni* (average size:
79 8.31 ± 0.039 mm standard length; 0.022 ± 0.0005 g mass, Fig 1A) do not have sufficient dorsal
80 muscle into which to inject elastomer (Fig 1B-E), which uses a 30-gauge needle because of the
81 viscosity of the substance. The bead (often cut in half to be lighter) and plastic tag (can also be
82 used alone) are too large and heavy for the juveniles.

83 The tattoo method injects nanoliter quantities (~100 nL) of tattoo ink into the dorsal
84 muscle using a pulled glass capillary tube needle. We can use this method with the youngest and
85 smallest fish, including larva that are still developing. This method is moderately long-lasting: up
86 to 4 weeks, with an average of 2.5-3 weeks under our laboratory conditions. This is similar to
87 injection methods in other species (Thresher and Gronell, 1978). This tag does not appear to
88 interfere with locomotion, and it is visible to the naked eye. Because fish of this early
89 developmental stage are especially sensitive to handling and prone to injury or death, we also

present physiological data on how anesthetized and injected juveniles recover from the procedure compared to anesthetized juveniles that were not injected (Sneddon, 2012).

The piercing method threads very thin fishing line through the dorsal muscle of the fish using a pin or needle appropriate to the fish's size. The line is then tied into a barbell or loop and remains in place. These tags are visible to the naked eye, visible on camera for video analysis (depending on the size of the tag and cameras), it can remain in place as the juvenile grows, and it is sufficiently long-lasting for the duration of our experiments (months). Because a physical tag might impede locomotion in the smallest fish, we also include data from an open field exploration behavior test using 1-day old juveniles. We chose these young fish because they are most likely to be physically impeded. Anesthesia recovery data are not included for this method.

Overall, we hope these methods will be useful to other researchers working with a variety of species and that they will continue to be developed, adapted, and improved. The ability to identify individuals over time, starting in early life, will make it more feasible to carry out developmental and longitudinal research when working with very small, young fish.

Tattoo ink injection

METHODS

Astatotilapia burtoni juveniles

We used *A. burtoni* juveniles from a laboratory population descended from a wild-caught stock (see lab strain from Hofmann and Renn: Pauquet et al., 2018). The individuals that bred the juveniles were housed in naturalistic social groups of males and females. Dominant males court gravid females that then lay eggs in his territory. The female then scoops up the eggs into her mouth, where the male fertilizes them. The mother orally incubates the larvae as they develop.

113 When the larvae are fully developed and ready to leave the mother's buccal cavity 10-13 days
114 after fertilization (Fernald and Hirata, 1979; Renn et al., 2009), the average standard length (SL)
115 in our laboratory populations is 8.31 ± 0.039 mm (n=356, max SL: 10.18 mm, min SL: 4.6 mm,
116 Fig 1A). All procedures were done in compliance with the Institutional Animal Care and Use
117 Committee (IACUC) of the W.M. Keck Science Department of Scripps, Pitzer, and Claremont
118 McKenna Colleges (protocol #19-001) and the IACUC of The University of Texas at Austin.
119
120 *Tattoo ink and Nanoject injection set-up*
121 We developed this method using commercially available sets of tattoo ink, including
122 seven color sets of Millennium Mom's Tattoo Ink (Amazon.com, $40 USD at the time of the
123 work) and HAWINK Tattoo Ink (Amazon.com, $20 USD at the time of the work). These sets
124 included vibrant colors such as red, yellow, green, and blue (Supplemental Fig 1), as well as
125 neutral colors such as white, gray, and black. We also used Keep It Wet (Eternal Tattoo Supply,
126 $5 USD at the time of the work) to keep the ink from drying as quickly, without affecting the
127 color.
128 Tattoo injections were performed under a dissection microscope using the Nanoject II
129 Auto-Nanoliter Injector (Drummond Scientific Company). The Nanoject was positioned on a
130 micromanipulator attached to a magnetic base, which was secured to the benchtop on a magnetic
131 board. See Supplemental Materials for detailed set-up information. Briefly, we used a puller
132 (World Precision Instruments PUL-1) to make glass capillary tube needles (1.2 mm x 68 mm, A-
133 M Systems). The very tip was broken off using forceps, we backfilled the needle with mineral
134 oil, and then we secured the needle onto the Nanoject (Figure 2A, Supplemental Fig 2). Using
135 the Nanoject controls, we emptied a portion of the mineral oil, and then filled the needle with

136 tattoo ink. We mixed the ink in a weigh boat or on Parafilm. Different ink colors and brands had
137 different consistencies and drying times in the needle, so we used a ratio ranging from 1:1 to 1:4
138 ink drops to water drops (or Keep It Wet additive). We performed multiple test injections
139 (Supplemental Fig 2F) at the start and between fish to ensure the ink was not dried, clogging the
140 needle.
141
142 *Injection of tattoo ink*
143 We removed the fish from the anesthetic (see below) and placed it on the injection
144 platform under the dissection scope on a paper towel wet with aquarium water (Fig 2A). If the
145 Nanoject is set-up to the right of the scope (for a right-handed scientist), we positioned the fish
146 with its head to the left and tail to the right so the needle inserted between or under the fish's
147 scales. Securing the fish's head between the left thumb and forefinger, pressing firmly down on
148 the paper towel but without squeezing the fish, we positioned the needle at the desired location
149 along the rostral-caudal axis of the dorsal muscle (Fig 2B). A ventral injection in or near the
150 abdominal cavity will not remain visible for long, and the tattoo ink may affect internal organs.
151 The needle was lowered using the micromanipulator until the skin was pierced and the tip
152 of the needle was in the muscle. Young *A. burtoni* juveniles are semi-transparent at this
153 developmental stage, so we could also visibly check the needle position (Fig 2B). There may be
154 some resistance to piercing the skin, which can indicate a dull needle that should be replaced.
155 Holding the fish as still as possible, we pressed "inject" twice for a total injection volume of
156 ~100 nL of ink. Waiting a couple seconds to remove the needle can help prevent the ink from
157 flowing out of the injection site. The ink is immediately visible at the injection site, as well as on
158 the opposite side of the fish (Fig 2C-E). Over time, the tag is usually most visible just on one

