Tagging Very Small Fish: Two Effective and Low Impact Methods

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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 procedures for how two tagging methods – a tattoo ink injection method and a fishing line piercing method – can be used with the youngest, smallest juveniles of the African cichlid fish, *Astatotilapia burtoni*, which are too small for the methods used with adults. With the tattoo method, we injected 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. With the piercing method, very thin fishing line is 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. Because a physical tag might impede normal movement in a very small fish, we also include data from an open field exploration test showing similar behavior between tagged and control (non-tagged) juveniles. 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 ethical 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 for external marking, 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; Lennox et al., 2025); 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) tags (e.g.,

67 Lewejohann et al., 2009; Weissbrod et al., 2013) and computer-vision systems (e.g., bleach-
68 marks on mouse fur, Ohayon et al., 2013). The spatial and temporal scales involved, and the
69 requirements and limitations of the study species and experiment, together determine the most
70 appropriate marking method.

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

83 The tattoo method adapts existing approaches that use injected or implanted dyes or
84 paints for identification (Lotrich and Meredith, 1974; Ryan, 1975; Hill and Grossman, 1987;
85 Chart and Bergersen, 1988) for use on juvenile fish that are at least an order of magnitude
86 smaller than the animals used in previous studies. We describe how to inject nanoliter quantities
87 (~100 nL) of tattoo ink into the dorsal muscle using a pulled glass capillary tube needle. We can
88 use this method with the youngest and smallest fish, including larva that are still developing.
89 This method is moderately long-lasting: up to 4 weeks, with an average of 2.5-3 weeks under our

laboratory conditions. This duration is similar to injection methods in other species (Thresher and Gronell, 1978). This tag does not appear to interfere with locomotion, and it is visible to the naked eye. Because fish of this early 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). All injected and control fish are expected to recover fully from anesthesia, and we hypothesize that tattoo injections would not significantly affect the timing of the stages of recovery from anesthesia.

The piercing method adapts existing approaches that use a physical tag for identification (e.g., Martin and Wainwright, 2013; Martin and Gould, 2020) for use in juvenile fish that are at least an order of magnitude smaller than the animals used in previous studies. Here, we describe how to thread very thin fishing line through the dorsal muscle of the fish using a minutien 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 test using fully developed juveniles, 1-day after their removal from the mother's buccal cavity. We chose fish this young because they are the most likely to be physically impeded by a tag and/or negatively affected by the procedure. We hypothesized that tagged juveniles would behave similarly to untagged fish in the open field, spending similar amounts of time in the different zones of the tank. Anesthesia recovery data are not included for this method.

Overall, we expect that these procedures 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.

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 adults that bred these juveniles were housed in naturalistic social groups of males and females. The mother orally incubates the larvae as they develop for 10-13 days after fertilization (Fernald and Hirata, 1979; Renn et al., 2009). When the larvae are fully developed and ready to leave the mother's buccal cavity, the average standard length (SL) in our laboratory populations is 8.31 ± 0.039 mm (n=356, max SL: 10.18 mm, min SL: 4.6 mm, Fig 1A). All procedures were done in compliance with the Institutional Animal Care and Use Committee (IACUC) of the Department of Natural Sciences of Pitzer and Scripps Colleges (protocol #19-001) and the IACUC of The University of Texas at Austin.

Tagging by tattoo ink injection

<u>Tattoo ink and Nanoject injection set-up</u>

We developed this procedure using commercially available sets of tattoo ink, including seven color sets of Millennium Mom's Tattoo Ink (Amazon.com, $40 USD at the time of the work) and HAWINK Tattoo Ink (Amazon.com, $20 USD at the time of the work). These sets

135 included vibrant colors such as red, yellow, green, and blue (Supplemental Fig 1), as well as
136 neutral colors such as white, gray, and black. We also used Keep It Wet (Eternal Tattoo Supply,
137 $5 USD at the time of the work) to keep the ink from drying as quickly, without affecting the
138 color.

139 Tattoo injections were performed under a dissection microscope using the Nanoject II
140 Auto-Nanoliter Injector (Drummond Scientific Company). The Nanoject was positioned on a
141 micromanipulator attached to a magnetic base, which was secured to the benchtop on a magnetic
142 board. See Supplemental Materials for detailed set-up information. Briefly, we used a puller
143 (World Precision Instruments PUL-1) to make glass capillary tube needles (1.2 mm x 68 mm, A-
144 M Systems). The tip of the needle was broken off using forceps, and then we backfilled the
145 needle with mineral oil and secured it onto the Nanoject (Fig. 2A, Supplemental Fig 2). After
146 emptying a portion of the mineral oil, we filled the needle with tattoo ink from a weigh boat or
147 Parafilm. We found that different ink colors and brands had different consistencies and drying
148 times in the needle, so we used a ratio ranging from 1:1 to 1:4 ink drops to water drops (or Keep
149 It Wet additive). We performed multiple test injections (Supplemental Fig 2F) at the start and
150 between fish to ensure the ink was not dried, clogging the needle.

