1   **A tagging method for very small fish**

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23   longitudinal study

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24 **ABSTRACT**

25 The ability to reliably identify individuals over time and across contexts is essential in numerous

26 areas of science. There are a variety of well-established methods for uniquely marking

27 individuals, such as using paint or dye, visible implant elastomer tags, numbers or barcodes

28 glued to the animal, passive integrated transponders, and more. For some species, life history

29 stages, and/or experiments, however, these existing tagging methods are not sufficient. Here, we

30 describe the method we developed for tagging juveniles of the African cichlid fish, *Astatotilapia*

31 *burtoni*, which are too small for the methods used to tag adults. We used fishing line threaded

32 through the needle of an insulin syringe to tie a loop of line through the dorsal muscle of

33 juveniles as small as 10 mm standard length. Unique color patterns on the line can be used to

34 distinguish among individuals. The tag is compatible with normal locomotion and social

35 behavior, discernible to the eye and on camera, durable enough to last at least months, and the

36 juvenile can grow with the tag. For *A. burtoni*, which is a model system in social neuroscience,

37 the lack of an appropriate tagging method for very small juveniles likely contributes to the

38 relative lack of early-life studies, and the same may be true for other small species. We expect

39 this method to be useful in a variety of species and will facilitate the integration of organismal

40 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.

64   Despite the range of existing options, suitable marking solutions are still lacking for some

65   species, life history stages, and/or experiments. Here, we describe the tagging method we

66   developed for juveniles of the African cichlid fish, *Astatotilapia burtoni*, a model system in

67   social neuroscience (Fernald and Maruska, 2012; Hofmann, 2003). The adults of this species are

68   routinely tagged using a visible implant elastomer tag or a colored bead secured with a plastic tag

69   through the dorsal muscle (as in Trainor and Hofmann, 2006). In juveniles, however, there is not

70   sufficient dorsal muscle into which to inject elastomer, and the bead and tag are too large and

71   heavy. We used fishing line threaded through the needle of an insulin syringe to tie a loop of line

72   through the dorsal muscle of fish as small as 10 mm standard length (SL). This tag does not

73   appear to impede the locomotion or social behavior of freely interacting fish, it is visible to the

74   naked eye and on camera for video analysis, it can remain in place as the animal grows, and it is

75   sufficiently long-lasting for the duration of our experiments (months). We expect this method

76   will be useful to other researchers in a variety of species and will make it feasible to incorporate

77   studies on organismal and behavioral development into more research programs that work with

78   small animals.

79

80   **METHODS**

81   **Making the tag**

82   The needle attached to the fishing line that we use is modeled on an eyeless suture, which

83   is expensive to purchase ($6-$24 per suture). Our alternative can be easily made and with

84   inexpensive materials. We used Berkley Nanofil Fishing Line, 0.006 in (0.15 mm) average

85   diameter, in the color clear mist (150 yards for $20) and BD Ultra-Fine[TM] Short Needle (8 mm,

86   31G) Insulin Syringes (3/10 mL) (100 for $75). Different fishing line and needle combinations

87 could also be used, as long as the average diameter of the line is smaller than the inner diameter

88 of the needle. We used the smallest, thinnest combination of materials that were available. The

89 procedure for making the tag is as follows:

90

91 1) To attach the fishing line to the needle, remove the plunger from the syringe and

92   thread the line through the sharp end of the needle. It is easiest to do under a

93   dissecting microscope, and it helps to work with a freshly cut end. Use a sharp, new

94   razor to avoid a messy cut, which can flatten the line and widen the diameter. If the

95   fishing line will clearly not thread into the needle, try discarding a length of line and

96   starting at a new place in the spool. The diameter of the line varies slightly

97   throughout, and it is easiest to work at an average or narrower section.

98 2) Once started, keep threading line through the needle until it has gone beyond the

99   length of the needle and is visible in the barrel of the syringe (Figure 1A).

100 3) Take a pair of forceps and grip the base of the needle, being careful not to dislodge

101   the line. Rock the needle back and forth with the forceps until it detaches from the

102   syringe (Figure 1B).

103 4) From the blunt side of the needle, pull the line through until it is sufficiently long. We

104   typically work with a length of line between 30 cm and 50 cm, which can be used to

105   tag ~4-10 fish.

106 5) Cut the length of line from the spool using a razor blade and pull the last bit of line

107   through the needle until is no longer sticking out beyond the sharp end. We do not

108   find it necessary to crimp the blunt end of the needle to keep the line in place.

