A tagging method for very small fish

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ABSTRACT

The ability to reliably identify individuals over time and across contexts is essential in numerous areas of science. There are a variety of well-established methods for uniquely marking individuals, such as using paint or dye, visible implant elastomer tags, numbers or barcodes glued to the animal, passive integrated transponders, and more. For some species, life history stages, and/or experiments, however, these existing tagging methods are not sufficient. Here, we describe the method we developed for tagging juveniles of the African cichlid fish, *Astatotilapia burtoni*, which are too small for the methods used to tag adults. We used fishing line threaded through the needle of an insulin syringe to tie a loop of line through the dorsal muscle of juveniles as small as 10 mm standard length. Unique color patterns on the line can be used to distinguish among individuals. The tag is compatible with normal locomotion and social behavior, discernible to the eye and on camera, durable enough to last at least months, and the juvenile can grow with the tag. For *A. burtoni*, which is a model system in social neuroscience, the lack of an appropriate tagging method for very small juveniles likely contributes to the relative lack of early-life studies, and the same may be true for other small species. We expect this method to be useful in a variety of species and will facilitate the integration of organismal and behavioral development into more research programs.

INTRODUCTION

The ability to uniquely identify individuals is essential to many scientific endeavors. Although natural patterns are sufficiently distinctive in some species, such as humpback whales (Katona et al., 1979), the African cichlid fish *Neolamprologus pulcher* (Balzarini et al., 2017; Kohda et al., 2015), the guppy *Poecilia reticulata* (Kemp et al., 2009), and the bluebanded goby *Lythrypnus dalli* (Reavis and Grober, 1999), it is usually necessary to mark individuals in order 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 is 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., 2008; 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 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., Campbell Grant, 2008; Malone et al., 1999; Thompson et al., 2005); 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); and more (e.g., Hasler and Faber, 2018; Volk et al., 1999). New automated tracking technology also influences marking methods (e.g., Lewejohann et al., 2009; Ohayon et al., 2013; Weissbrod et al., 2013). Certain methods will be more appropriate than others depending on the spatial and temporal scales involved and the requirements and limitations of the study species and experiment.

Despite the range of existing options, suitable marking solutions are still lacking for some species, life history stages, and/or experiments. Here, we describe the tagging method we developed for juveniles of the African cichlid fish, *Astatotilapia burtoni*, a model system in social neuroscience (Fernald and Maruska, 2012; Hofmann, 2003). The adults of this species are routinely tagged using a visible implant elastomer tag or a colored bead secured with a plastic tag through the dorsal muscle (as in Trainor and Hofmann, 2006). In juveniles, however, there is not sufficient dorsal muscle into which to inject elastomer, and the bead and tag are too large and heavy. We used fishing line threaded through the needle of an insulin syringe to tie a loop of line through the dorsal muscle of fish as small as 10 mm standard length (SL). This tag does not appear to impede the locomotion or social behavior of freely interacting fish, it is visible to the naked eye and on camera for video analysis, it can remain in place as the animal grows, and it is sufficiently long-lasting for the duration of our experiments (months). We expect this method will be useful to other researchers in a variety of species and will make it feasible to incorporate studies on organismal and behavioral development into more research programs that work with small animals.

METHODS

Making the tag

The needle attached to the fishing line that we use is modeled on an eyeless suture, which is expensive to purchase ($6-$24 per suture). Our alternative can be easily made and with inexpensive materials. We used Berkley Nanofil Fishing Line, 0.006 in (0.15 mm) average diameter, in the color clear mist (150 yards for $20) and BD Ultra-Fine™ Short Needle (8 mm, 31G) Insulin Syringes (3/10 mL) (100 for $75). Different fishing line and needle combinations

could also be used, as long as the average diameter of the line is smaller than the inner diameter of the needle. We used the smallest, thinnest combination of materials that were available. The procedure for making the tag is as follows:

1) To attach the fishing line to the needle, remove the plunger from the syringe and thread the line through the sharp end of the needle. It is easiest to do under a dissecting microscope, and it helps to work with a freshly cut end. Use a sharp, new razor to avoid a messy cut, which can flatten the line and widen the diameter. If the fishing line will clearly not thread into the needle, try discarding a length of line and starting at a new place in the spool. The diameter of the line varies slightly throughout, and it is easiest to work at an average or narrower section.
2) Once started, keep threading line through the needle until it has gone beyond the length of the needle and is visible in the barrel of the syringe (Figure 1A).
3) Take a pair of forceps and grip the base of the needle, being careful not to dislodge the line. Rock the needle back and forth with the forceps until it detaches from the syringe (Figure 1B).
4) From the blunt side of the needle, pull the line through until it is sufficiently long. We typically work with a length of line between 30 cm and 50 cm, which can be used to tag ~4-10 fish.
5) Cut the length of line from the spool using a razor blade and pull the last bit of line through the needle until is no longer sticking out beyond the sharp end. We do not find it necessary to crimp the blunt end of the needle to keep the line in place.