151

152 <u>Injection of tattoo ink</u>

153 We removed the fish from the anesthetic (see below) and placed it on the injection
154 platform under the dissection scope on a paper towel wet with aquarium water (Fig 2A). We
155 positioned the fish so the needle could be inserted (using the micromanipulator) between scales
156 and into the dorsal muscle, at the desired location along the rostral-caudal axis (Fig 2B). Young
157 *A. burtoni* juveniles are semi-transparent at this developmental stage; therefore, the needle

158 position was also checked visually prior to injection (Fig 2B). We then injected ~100 nL of ink
159 with 2 sequential injections of 50.6 nL (the largest volume for the Nanoject II model). The
160 'tattoo' was immediately visible at the injection site, as well as on the opposite side of the fish
161 (Fig 2C-E). We kept the needle in place for ~5 s to help prevent the ink from flowing out of the
162 injection site. We then carefully placed the fish into a beaker of fresh aquarium water and aided
163 recovery from anesthesia using artificial ventilation (see below). We wiped the needle with
164 ethanol on a Kimwipe (Kimberly-Clark) between injections and changed the needle every 4-8
165 animals, as the needle dulled, if the needle clogged, or to change ink color.
166
167 Anesthesia and post-tattoo ink injection recovery
168 We anesthetized juveniles less than two weeks old in tricaine methanesulfonate (MS-222,
169 Sigma Aldrich) at a dose of 0.0003 g / mL aquarium water, buffered with sodium hydroxide to
170 pH 7-7.5. During anesthetization, we recorded the time it took for body movement to stop; loss
171 of equilibrium, when the fish's dorsal side first rolled away from an upright orientation; and
172 when ventilation stopped, indicated by movement of the opercula ceasing fully. We removed fish
173 from the MS-222 immediately after ventilation stopped.
174 After tagging (see above), the fish was placed in a 200 mL plastic beaker of fresh
175 aquarium water to recover. For experiments that require strict procedural standardization, we
176 recommend keeping the amount of time out of water consistent across fish (e.g., 1 min for
177 tattooing only; 2 min for tattooing and an additional procedure, such as drug administration).
178 Otherwise, we placed the fish back in aquarium water for recovery as quickly as possible.
179 We immediately began artificial ventilation using a plastic pipette to gently push water
180 over the gills. Recovery from MS-222 is a highly stereotyped process (McFarland, 1959). We

recorded the time it took for the fish to first move its body; first move the opercula; ventilate independently without the artificial ventilation of the pipette; initial regaining of equilibrium, when the dorsal side of the fish first returned to an upright position; and sustained equilibrium, when the fish was permanently upright. We also recorded when the fish looked fully recovered, which tended to happen suddenly. Fully recovered fish swam smoothly and with steady ventilation and no wobbles in equilibrium.

Tagging by piercing with fishing line

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, although this color fades over a period of weeks (3-6). “Beads” of different colors, sizes, or patterns can also be made along the length of the line by tying a knot (or more than one) and using a drop of super glue to preserve the color of the permanent marker. Non-toxic nail polish can also be used over knot(s) in the line for additional colors and patterns of “beads,” which are larger and more visible on camera than knots in the line alone.

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

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

To attach the fishing line to the needle, we removed the plunger from the syringe and threaded the line through the sharp end of the needle under a dissection scope. Line that was freshly and smoothly cut with a new razor blade was the easiest to work with. If the fishing line did not easily fit into the needle, we discarded a length of line and started at a new place along the spool. The diameter of the line varies slightly throughout, and it was easiest to work at an average or narrower section. Once started, we kept threading the line through the needle until it was visible in the barrel of the syringe (Supplemental Fig 3A). We then used forceps to grip the base of the needle, being careful not to dislodge the fishing line. We rocked the needle back and forth with the forceps until it detached from the syringe (Supplemental Fig 3B). From the blunt side of the needle, we then pulled the line through until it was sufficiently long (30-50 cm of line can be used to tag ~4-10 fish, depending on the efficiency of the tagger). Finally, we cut the line from the spool using a razor blade and pulled the last bit of line through the needle until it was no longer sticking out beyond the sharp end. We did not find it necessary to crimp the blunt end of the needle to keep the line in place.

227

228 Anesthesia and recovery

229 We anesthetized juveniles less than 2 weeks old as described above. Older, larger
230 juveniles were anesthetized in MS-222 at a dose of 0.0006 g / mL aquarium water, buffered to
231 pH 7-7.5. We removed the larger juveniles from the MS-222 immediately after they stopped
232 responding to touch, which occurred after losing equilibrium and after gill ventilation stopped.
233 We aided fish in their recovery from anesthesia using artificial ventilation, as described above.

234

235 Tagging the fish

236 For the smallest fish (Fig 4A, B), we placed the fish on a paper towel wet with aquarium
237 water under a dissection scope. Using forceps to hold the minutien pin perpendicular to the fish,
238 we pushed the pin into the dorsal muscle. With the pin still in place, we repositioned the fish to
239 be upright and pulled the pin through the other side of the fish. Using forceps to hold the fishing
240 line, we immediately pushed the line through the hole in the muscle made by the pin. If the tag
241 was marked by knots or nail polish "beads," we were careful not to pull those through the fish,
242 causing more tissue damage. For larger fish (Fig 4C), where the needle can be attached to the
243 fishing line, we followed a similar procedure. With the fish lying flat on a paper towel wet with
244 aquarium water, we used forceps to hold the needle perpendicular to the fish and pierced the
245 dorsal muscle. Shifting the fish to an upright position, we pushed the needle through the muscle
246 (Fig 4D), pulling the needle and the line through the other side of the fish until 3-4 cm of line
247 remained (Fig 4E). A sharp razor blade was used to trim the excess fishing line.