109

110   *Astatotilapia burtoni* **juveniles**

111   We used *A. burtoni* juveniles from a laboratory population descended from a wild-caught

112   stock. The individuals that bred the juveniles were housed in naturalistic social groups of males

113   and females. Dominant males court gravid females that then lay eggs in his territory. The female

114   then scoops up the eggs into her mouth, where the male fertilizes them. The mother orally

115   incubates the larvae as they develop (Fernald and Hirata, 1979; Renn et al., 2009). When the

116   larvae are fully developed and ready to leave the mother's mouth 10-13 days after fertilization

117   (Fernald and Hirata, 1979; Renn et al., 2009), the average SL is 8.31 ± 0.039 mm (n=356, max

118   SL: 10.18 mm, min SL: 4.6 mm). The smallest juveniles we have successfully tagged were 10

119   mm SL (Figure 2A); therefore, even with this technique, it is not yet possible to tag juveniles

120   immediately upon release by the mother.

121

122   **Tagging the fish**

123   We anesthetized juveniles in tricaine methanesulfonate (MS-222, Sigma Aldrich) at a

124   dose of 0.0006 g / mL aquarium water, buffered with sodium bicarbonate to pH 7-7.5. We

125   removed fish from the MS-222 immediately after they stopped responding to touch, which

126   occurred after losing equilibrium and ceasing ventilation (opercular movement). Juveniles were

127   then positioned on a wet paper towel flat on their side and tagged as follows:

128

129   1) Holding the needle with forceps perpendicular to the fish, push the needle through the

130   dorsal muscle (Figure 2B).

131   2) Pull the needle, followed by the line, through the dorsal muscle with the forceps until 3-4

132   cm of line remains (Figure 2C). Use a razor blade to cut the line on the needle side,

133   leaving another 3-4 cm.

134   3) Using forceps, tie a square knot in the line (Figure 2D, E), and trim the excess using a

135   razor blade. The knot can also be tied by hand, although this method uses more line.

136   4) Make the loop large enough for the fish to raise its dorsal fin and to grow over the course

137   of the experiment. For longer experiments, place a drop of super glue on the knot to

138   ensure it stays tied.

139   5) As soon as possible, place the fish in water to recover. The glue should dry sufficiently in

140   less than a minute, and during this time (and throughout), the fish's gills / body can be

141   kept wet. Once in the water, if the fish does not start ventilating on its own, we use a

142   transfer pipette to gently push water over the gills until the opercula move regularly.

143

144   Anesthetizing and tagging one fish takes 2 min. The tag can be removed quickly, and

145 without anesthesia, by cutting the line with a sharp razor blade.

146

147 **Uniquely identify individuals**

148   To distinguish between multiple tagged fish in the same group or enclosure, we use

149 permanent markers to uniquely color the white fishing line (before the line is threaded through

150 the fish). For a short experiment (<1week), the color(s) remain vibrant under our aquarium

151 conditions, but over time, the colors fade. For long-term experiments, we color the line and then

152 add a small drop of super glue to different places along the loop to create a unique pattern. The

153 color under the super glue "bead" lasts at least two months. It may also be possible to thread a

154   seed bead onto the loop for unique identification. Adding a seed bead to the loop may also be

155   useful as a unique identifier. Although we have not yet tried this, seed beads are very small,

156   readily available in an array of colors, and inexpensive.

157

158   **CONCLUSIONS**

159   The tagging method described here has made it possible to study the behavioral

160   development and the underlying neuromolecular mechanisms of juvenile *A. burtoni*, a species

161   that is well studied in adulthood but strikingly understudied during development. The lack of an

162   appropriate method for identifying small juveniles likely contributes to the relative paucity of

163   early-life studies, in this and other small species. In social neuroscience, behavioral ecology,

164   animal behavior, and related fields, experiments that follow individuals through development are

165   critical to uncovering the emergence of individual phenotypic variation, as well as the underlying

166   mechanisms and fitness consequences (Taborsky, 2016). We expect this tagging method for very

167   small fish will be broadly useful and hope that other researchers will continue to improve upon

168   the technique.