159 side of the body (Fig 2E). We then carefully placed the fish into a beaker of fresh aquarium
160 water and began the resuscitation protocol (see below). The needle was cleaned with ethanol on a
161 Kimwipe (Kimberly-Clark) between injections. We changed the needle approximately every 4-8
162 animals, as the needle dulled, if the needle clogged, or to change ink color.
163 For experiments that require strict standardization of the procedure, we recommend
164 keeping the amount of time out of water consistent across fish (e.g., 1 min for tattooing only; 2
165 min for tattooing and an additional procedure, such as drug administration). Otherwise, we place
166 the fish back in water for recovery as quickly as possible. The procedure can take less than 30 s
167 once the fish is anesthetized.
168
169 *Anesthesia and post-tagging resuscitation*
170 We anesthetized juveniles less than two weeks old in tricaine methanesulfonate (MS-222,
171 Sigma Aldrich) at a dose of 0.0003 g / mL aquarium water, buffered with sodium hydroxide to
172 pH 7-7.5. During anesthetization, we recorded the time it took for body movement to stop; loss
173 of equilibrium, when the fish's dorsal side first rolled away from an upright orientation; and
174 when breathing stop, indicated by movement of the opercula stopping fully. We removed fish
175 from the MS-222 immediately after breathing stopped. When tagging fish without collecting
176 anesthetization and/or recovery data, we remove fish from the MS-222 immediately after
177 breathing stopped or after 30-35 s, whichever happens earlier.
178 After tagging (see above), the fish was placed in a 200 mL plastic beaker of fresh
179 aquarium water to recover. We immediately began resuscitating the fish using a plastic pipette to
180 gently push water over the gills. Recovery from MS-222 is a highly stereotyped process
181 (McFarland, 1959). We recorded the time it took for the fish to first move its body; first move

182 the opercula; breathe independently without resuscitation; initial regaining of equilibrium, when
183 the dorsal side of the fish first returned to an upright position; and sustained equilibrium, when
184 the fish was permanently upright. We also recorded when the fish looked fully recovered, which
185 tended to happen suddenly. Fully recovered fish swam smoothly and breathed steadily, with no
186 wobbles in equilibrium.
187
188 *Statistics*
189 All statistical analyses were conducted using R Studio (R version 4.4.0) (RStudio Team,
190 2022). Results were considered significant at the $p<0.05$ level, and averages ± standard error of
191 the mean are included in the text. To compare anesthetization and recovery in control and tagged
192 juveniles, we used t-tests for data that met the assumptions of parametric statistics. A Wilcoxon
193 signed ranks test was used for data that did not meet those assumptions. Cohen's d is reported to
194 estimate effect size for significant pairwise differences (small effect: $0.2 < d < 0.5$; medium: 0.5
195 $< d < 0.8$; large: $0.8 < d$). Pearson correlations were used to test for associations among recovery
196 metrics in control and tagged fish.
197
198 **RESULTS**
199 *Recovery from anesthesia and injection*
200 To investigate the safety and efficacy of this procedure on very young, very small
201 juveniles (< 2 weeks after removal from the mother's buccal cavity), we compared the
202 anesthetization and recovery of fish that were both anesthetized and tagged (tagged: n=8) to fish
203 that were anesthetized but not injected (control: n=8). Fish in both treatment groups were

204 removed from the MS-222 immediately after breathing stopped and kept out of water for 1 min
205 on a wet paper towel. Tagged fish were injected during the time out of water.
206 While being anesthetized, juveniles first lost equilibrium (30.6 ± 2.9 s), after which they
207 stopped moving their bodies (52.5 ± 3.0 s) and stopped breathing (54.1 ± 3.5 s) in close
208 succession. Some individuals appeared to stop breathing first, and others appeared to stop
209 moving first. The treatment groups did not differ in time to lose equilibrium or time to stop
210 moving (Table 1, Supplemental Fig 3A, B). Fish in the tagged group (although they had not been
211 tagged yet) took significantly longer to stop breathing (Supplemental Fig 3C), but overall, the
212 groups did not differ in the total time spent in MS-222 (Table 1, Supplemental Fig 3D).
213 All (100%) of the fish in this cohort successfully recovered. After being placed in the
214 recovery beaker, opercular movement during resuscitation occurred first (40.4 ± 9.5 s), followed
215 by independent breathing without resuscitation (107.9 ± 18 s). First body movement (128.4 ±
216 17.2 s), initial regained equilibrium (145.3 ± 21.9 s), and sustained regained equilibrium (162.6 ±
217 26.8 s) occurred next, in close succession. By definition, full recovery occurred last (407.3 ± 51
218 s). There were no significant differences for any recovery metric between fish that were
219 anesthetized compared to those that were anesthetized and tagged (Table 1, Fig 3A, B). Some
220 recovery metrics were good predictors of later recovery (Table 2, Supplemental Fig 4).
221 Independent breathing was significantly and positively associated with first body movement (Fig
222 3C) and initial regained equilibrium for both control and tagged fish, as well as sustained
223 regained equilibrium in control fish. Initial body movement was significantly and positively
224 associated with initial regained equilibrium in control and tagged fish, as well as sustained
225 regained equilibrium in control fish. Initial regained equilibrium was significantly and positively
226 associated with sustained regained equilibrium in control and tagged fish. Initial (Fig 3D) and

227 sustained equilibrium were the only metrics associated with full recovery, and the associations
228 were significant only for control fish. Initial breathing during resuscitation did not predict any
229 later recovery points (Table 2).
230

231 **DISCUSSION**

232 Injecting tattoo ink into the dorsal muscle of very young, small fish immediately creates a
233 vibrant visual mark, allowing for individual identification. Tattoo ink is inexpensive and
234 commercially available in an array of colors. Mixing inks may also be useful in creating unique
235 colors to track more individuals within a group. Depending on the size of the fish, injection
236 location may also be a useful unique identifier. The main limitation of this technique in our
237 hands is that the colors fade over time, with lighter colors (e.g., yellow) appearing to fade more
238 quickly than darker colors (e.g., black, blue). Our longest lasting tattoo tags lasted 3-4 weeks
239 (Fig 2E). Different colored inks, and different manufactures, have different compositions
240 (Moseman et al., 2024), which could underlie the differences in tag visibility we observed. The
241 different ink components could also have differential health effects (Grant et al., 2015; Moseman
242 et al., 2024). In humans, tattoo ink is injected into the skin, specifically the papillary layer of the
243 dermis. Over time, ink particles move deeper into the reticular dermis, and the tattoo persists
244 because sufficient pigment stays in the skin (Grant et al., 2015; Moseman et al., 2024). Here, we
245 injected ink into the dorsal muscle, which resulted in a moderately long-lasting tag, but the mark
246 was not permanent. Pigment may disperse more readily, or fail to accumulate, in muscle
247 compared to skin. Muscle can also change dramatically during fish development, including in
248 muscle fiber type, size, and number (Patruno et al., 1998; Johnston, 1999).