248 Once the fishing line was through the dorsal muscle of the fish, we tied it into a barbell
249 (Fig 4B) or into a loop (Fig 4A,C) using a square knot (Fig 4F,G). If using a loop, we were sure

250 to tie a large enough loop to allow the dorsal fin to raise and lower. However, a loop that was too
251 large could get tangled in the tags of other fish or tank features (e.g., plastic plants). For the
252 smallest fish, we found it safest to tie the barbell knot with two people. While one person
253 pinched the line with forceps on the side of the fish where the second knot was to-be-tied, the
254 second person tied a knot in the free line and slid the second knot into position using forceps.
255 In general, if the knots were challenging to tie, we loosely pre-tied and then untied a knot in the
256 line prior to threading it through the fish. For longer experiments, we placed a drop of super glue
257 on the knot(s) to ensure they stayed tied, which dried in less than a minute. We kept the fish's
258 gills and body away from any glue and applied aquarium water to the gills, as needed, using a
259 transfer pipette. As soon as possible, the fish was placed in a beaker of fresh aquarium water to
260 recover. A fishing line tag can be removed quickly, and without anesthesia, by briefly removing
261 the fish from its tank and cutting the line with a sharp razor blade.
262
263 Open field exploration
264 A physical tag may be more likely to affect locomotion and behavior, particularly in the
265 smallest juveniles. We used an open field exploration to compare between juveniles that were
266 anesthetized and tagged (n=7; one animal died between recovery and the behavioral test on the
267 subsequent day) and those that were anesthetized but not tagged (n=8). This test is commonly
268 used across species to assess movement, activity, and anxiety-like behavior (Prut and Belzung,
269 2003; Cachat et al., 2010; Solomon-Lane and Hofmann, 2019). We tagged the juveniles less than
270 2 hrs after being removed from the mother's buccal cavity, and the open field test was carried out
271 the next day (~20 hrs later). The time it took to complete each tagging surgery was recorded
272 (average 257 ± 22 s), and each control fish was kept out of water and manipulated with forceps

273 for the same amount of time as one of the tagged fish. Additional MS-222 (or water) was applied
274 to the fish during the procedure, as needed.
275 For the open field exploration, we placed fish individually in a small, novel aquarium
276 (22.9 x 15.2 x 15.2 cm) without a cover for 30 min. For analysis, aquaria were divided into 4
277 zones: the territory zone, which contained a small terracotta pot territory / shelter, the close zone,
278 the far zone, and the investigate zone (Fig 5A). Video cameras (Warrior 4.0, Security Camera
279 Warehouse, Asheville, NC) recorded behavior from above, and we used BORIS (Friard and
280 Gamba, 2016) to score the number of times fish entered each zone of the aquarium, and how
281 long fish spent in each zone of the tank during a 10 min observation (minutes 20-30 of the test).
282
283 **Statistics**
284 All statistical analyses were conducted using R Studio (R version 4.4.0) (RStudio Team,
285 2022). Results were considered significant at the $p<0.05$ level, and averages ± standard error of
286 the mean are included in the text. To compare anesthetization and recovery in control and
287 tattooed juveniles, we used t-tests for data that met the assumptions of parametric statistics. A
288 Wilcoxon signed ranks test was used for data that did not meet those assumptions. Cohen's d was
289 reported to estimate effect size for significant pairwise differences (small effect: $0.2 < d < 0.5$;
290 medium: $0.5 < d < 0.8$; large: $0.8 < d$). Pearson correlations were used to test for associations
291 among recovery metrics in control and tattooed fish. To compare locomotion and behavior in the
292 open field exploration between control and fishing line tagged juveniles, we used a two-way
293 mixed ANOVAs, with tagging treatment as the independent measure and aquarium zone
294 (frequency entering, time in) as the repeated measure. Tukey's HSD test was used for *post hoc*
295 analysis of significant ANOVA results. Eta-squared was reported to estimate effect size for

296 significant ANOVA results (small effect: $0 < \eta^2 < 0.01$; moderate: $0.01 < \eta^2 < 0.06$; large: $0.06 <$
297 η^2).
298
299 **RESULTS**
300 **Recovery from anesthesia and injection of tattoo ink**
301 To investigate the safety and efficacy of the tattoo ink injection procedure on very young,
302 very small juveniles (< 2 weeks after removal from the mother's buccal cavity), we compared the
303 anesthetization and recovery of fish that were both anesthetized and tagged (tagged: n=8) to fish
304 that were anesthetized but not injected (control: n=8). Fish in both treatment groups were
305 removed from the MS-222 immediately after ventilation stopped and kept out of water for 1 min
306 on a paper towel wet with aquarium water. Tagged fish were injected during the time out of
307 water.
308 While being anesthetized, juveniles first lost equilibrium (30.6 ± 2.9 s), after which, body
309 movement (52.5 ± 3.0 s) and gill ventilation (54.1 ± 3.5 s) stopped in close succession. Some
310 individuals appeared to stop ventilating first, while others appeared to stop moving first. The
311 treatment groups did not differ in time to lose equilibrium or time to stop moving (Table 1,
312 Supplemental Fig 4A, B). Fish in the tagged group (although they had not been tagged yet) took
313 significantly longer to stop ventilating (Supplemental Fig 4C), but overall, the groups did not
314 differ in the total time spent in MS-222 (Table 1, Supplemental Fig 4D).
315 All of the fish in this cohort successfully recovered. After being placed in the recovery
316 beaker, opercular movement during artificial ventilation occurred first (40.4 ± 9.5 s), followed by
317 independent ventilation (without the aid of artificial ventilation, 107.9 ± 18 s). Initial body
318 movement (128.4 ± 17.2 s), initial regained equilibrium (145.3 ± 21.9 s), and sustained regained

319 equilibrium (162.6 ± 26.8 s) occurred next, in close succession. By definition, full recovery
320 occurred last (407.3 ± 51 s). There were no significant differences for any recovery metric
321 between fish that were anesthetized compared to those that were anesthetized and tagged (Table
322 1, Fig 3A, B).