Astatotilapia burtoni juveniles

We used *A. burtoni* juveniles from a laboratory population descended from a wild-caught stock. 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 (Fernald and Hirata, 1979; Renn et al., 2009). When the larvae are fully developed and ready to leave the mother's mouth 10-13 days after fertilization (Fernald and Hirata, 1979; Renn et al., 2009), the average SL is 8.31 ± 0.039 mm (n=356, max SL: 10.18 mm, min SL: 4.6 mm). The smallest juveniles we have successfully tagged were 10 mm SL (Figure 2A); therefore, even with this technique, it is not yet possible to tag juveniles immediately upon release by the mother.

Tagging the fish

We anesthetized juveniles in tricaine methanesulfonate (MS-222, Sigma Aldrich) at a dose of 0.0006 g / mL aquarium water, buffered with sodium bicarbonate to pH 7-7.5. We removed fish from the MS-222 immediately after they stopped responding to touch, which occurred after losing equilibrium and ceasing ventilation (opercular movement). Juveniles were then positioned on a wet paper towel flat on their side and tagged as follows:

1) Holding the needle with forceps perpendicular to the fish, push the needle through the dorsal muscle (Figure 2B).

2) Pull the needle, followed by the line, through the dorsal muscle with the forceps until 3-4 cm of line remains (Figure 2C). Use a razor blade to cut the line on the needle side, leaving another 3-4 cm.
3) Using forceps, tie a square knot in the line (Figure 2D, E), and trim the excess using a razor blade. The knot can also be tied by hand, although this method uses more line.
4) Make the loop large enough for the fish to raise its dorsal fin and to grow over the course of the experiment. For longer experiments, place a drop of super glue on the knot to ensure it stays tied.
5) As soon as possible, place the fish in 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. Once in the water, if the fish does not start ventilating on its own, we use a transfer pipette to gently push water over the gills until the opercula move regularly.

Anesthetizing and tagging one fish takes 2 min. The tag can be removed quickly, and without anesthesia, by cutting the line with a sharp razor blade.

Uniquely identify individuals

To distinguish between multiple tagged fish in the same group or enclosure, we use permanent markers to uniquely color the white fishing line (before the line is threaded through the fish). For a short experiment (<1week), the color(s) remain vibrant under our aquarium conditions, but over time, the colors fade. For long-term experiments, we color the line and then add a small drop of super glue to different places along the loop to create a unique pattern. The color under the super glue "bead" lasts at least two months. It may also be possible to thread a

seed bead onto the loop for unique identification. Adding a seed bead to the loop may also be useful as a unique identifier. Although we have not yet tried this, seed beads are very small, readily available in an array of colors, and inexpensive.

CONCLUSIONS

The tagging method described here has made it possible to study the behavioral development and the underlying neuromolecular mechanisms of juvenile *A. burtoni*, a species that is well studied in adulthood but strikingly understudied during development. The lack of an appropriate method for identifying small juveniles likely contributes to the relative paucity of early-life studies, in this and other small species. In social neuroscience, behavioral ecology, animal behavior, 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). We expect this tagging method for very small fish will be broadly useful and hope that other researchers will continue to improve upon the technique.