249 Although tagging can take less than 30 s, anesthesia is necessary for the welfare and
250 recovery of the juvenile fish. We chose to use MS-222 (pH buffered), which is a common and
251 safe fish anesthetic (Wagner et al., 2003; Kiessling et al., 2009; Sneddon, 2012; Zahl et al., 2012;
252 Topic Popovic et al., 2012) that can be cleared from circulation in less than 300 s (Kiessling et
253 al., 2009). Observing individuals during anesthetization and recovery is highly informative for
254 establishing an effective anesthetic dose, determining the optimal procedural timing, and
255 ensuring full recovery (Sneddon, 2012). For example, some fish species will recovery unaided
256 from MS-222 (e.g., bluebanded gobies, *Lythrypnus dalli*, Solomon-Lane and Grober, 2012);
257 however, *A. burtoni* juveniles recover best with resuscitation and continued monitoring until full
258 recovery. These data can also be used to identify unintended biases. Here, we found that
259 juveniles in the tagged group took longer to stop breathing, even though they had not yet
260 experienced anything different from the control fish (i.e., no fish had yet been tagged). With this
261 information, we can test for downstream consequences. For example, despite this significant
262 difference, tagged fish did not spend more total time in MS-222.
263 We found no significant differences in recovery timing between anesthetized control fish
264 compared to anesthetized and tagged fish. This suggests that, overall, tattoo ink injections are
265 safe, with limited negative physiological consequences. We did identify some effects of tagging
266 on the temporal progression of recovery. In control fish, for example, the timing of initial and
267 sustained regained equilibrium was significantly associated with the timing of full recovery. In
268 contrast, for tagged fish, no recovery metric predicted the timing of full recovery. These
269 differences support our protocol of using recovery data as an indicator of how an individual was
270 impacted by the procedure and if it is ready to return to its home aquarium or engage in an
271 experimental test (e.g., a behavioral assay) (Solomon-Lane and Grober, 2012). For example, we

found that the timing of first opercular movement (initial breathing) during resuscitation was not a good predictor of subsequent stages of recovery. In contrast, independent breathing and body movement, which occurred in close succession, predicted when equilibrium was regained. Additional validations may be needed, depending on the species or experiment. For example, in our future studies related to social behavior, we will test whether tag color affects how fish behave or are treated by other members of their social group.

We also tested the efficacy of injecting acrylic paint (Lotrich and Meredith, 1974), thinned slightly with Liquidtex® Flow Aid™ to prevent the paint from drying rapidly in the needle. These acrylic paint tags lasted approximately two weeks, with the longest tag lasting four weeks, under our conditions. Acrylic paint is non-toxic, inexpensive, and available in many colors, making it a potentially good option, in addition to tattoo ink. Injections of Alcian blue (Thermo Scientific Chemicals) lasted approximately three weeks. With no color options other than blue, this did not meet our needs for distinguishing among multiple juveniles in a social group. Injections of food coloring lasted only a couple of days (data not shown).

Fishing line piercing method

METHODS

Making the fishing line tag

Unique tags can be made from fishing line by coloring or dying the line, or purchasing fishing line manufactured in different colors (options may be limited). We used permanent markers (Sharpie) to uniquely color white fishing line (before the line is threaded through the fish). “Beads” of different colors, sizes, or patterns can also be made along the length of the line

295 by tying a knot (or more than one) and using a drop of super glue to preserve the color. Nail
296 polish can also be used over the knot(s) for additional color combinations. Note that these beads
297 should be on a portion of the tag that does not get threaded through the body of the fish, as they
298 would cause more tissue damage.

299 For the juveniles that are too small for a 31-gauge insulin needle (or the smallest
300 commercially available needle) to pass through the dorsal muscle (Fig 1D, E), we use fishing
301 line in combination with a minutien pin (Fine Science Tools, 0.1mm diameter, 500 pins for $32
302 USD at the time of the work). We used the thinnest line available, which was Berkley NanoFil
303 Uni-filament fishing line (2 lb break weight, 0.051 mm average diameter, 150 yards for $20 USD
304 at the time of the work). For this method, the fishing line and pin are not physically attached.

305 For juveniles that are large enough for a 31-gauge insulin needle (body size > ~12 mm
306 SL, ~0.08 g mass for *A. burtoni*), we find it easiest to tag many fish at once by attaching the
307 fishing line to a needle. This design is modeled on an eyeless suture, which can be expensive to
308 purchase. Our alternative is easy and inexpensive to make. We used Berkley NanoFil Uni-
309 filament Fishing Line (4lb break weight, 0.1 mm average diameter, in the color clear mist; 150
310 yards for $20 USD at the time of the work) and BD Ultra-Fine™ Short Needle (8 mm, 31G)
311 Insulin Syringes (3/10 mL) (90 count for $21 USD at the time of the work). Any fishing line and
312 needle combination can be used, as long as the average diameter of the line is smaller than the
313 inner diameter of the needle.