323 Some recovery metrics were good predictors of later recovery (Table 2, Supplemental Fig
324 5). The timing of independent ventilation was significantly and positively associated with first
325 body movement (Fig 3C) and initial regained equilibrium for both control and tagged fish, as
326 well as sustained regained equilibrium in control fish. Initial body movement was significantly
327 and positively associated with initial regained equilibrium in control and tagged fish, as well as
328 sustained regained equilibrium in control fish. Initial regained equilibrium was significantly and
329 positively associated with sustained regained equilibrium in control and tagged fish. Initial (Fig
330 3D) and sustained equilibrium were the only metrics associated with full recovery, and the
331 associations were significant only for control fish. The timing of initial ventilation (while
332 artificial ventilation was ongoing) did not predict any later recovery points (Table 2).
333

334 **Open field exploration behavior with a fishing line piercing tag**

335 We measured the time spent in each tank zone (Fig 5A), as well as the frequency entering
336 each aquarium zone, for control and juveniles tagged with fishing line. We found a significant
337 main effect of aquarium zone for time spent in each zone ($F(1.46, 13.13)=13.44$, $p=0.001$), but
338 no main effect of treatment ($F(1,9)=1.20$, $p=0.30$), and no interaction effect ($F(1.46,$
339 $13.12)=1.35$, $p=0.28$). *Post hoc* analysis showed that juveniles spent significantly less time in the
340 investigate zone compared to the territory ($p=0.00026$), close ($p<0.0001$), and far zones
341 ($p=0.00029$). The other pairwise comparisons were not significant (close-territory: $p=0.39$, far-

territory: p=1.00, far-close: 0.31) (Fig 5B). Similarly for the frequency of entering each zone, we found a significant main effect of aquarium zone ($F(1.13, 10.2)=16.79$, $p=0.002$), but no main effect of treatment ($F(1,9)=1.90$, $p=0.20$), and no interaction effect ($F(1.13, 10.2)=0.48$, $p=0.53$). Juveniles spent significantly less time in the investigate zone compared to the close zone ($p=0.0004$). The other pairwise comparisons were not significant (close-territory: p=0.23, far-territory: p=1.00, investigate-territory: p=0.088, far-close: p=0.21, investigate-far: p=0.082) (Fig 5C).

DISCUSSION

Although there are a variety of approaches used to tag fish and other organisms, until the present study, no appropriate procedures were available for using these techniques to the youngest, and smallest, juveniles. We expect other researchers working with small organisms have encountered similar challenges. The procedures we describe here have allowed us to utilize two types of tags for the first time: an injected, subcutaneous tag (tattoo method) and a physical tag embedded in the body that remains externally visible (piercing method). These tags 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).

Tattoo ink injection tagging

Injecting tattoo ink into the dorsal muscle of very young, small fish immediately creates a vibrant visual mark, allowing for individual identification. Initially, the tag was visible on both sides of the fish, but over time, the tag was visible for most fish just on one side of the body (e.g., Fig 2E). Tattoo ink is inexpensive and commercially available in an array of colors and mixing

365 inks may be useful in creating more unique colors. Depending on the size of the fish, injection
366 location may also be a useful unique identifier. We found the main limitation to be the fading of
367 the colored mark over time, with lighter colors (e.g., yellow) appearing to fade more quickly than
368 darker colors (e.g., black, blue). Our longest lasting tattoo tags persisted for 3-4 weeks (Fig 2E).
369 Different colored inks, and different manufactures, have different compositions (Moseman et al.,
370 2024), which could underlie the differences in tag visibility we observed. The different ink
371 components could also have differential health effects (Grant et al., 2015; Moseman et al., 2024).
372 In humans, tattoo ink is injected into the skin, specifically the papillary layer of the dermis. Over
373 time, ink particles move deeper into the reticular dermis, and the tattoo persists nearly
374 permanently because sufficient pigment stays in the skin (Grant et al., 2015; Moseman et al.,
375 2024). Here, we injected ink into the dorsal muscle, which resulted in a moderately long-lasting
376 tag, but it was not permanent. Pigment may disperse more readily, or fail to accumulate, in
377 muscle compared to skin. Muscle can also change dramatically during fish development,
378 including in muscle fiber type, size, and number (Patruno et al., 1998; Johnston, 1999). Once fish
379 have sufficient dorsal muscle, a visible implant elastomer tag is a more permanent option than
380 tattoo ink.