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FIGURES

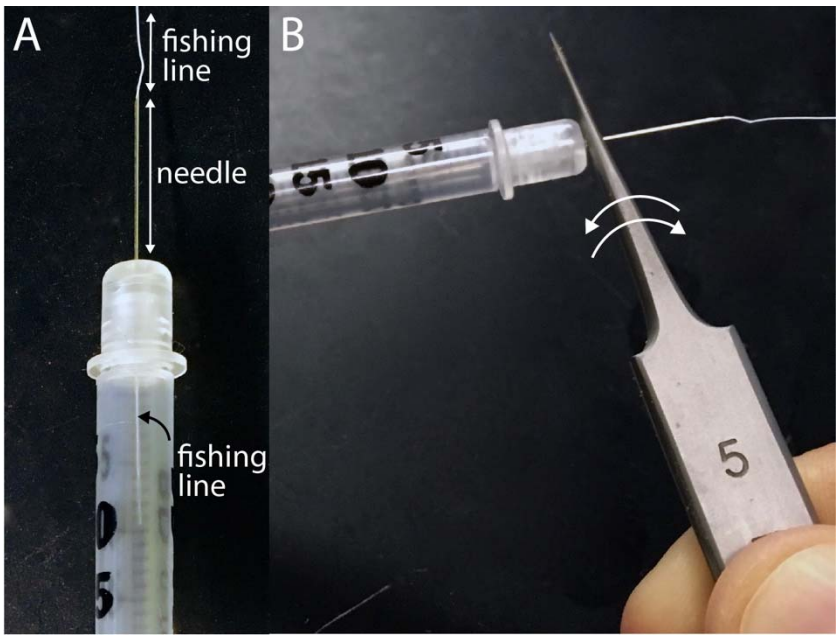

Figure 1: Making the tag. A) Thread the fishing line through the needle of an insulin syringe until it is visible in the barrel of the syringe. B) Use forceps to grasp the base of the needle. Rock back and forth (arrows) until the needle breaks off from the syringe. Not shown: From the blunt side of 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.

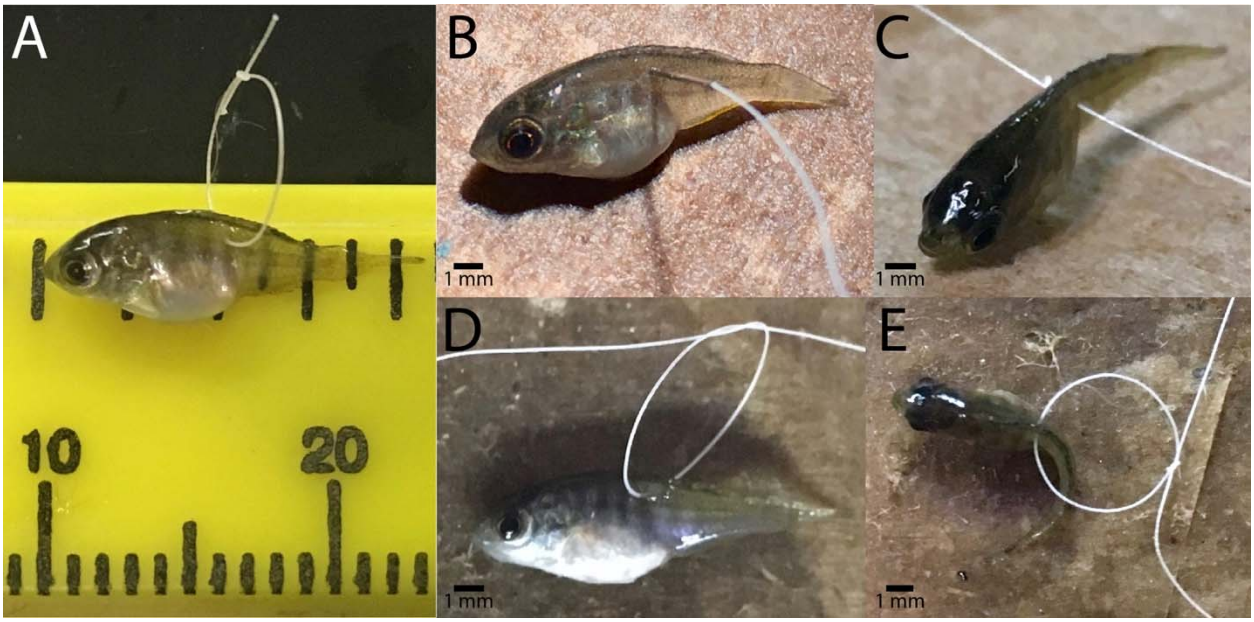

Figure 2: Tagging fish. A) Image of a tagged juvenile *A. burtoni* (10.5 mm standard length) on a ruler (inches top, mm bottom). B) Pierce the dorsal muscle with the needle. C) Pull the needle and line through the dorsal muscle. D) Make a loop in the line and tie the first half of a square knot. Adjust the loop to the desired size. E) Finish the square knot, and cut the excess line using a razor blade. Note: The same individual is pictured in each photograph.