314 To attach the fishing line to the needle, remove the plunger from the syringe and thread
315 the line through the sharp end of the needle. It is easiest to do under a dissection microscope, and
316 it helps to work with a freshly cut end. Use a sharp, new razor blade to avoid a messy cut, which
317 can flatten the line and widen the diameter. If the fishing line will clearly not thread into the

318 needle, try discarding a length of line and starting at a new place along the spool. The diameter
319 of the line varies slightly throughout, and it is easiest to work at an average or narrower section.
320 Once started, keep threading the line through the needle until it has gone beyond the length of the
321 needle and is visible in the barrel of the syringe (Figure 4A). Then, take a pair of forceps and
322 grip the base of the needle, being careful not to dislodge the line. Rock the needle back and forth
323 with the forceps until it detaches from the syringe (Figure 4B). From the blunt side of the needle,
324 pull the line through until it is sufficiently long. We typically work with a length of line between
325 30 cm and 50 cm, which can be used to tag ~4-10 fish. Cut the length of line from the spool
326 using a razor blade and pull the last bit of line through the needle until it is no longer sticking out
327 beyond the sharp end. We do not find it necessary to crimp the blunt end of the needle to keep
328 the line in place.
329
330 *Anesthesia and resuscitation*
331 We anesthetized juveniles less than 2 weeks old as described above. Older, larger
332 juveniles were anesthetized in MS-222 at a dose of 0.0006 g / mL aquarium water, buffered to
333 pH 7-7.5. We removed the larger juveniles from the MS-222 immediately after they stopped
334 responding to touch, which occurred after losing equilibrium and after breathing stopped. Fish
335 were resuscitated as above.
336
337 *Tagging the fish*
338 For the smallest fish (Fig 5A, B), place the fish on a wet paper towel under a dissection
339 scope. Using forceps to hold the minutien pin perpendicular to the fish, push the needle into and
340 through the dorsal muscle. With the pin still in place, reposition the fish to be upright, and pull

the pin through the other side of the fish. Using forceps to hold the fishing line, immediately push the line through the hole made by the pin. If there are knots on the line, be careful not to pull those through the fish. For larger fish (Fig 5C), follow a similar procedure with the needle attached to the fishing line. With the fish lying flat on a wet paper towel, use forceps to hold the needle perpendicular to the fish. Shifting the fish to an upright position, push the needle through the dorsal muscle (Figure 5D). Pull the needle, followed by the line, through the dorsal muscle with the forceps until 3-4 cm of line remains (Figure 5E). Use a razor blade to cut the line on the needle side, leaving another 3-4 cm.

Once the fishing line is through the dorsal muscle of the fish, the fishing line can be tied into a barbell (Fig 5B) or into a loop (Fig5 A,C) using a square knot (Fig 5F,G). If using a loop, be sure to tie it large enough for the fish to raise its dorsal fin and to grow over the course of the experiment. However, a loop that is too large could get tangled in the tags of other fish or tank elements (e.g., plastic plants). The knots can be tied by hand, using forceps, or a combination. If the knots are challenging to tie, we find that loosely pre-tying and then untying a knot in the line prior threading it through the fish can make this step much easier, as well as gentler for the fish. Trim the excess fishing line using a razor blade. We recommend placing a drop of super glue on the knot(s) to ensure they stay tied. As soon as possible, place the fish in a beaker of fresh aquarium water to recover. The glue should dry sufficiently in less than a minute, and during this time (and throughout), the fish's gills / body can be kept wet (with aquarium water or MS-222, as needed) using a transfer pipette. The tag can be removed quickly, and without anesthesia, by cutting the line with a sharp razor blade.

Open field exploration

364 A physical tag may be more likely to affect locomotion and behavior, particularly in the
365 smallest juveniles. We used an open field exploration to compare between juveniles that were
366 anesthetized and tagged (n=7) and those that were anesthetized but not tagged (n=8). This test is
367 commonly used across species to assess movement, activity, and anxiety-like behavior (Prut and
368 Belzung, 2003; Cachat et al., 2010; Solomon-Lane and Hofmann, 2019). We tagged the juveniles
369 less than 2 hrs after being removed from the mother's buccal cavity, and the open field test was
370 carried out the next day (~20 hrs later). The time it took to complete each tagging surgery was
371 recorded (average 257 ± 22 s), and each control fish was kept out of water and manipulated with
372 forceps for the same amount of time as one of the tagged fish. Additional MS-222 (or water) was
373 applied to the fish during the procedure, as needed.
374 For the open field exploration, we placed fish individually in a small, novel aquarium
375 (22.9 x 15.2 x 15.2 cm) without a cover for 30 min. For analysis, aquaria were divided into 4
376 zones: the territory zone, which contained a small terracotta pot territory / shelter, the close zone,
377 the far zone, and the investigate zone (Fig 6A). Video cameras (Warrior 4.0, Security Camera
378 Warehouse, Asheville, NC) recorded behavior from above, and we used BORIS (Friard and
379 Gamba, 2016) to score the number of times fish entered each zone of the aquarium, and how
380 long fish spent in each zone of the tank during a 10 min observation (minutes 20-30 of the test).
381
382 *Statistics*
383 To compare locomotion and behavior in the open field exploration between control and
384 tagged juveniles, we used a two-way mixed ANOVAs, with tagging treatment as the independent
385 measure and aquarium zone (frequency entering, time in) as the repeated measure. Tukey's HSD
386 test was used for *post hoc* analysis of significant ANOVA results. Eta-squared is reported to

387 estimate effect size for significant ANOVA results (small effect: $0 < \eta^2 < 0.01$; moderate: $0.01 <$

388 $\eta^2 < 0.06$; large: $0.06 < \eta^2$).

389

390 **RESULTS**

391 *Open field exploration*

392 We measured the time spent in each tank zone, as well as the frequency entering each

393 aquarium zone, for control and tagged juveniles. For the time in each zone, we found a

394 significant main effect of aquarium zone ($F(1.46, 13.13)=13.44$, $p=0.001$), but no main effect of

395 treatment ($F(1,9)=1.20$, $p=0.30$), and no interaction effect ($F(1.46, 13.12)=1.35$, $p=0.28$). *Post*

396 *hoc* analysis showed that juveniles spent significantly less time in the investigate zone compared

397 to the territory ($p=0.00026$), close ($p<0.0001$), and far zones ($p=0.00029$). The other pairwise

398 comparisons were not significant (close-territory: $p=0.39$, far-territory: $p=1.00$, far-close: 0.31)

399 (Fig 6B). Similarly for the frequency of entering each zone, we found a significant main effect of

400 aquarium zone ($F(1.13, 10.2)=16.79$, $p=0.002$), but no main effect of treatment ($F(1,9)=1.90$,

401 $p=0.20$), and no interaction effect ($F(1.13, 10.2)=0.48$, $p=0.53$). Juveniles spent significantly less

402 time in the investigate zone compared to the close zone ($p=0.0004$). The other pairwise

403 comparisons were not significant (close-territory: $p=0.23$, far-territory: $p=1.00$, investigate-

404 territory: $p=0.088$, far-close: $p=0.21$, investigate-far: $p=0.082$) (Fig 6C).