381 This procedure is fast and effective, and an experienced practitioner can tag a fish in less
382 than 30 s. The use of anesthesia is necessary for the welfare and recovery of the juvenile fish,
383 and we chose to use MS-222, a common and safe fish anesthetic (Wagner et al., 2003; Kiessling
384 et al., 2009; Sneddon, 2012; Topic Popovic et al., 2012; Zahl et al., 2012) that can be cleared
385 from circulation in less than 300 s (Kiessling et al., 2009). Observing individuals during
386 anesthetization and recovery is highly informative for establishing an effective anesthetic dose,
387 determining the optimal procedural timing, and ensuring full recovery (Sneddon, 2012). For

388 example, some fish species will recover unaided from MS-222 (e.g., bluebanded gobies,
389 *Lythrypnus dalli*, Solomon-Lane and Grober, 2012); however, *A. burtoni* juveniles recover best
390 with artificial ventilation and continuous monitoring until full recovery. These data can also be
391 used to identify and/or control for unintended biases. Here, we found that juveniles in the tagged
392 group took longer to stop ventilating, even though they had not yet experienced anything
393 different from the control fish (i.e., no fish had yet been tagged). With this information, we can
394 test for downstream consequences. For example, despite this significant difference, tagged fish
395 did not spend more total time in MS-222.

396 We found no significant differences in recovery timing between anesthetized control fish
397 compared to anesthetized and tagged fish. This suggests that, overall, tattoo ink injections are
398 safe, with limited negative physiological consequences. We did identify some effects of tagging
399 on the temporal progression of recovery. In control fish, for example, the timing of initial and
400 sustained regained equilibrium was significantly associated with the timing of full recovery. In
401 contrast, for tagged fish, no recovery metric predicted the timing of full recovery. These
402 differences support our approach of using recovery data as an indicator of how an individual was
403 impacted by the procedure and if it is ready to return to its home aquarium or engage in an
404 experimental test (Solomon-Lane and Grober, 2012). For example, we found that the timing of
405 first opercular movement (initial ventilation) during artificial ventilation was not a predictor of
406 subsequent stages of recovery. In contrast, independent ventilation and body movement, which
407 occurred in close succession, predicted when equilibrium was regained. Additional validations of
408 this procedure may be needed, depending on the species or experiment. For example, in our
409 future studies related to social behavior, we will test whether tag color affects how fish behave or
410 are treated by other members of their social group.

In addition to tattoo ink, 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. 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. 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 tagging

Piercing the dorsal muscle with a fishing line tag is a highly effective way of individually marking juvenile fish that can last for months. The materials are inexpensive and commercially available, there are multiple methods for creating unique tags (e.g., line color, “bead” number, placement, and/or color(s)), and the surgery is quick, taking an experienced researcher less than 2 min to anesthetize and tag a fish. Recovery and survival is extremely high in older juveniles (e.g., Solomon-Lane et al., 2022), and although very young, small juveniles are more fragile, recovery and survival were also high in this cohort. 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 remained vibrant under our aquarium conditions, but the colors did fade after 3-4 weeks. When super glue was added over a knot, the color under the glue remained visible for much longer (at least 8 weeks).

A physical tag may be more likely to affect locomotion and behavior than a fully internal tag (e.g., tattoo ink); therefore, we tested fish in an open field exploration under conditions that we considered most likely to reveal potential negative effects on behavior. 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, therefore, we plan to measure locomotion as a control in future studies. Depending on the experiment, species, and developmental stage, a longer (or shorter) 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.

Conclusion

Overall, the lack of appropriate procedures for individually 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). In describing these procedures, we hope they will be useful to other researchers and will continue to be improved in the future.

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CONFLICT OF INTERESTS: None to declare.

ETHICS

All protocols and procedures employed in this study adhered to the ARRIVE guidelines for reporting study design and statistical analysis; experimental procedures; experimental animals and housing and husbandry (Percie du Sert et al., 2020) and in compliance with the US National

480 Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health
481 Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and
482 Use of Laboratory Animals. All procedures were ethically reviewed and approved by the
483 Institutional Animal Care and Use Committee (IACUC) of the Department of Natural Sciences
484 of Pitzer and Scripps Colleges (protocol #19-001) and the IACUC of The University of Texas at
485 Austin (protocol # AUP-2015-00153).
486
487 **DATA AVAILABILITY:** All relevant data are available for reviewers at
488 http://datadryad.org/share/siBW2kDGrSr-wVZ-hUoRsYuk-sL55N0ErGK89YRoKjQ. These
489 data will be made public upon acceptance for publication.

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624

625 **TABLES**

626 **Table 1:** Treatment differences in anesthetization and recovery between juveniles that were
627 anesthetized (control) vs. juveniles that were anesthetized and tattooed. All juveniles remained
628 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 ventilating	**9.01**	**t = -2.42**	**0.039**	**-1.3**
Total time in MS-222	10.07	t = -1.388	0.20	
Recovery				
Initial ventilation during artificial ventilation	12.13	t = 0.51	0.62	
Independent ventilation	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	

629

630 Results of pairwise comparisons using t-tests (t) or a Mann-Whitney Wilcoxon test (W). Cohen's
631 d is reported for effect size of significant results. Significant results are in bold.