405

406 **DISCUSSION**

407 Our fishing line piercing method is a highly effective way of individually marking

408 juvenile fish that can last months. Across multiple experiments using older juveniles (e.g.,

409 (Solomon-Lane et al., 2022), all fish have survived the procedure and recovered very well. An

experienced researcher can also carry out these surgeries quickly, with anesthetization and tagging taking ~2 min for the larger juveniles. All the tagged juveniles in this sample also survived and recovered well; however, juveniles of this small size and young age are more sensitive than older, larger fish. As needles and fishing line (or similar, e.g., 44-gauge stainless steel wire, Remington Industries) continue to get thinner and lighter, we expect this method can become even less invasive for very small fish. For a short experiment (< 1 week), line colored with permanent marker remains vibrant under our aquarium conditions. Over a period of 3-4 weeks, the colors do fade. If super glue is added to the knot or to "beads" along the length of the tag, the color under the glue remains visible for longer (at least 8 weeks). It may also be possible to thread a seed bead onto the line for unique identification. Although we have not tested this yet, seed beads are small, readily available in an array of colors, and inexpensive.

A physical tag may be more likely to affect locomotion and behavior than the tattoo method. We tested fish in an open field exploration under conditions that we considered most likely to reveal potential negative effects on behavior, while allowing juveniles time to recover from the surgery. Juveniles were tagged less than 2 hrs after being removed from the mother's buccal cavity, and at the time of the open field test, they had less than 24 hrs experience as freely-swimming fish. Tagging did not have a significant effect on where juveniles spent time in the aquarium, or how frequently they entered the different aquarium zones. Interestingly, tagged juveniles entered the territory zone and close zone fewer times than control fish. Remaining in or near the territory can be associated with shyness (Harmon et al., 2024), and injured fish may be more likely to hide in the territory. However, the effect we saw was in the opposite direction. This behavior pattern could indicate that locomotion decreases for a period of time after tagging, and we plan to measure locomotion as a control in future studies. Depending on the experiment,

species, and developmental stage, a longer recovery period may be needed, and testing relevant behaviors on subsequent days post-surgery will be valuable for determining appropriate experimental timing. Overall, we were impressed by the quick recovery of these very small, very young juveniles.

CONCLUSIONS

The tagging methods described here have made it possible for us to study the behavioral development and underlying neuromolecular mechanisms of juvenile *A. burtoni* (e.g., Solomon-Lane et al., 2022). The tattoo method can be used with the smallest, youngest juveniles and it has multiple benefits: the procedure is fast, keeping the fish out of water for ~30 s, it uses the smallest needle (pulled glass capillary tube), and the tag is fully internal. We have not yet tested this method with larger juvenile or adult *A. burtoni*; however, once fish have sufficient dorsal muscle, a visible implant elastomer tag or colored beads secured with a plastic tag (e.g., Trainor and Hofmann, 2006) may become better options. The piercing method can be used with fish of all sizes, including the smallest and youngest juveniles. The benefits of this method include being longer-lasting than the tattoo method, and the tags can be visible on video for behavior analysis (visibility will depend on the size of the tag and the camera system). Overall, the lack of an appropriate method for identifying juveniles this small and young likely has contributed to the relative paucity of early-life studies, in this and other small fish species. In social neuroscience, behavioral ecology, animal behavior, developmental biology, fisheries research, and related fields, experiments that follow individuals through development are critical to uncovering the emergence of individual phenotypic variation, as well as the underlying mechanisms and fitness consequences (Taborsky, 2016).

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547

548 **TABLES**

549 **Table 1:** Treatment differences in anesthetization and recovery between juveniles that were

550 anesthetized (control) vs. juveniles that were anesthetized and tattooed. All juveniles remained

551 out of the water for 1 min, whether or not they were tagged.

Behavior	DF	Test Statistic	p-value	Effect size
Anesthetization in MS-222				
Loss of equilibrium	10.963	t = -0.35	0.73	
Stop moving	9.50	t = -1.07	0.31	
Stop breathing	**9.01**	**t = -2.42**	**0.039**	**-1.3**
Total time in MS-222	10.07	t = -1.388	0.20	
Recovery				
Initial breathing during resuscitation	12.13	t = 0.51	0.62	
Independent breathing	12.9	t = 0.11	0.91	
Body movement	13.47	t = 0.90	0.38	
Initial regained equilibrium	12.82	t = 0.10	0.92	
Sustained regained equilibrium	12.57	t = -0.52	0.61	
Full recovery	1	W = 18	0.28	

552

553 Results of pairwise comparisons using t-tests (t) or a Mann-Whitney Wilcoxon test (W). Cohen's

554 d is reported for effect size of significant results. Significant results are in bold.

555 **Table 2:** Associations between recovery metrics following anesthesia.

	Initial breathing during resuscitation	**Independent breathing**	**Body movement**	**Initial regained equilibrium**	**Sustained regained equilibrium**	**Full recovery**
Initial breathing during resuscitation		p=0.60	p=0.65	p=0.57	p=0.57	p=0.95
Independent breathing	p=0.59		**p=0.005, r^2=0.72**	**p=0.0036, r^2=0.81**	**p=0.0036, r^2=0.81**	p=0.14
Body movement	p=0.57	**p=0.011, r^2=0.63**		**p<0.0001, r^2=0.96**	**p<0.0001, r^2=0.96**	p=0.14
Initial regained equilibrium	p=0.12	**p=0.046, r^2=0.43**	**p=0.015, r^2=0.60**		**p<0.0001, r^2=1.00**	**p=0.034, r^2=0.55**
Sustained regained equilibrium	p=0.052	p=0.51	p=0.31	**p=0.0082, r^2=0.67**		**p=0.034, r^2=0.55**
Full recovery	p=1.00	p=0.22	p=0.37	p=0.92	p=0.95	

556

557 Correlations for control fish in the upper triangular (black text). Correlations for tattooed fish in

558 the lower triangular (grey text). Significant associations in bold. Adjusted r^2 values shown for

559 significant results.