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

	Initial ventilation during artificial ventilation	**Independent ventilation**	**Body movement**	**Initial regained equilibrium**	**Sustained regained equilibrium**	**Full recovery**
Initial ventilation during artificial ventilation		p=0.60	p=0.65	p=0.57	p=0.57	p=0.95
Independent ventilation	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	

633

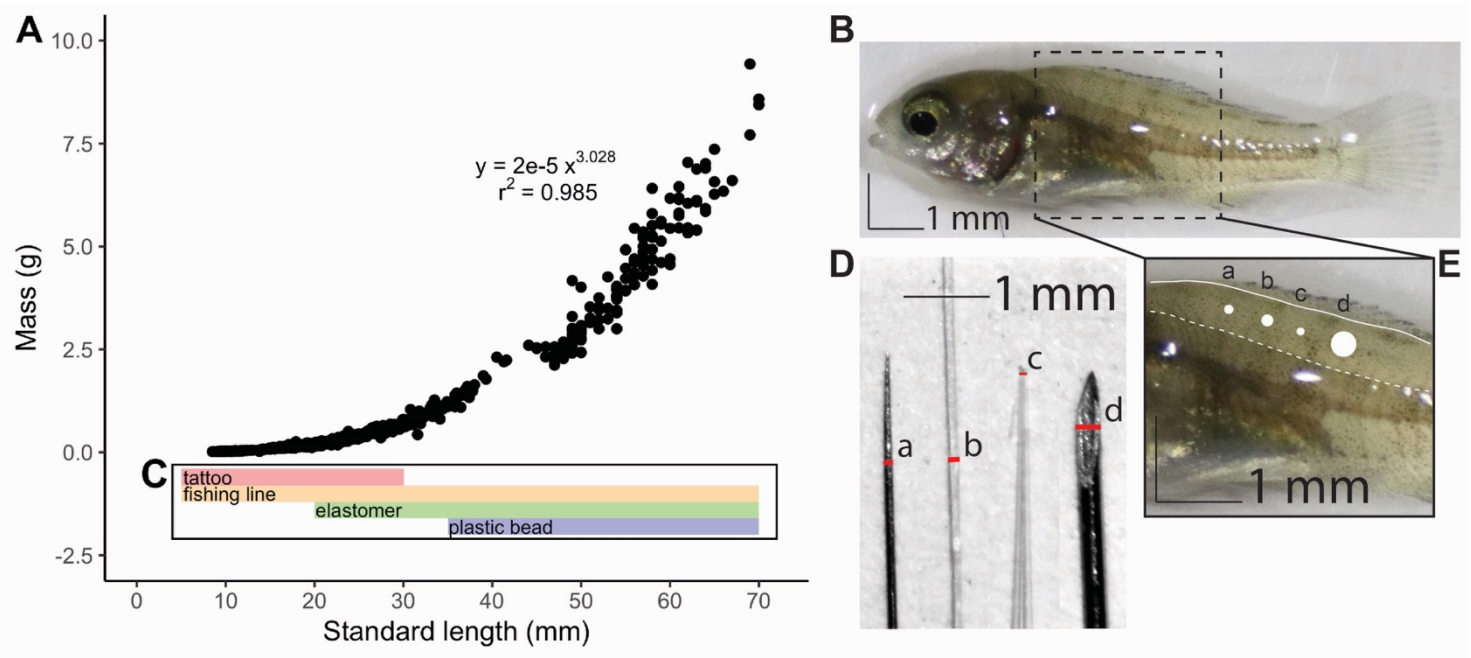
634 Correlations for control fish in the upper triangular (black text). Correlations for tattooed fish in

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

636 significant results.