FIGURES

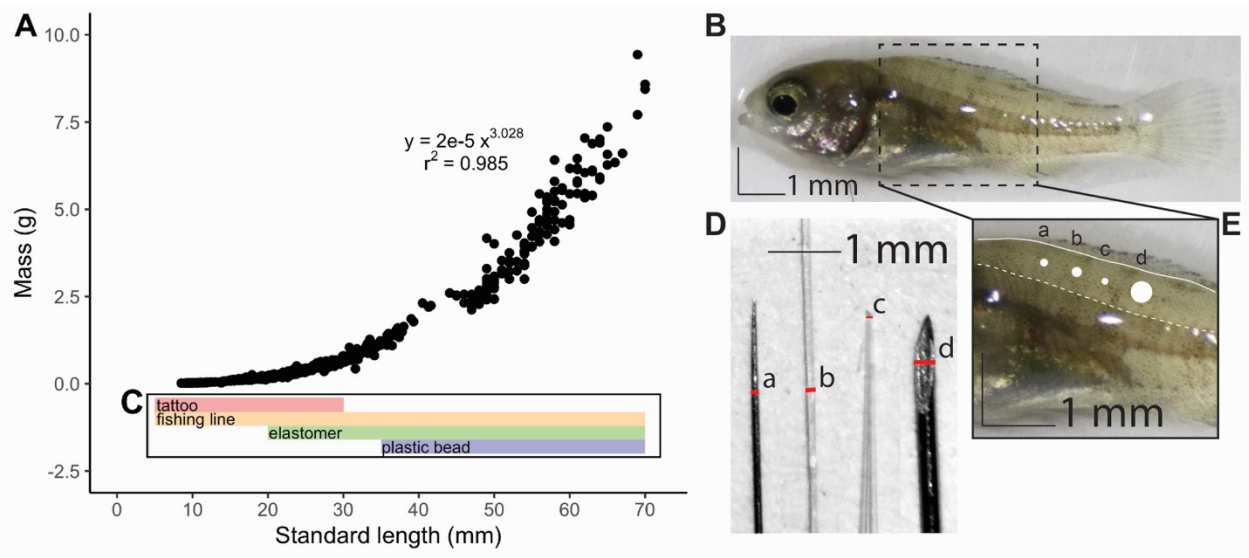

Figure 1: Summary of appropriate tagging methods for *Astatotilapia burtoni* of different body sizes. A) Association between standard length (mm) and mass (g) (n=729). B) Image of 1-day old juvenile (8.8 mm standard length). C) Four methods used for tagging *A. burtoni* at different body sizes and life history stages, including the two methods described here: tattooing and a fishing line piercing through the dorsal muscle. The size upper ranges for the tagging methods are approximate and may vary depending on the experiment (e.g., short- vs. long-term, live vs. video behavior scoring) and the species. D) Image of a 0.1 mm diameter minutien pin (a: for piercing the smallest juveniles), fishing line (b: for tagging the smallest juveniles), pulled glass capillary tube needle (c: for tattooing), and a 31-gauge needle (d: for piercing larger juveniles). The red lines indicating diameter are the same diameter as the white circles in (E) (D and E are at the same scale). E) Cropped image of the fish in (B). The dorsal edge of the spinal cord is edged in dashed white lines. The dorsal edge of the fish is shown in the sold white line. White circles show the diameter of the minutien pin (a), fishing line (b), capillary tube needle (c), and 31-g needle (d) shown in (D).

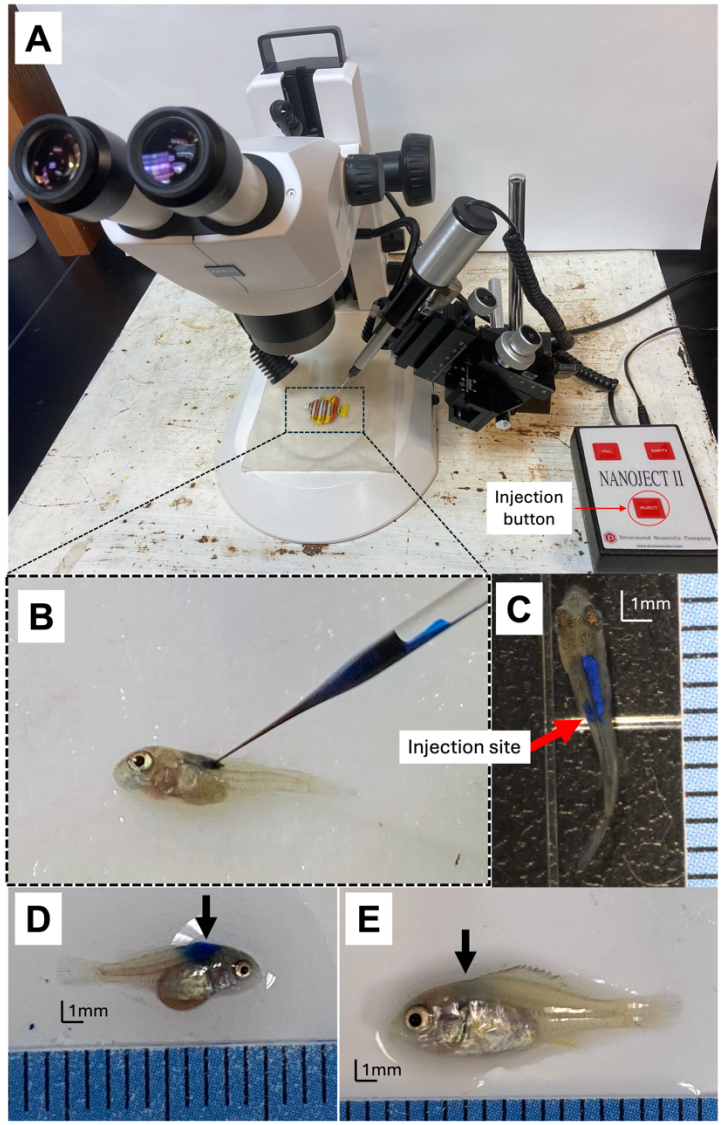

Figure 2: Method 1: Tattooing: A) Image of the Nanoject setup under a dissection scope, with a (plastic) fish placed on a wet napkin on the platform, prepped for injection. The injection button is labeled on the Nanoject controller. **B)** A juvenile fish being injected with blue ink using a pulled glass capillary tube needle. **C)** Fish immediately after injection with tattoo ink. The injection site is shown on the left, with the dye spreading on the right side of the fish. **D)** A juvenile fish 2 days after injection. **E)** The same juvenile fish as in (D, at the same scale) 36 days after injection. See Supplemental Information for pictures of additional tag colors.

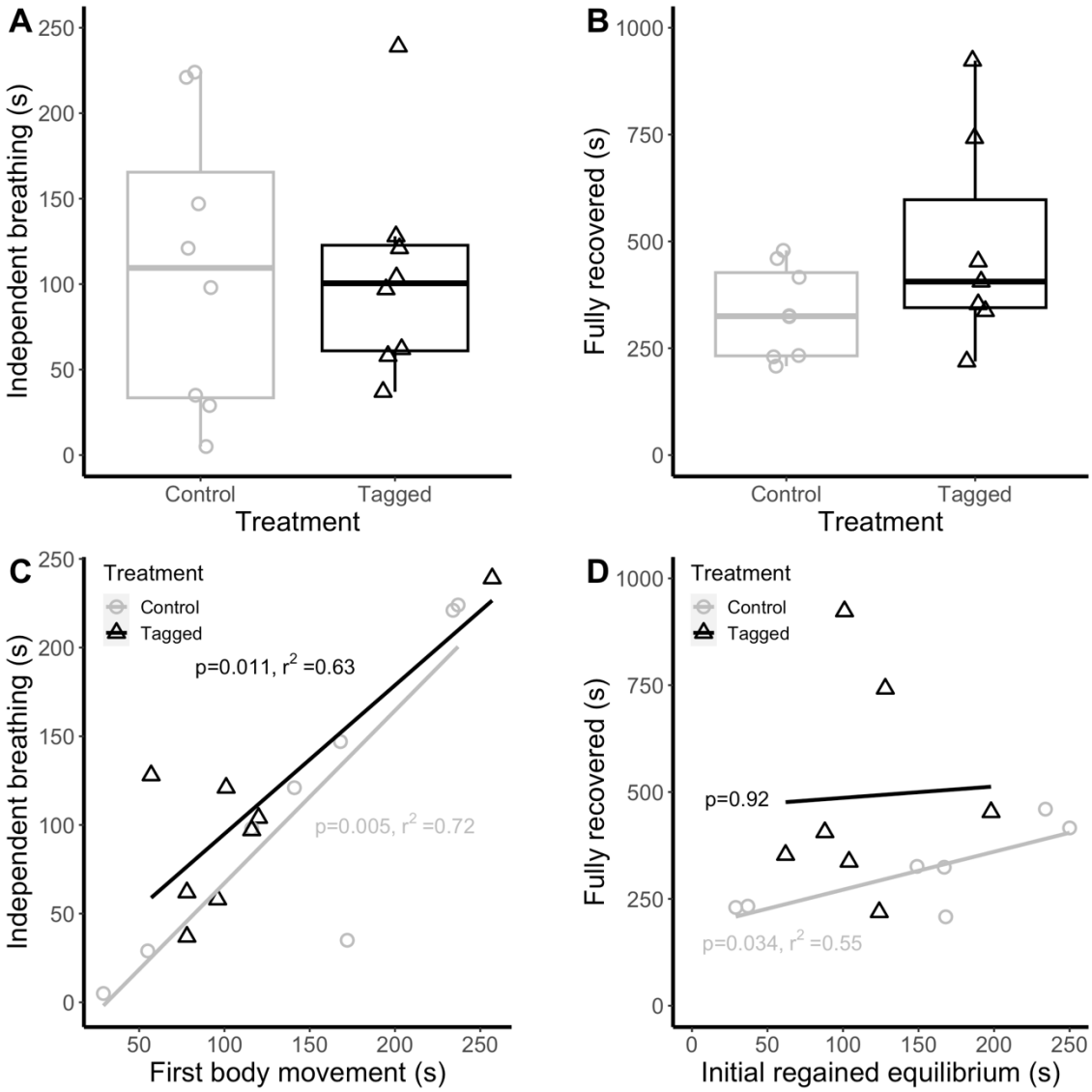

Figure 3: Recovery from anesthesia following tattooing: Time until A) breathing independently and B) full recovery from anesthetization in control and tagged juveniles. C) Association between initial body movement and independent breathing. D) Association between initial regained equilibrium and full recovery. All recovery times start when the fish was placed in the recovery beaker, following anesthetization and 1 min out of water. Tagged fish were injected with dye during the 1 min out of water.