637

638 **FIGURES**

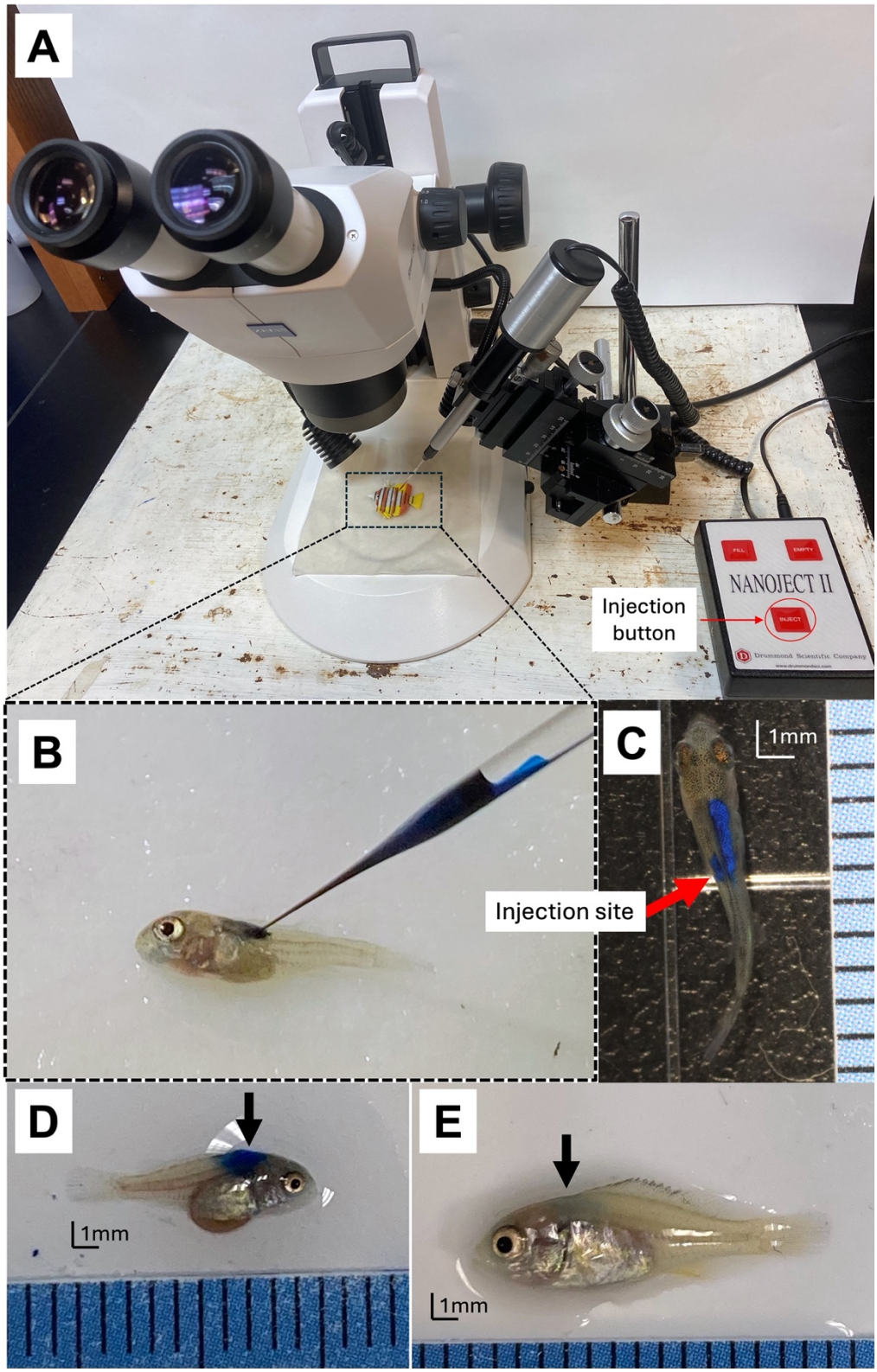


639

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

652 show the diameter of the minutien pin (a), fishing line (b), capillary tube needle (c), and 31-g

653 needle (d) shown in (D).



675 **Figure 2: Tattoo ink injection tagging. A)** Image of the Nanoject setup under a dissection

676 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.

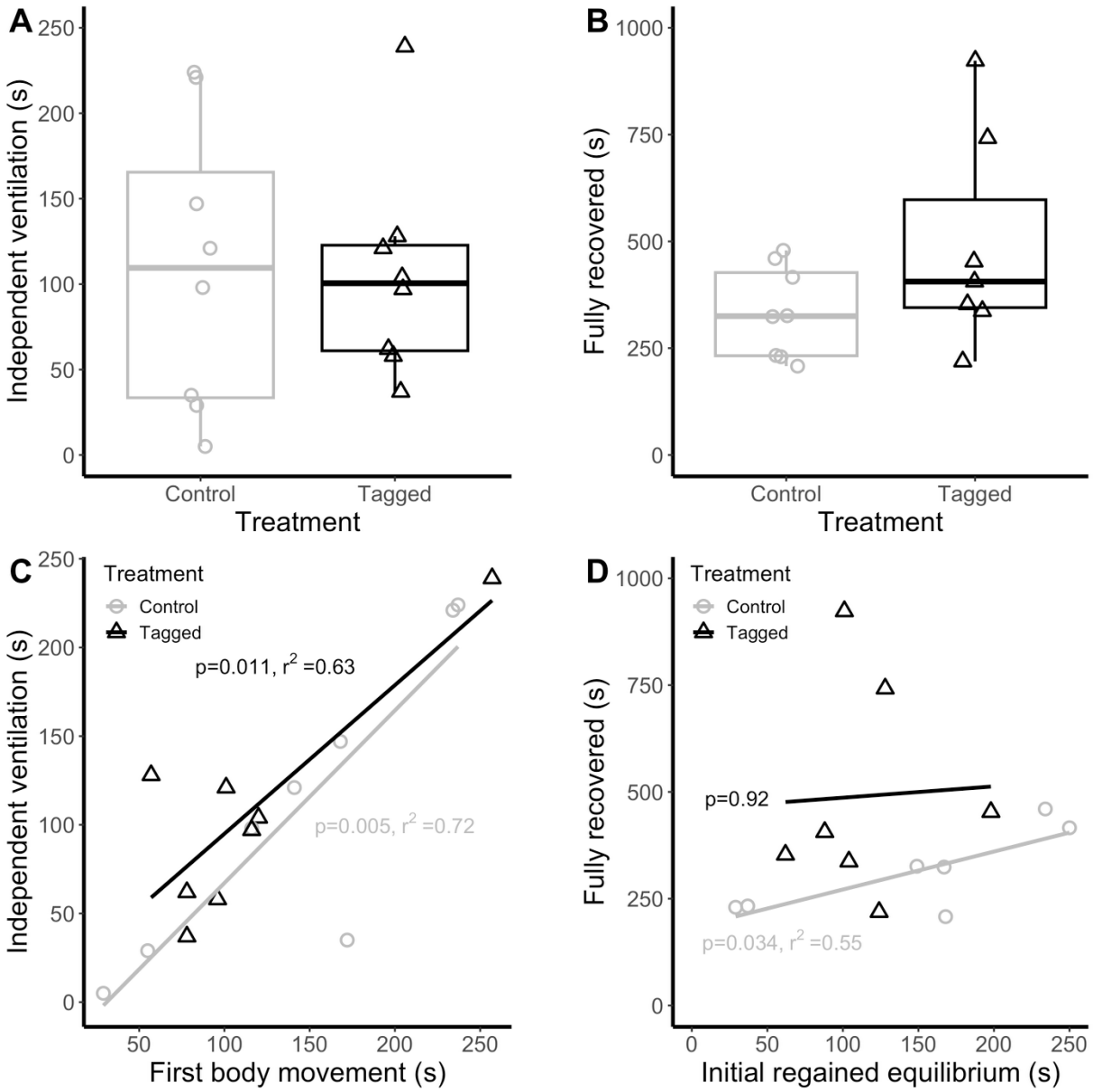


Figure 3: Recovery from anesthesia following tattoo ink injections: Time until A) ventilating independently and B) full recovery from anesthetization in control and tagged juveniles. C) Association between initial body movement and independent ventilation. D) Association between initial regained equilibrium 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.