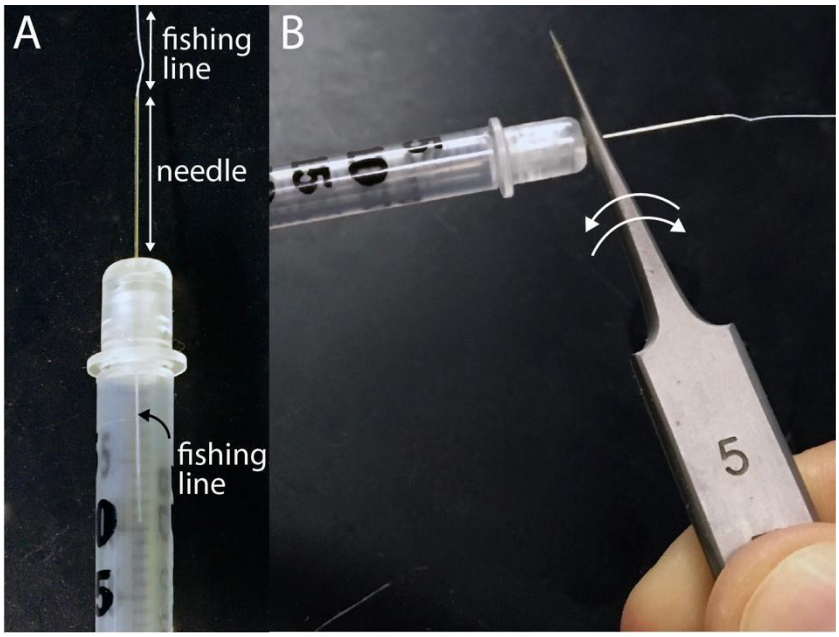

Figure 4: Making the fishing line tag. A) Thread the fishing line through the needle of an insulin syringe until it is visible in the barrel of the syringe. B) Use forceps to grasp the base of the needle. Rock back and forth (arrows) until the needle breaks off from the syringe. Not shown: From the blunt side of the needle, pull the line through, until it no longer sticks out beyond the needle tip. Use a razor blade to cut off excess line.

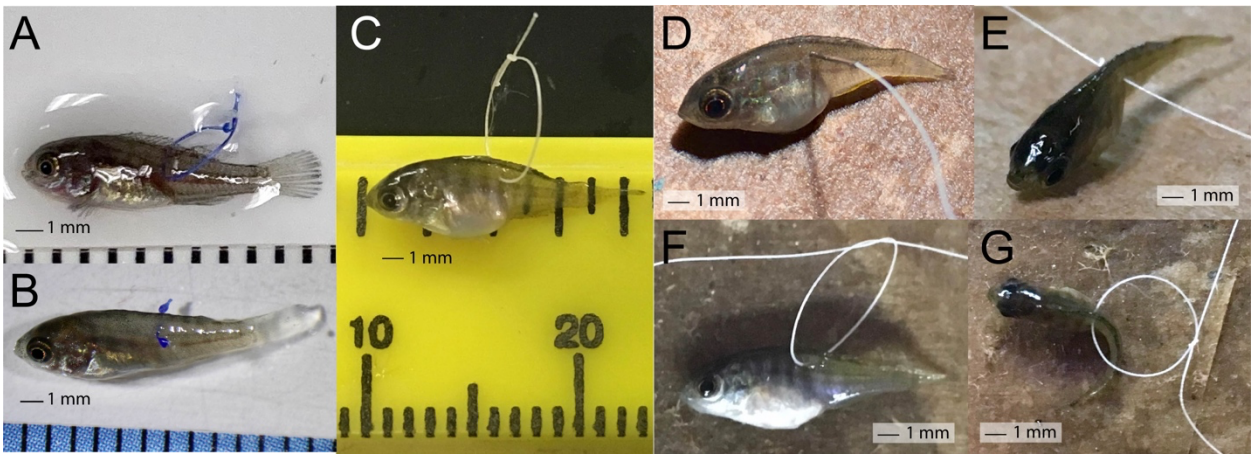

Figure 5: Tagging with a fishing line piercing. A) A juvenile tagged with a blue fishing line loop 1 hr after being removed from the mother's buccal cavity (8.88 mm standard length, SL). B) A young juvenile (4 days post-removal from the mother's buccal cavity) tagged with a blue fishing line barbell (10.08 mm SL). C) An older juvenile tagged with a fishing line loop (10.5 mm SL) on a ruler (inches top, mm bottom). Procedure for tagging larger juveniles: D) Pierce the dorsal muscle with the needle. E) Pull the needle and line through the dorsal muscle. F) Make a loop in the line and tie the first half of a square knot. Adjust the loop to the desired size. G) Finish the square knot. Cut the excess line using a razor blade. The same individual fish is pictured in panels C-G.

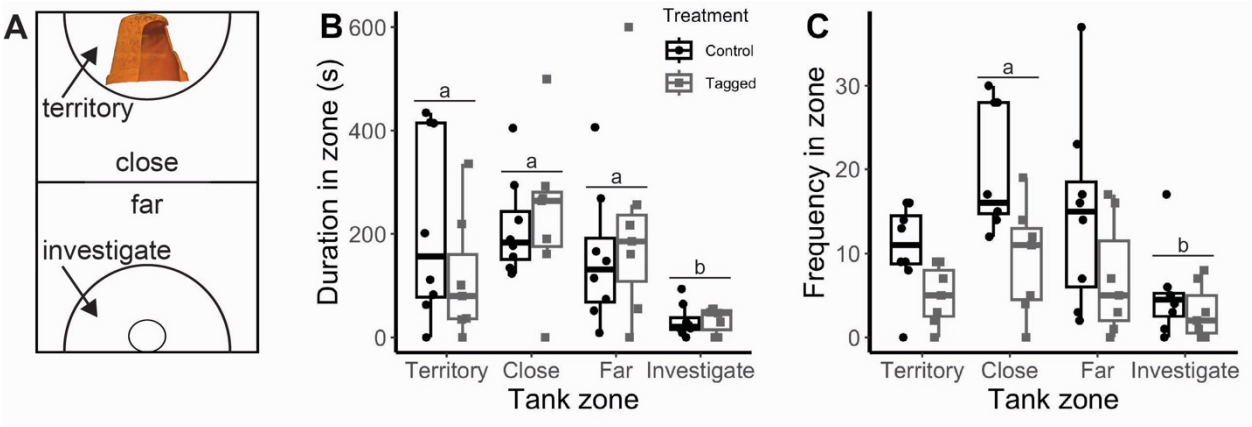

Figure 6: Open field exploration. A) Tank layout of the open field exploration, with the territory, close, far, and investigate zones. The territory zone has a small terracotta pot territory / shelter. **B)** Time spent (s) in each tank zone. **C)** Frequency entering each tank zone. Tagged fish (n=7) were anesthetized and tagged with a fishing line piercing less than 2 hrs after being removed from the mother's buccal cavity. Control fish (n=8) were anesthetized but not tagged. The behavior test took place ~20 hrs post-surgery. Different letters indicate significant differences.