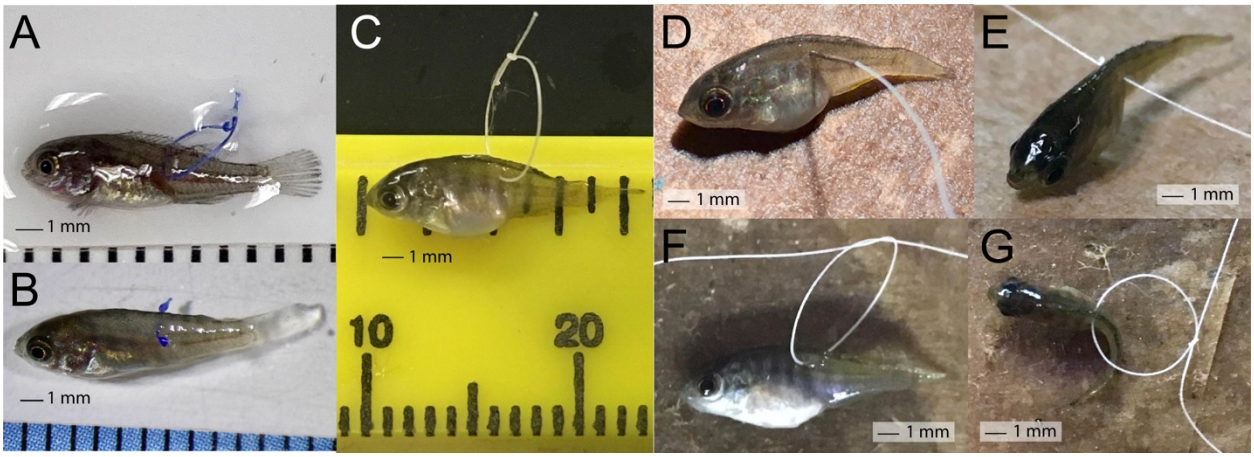


Figure 4: Tagging with a fishing line piercing. A) A juvenile tagged with a blue fishing line loop 1 hr after being removed from the mother's buccal cavity (8.88 mm standard length, SL). B) A young juvenile (4 days post-removal from the mother's buccal cavity) tagged with a blue fishing line barbell (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.

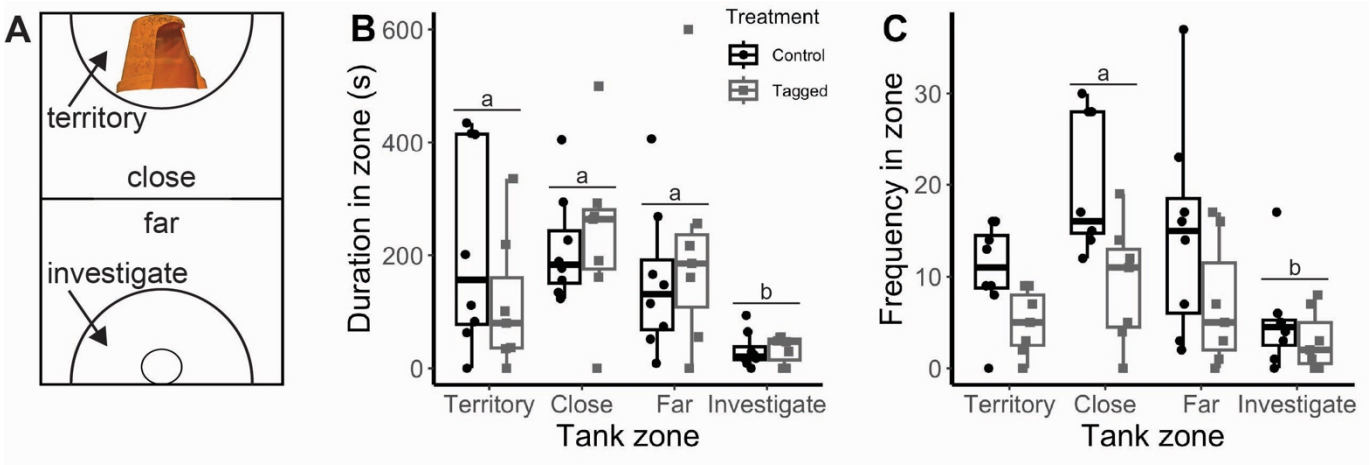


Figure 5: Open field exploration. A) Tank layout of the open field exploration, with the territory, close, far, and investigate zones. The territory zone has a small terracotta pot territory / shelter. **B)** Time spent (s) in each tank zone. **C)** Frequency entering each tank zone. Tagged fish (n=7) 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.