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251 **FIGURES**
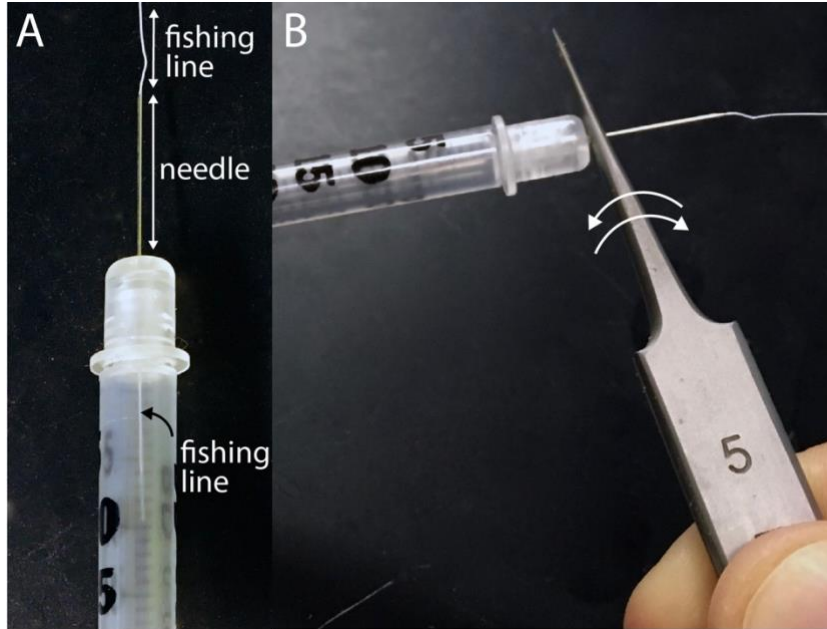
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254 **Figure 1: Making the tag.** A) Thread the fishing line through the needle of an insulin syringe

255 until it is visible in the barrel of the syringe. B) Use forceps to grasp the base of the needle. Rock

256 back and forth (arrows) until the needle breaks off from the syringe. Not shown: From the blunt

257 side of the needle, pull the line through, until it no longer sticks out beyond the needle tip. Use a

258 razor blade to cut off excess line.
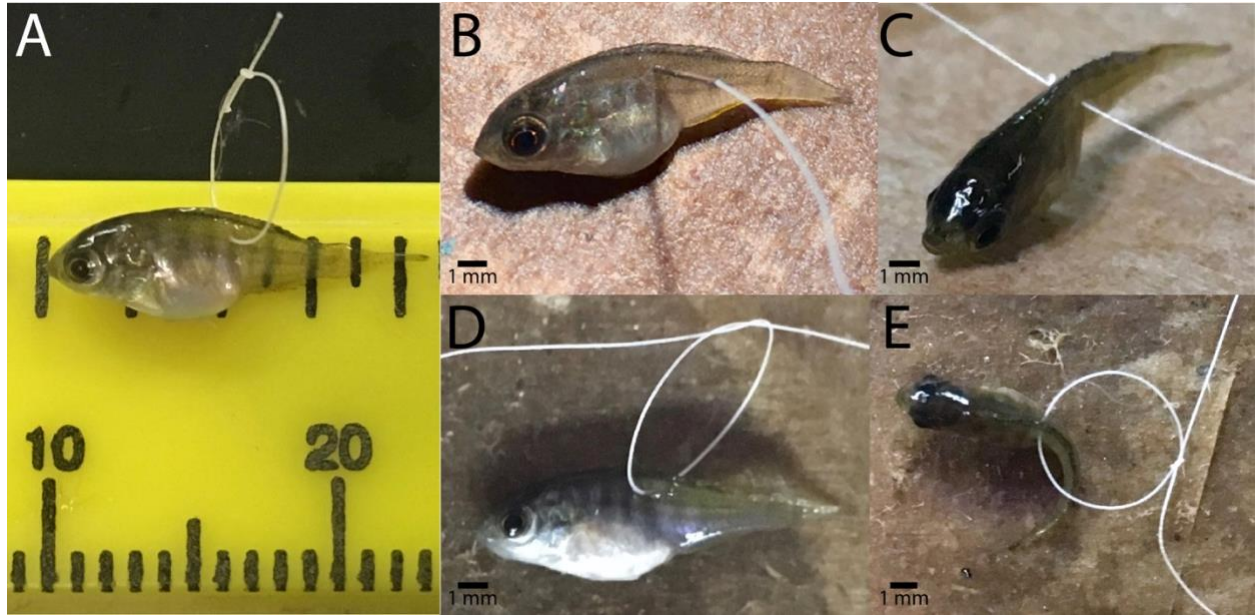
Figure 2: Tagging fish. A) Image of a tagged juvenile *A. burtoni* (10.5 mm standard length) on a ruler (inches top, mm bottom). B) Pierce the dorsal muscle with the needle. C) Pull the needle and line through the dorsal muscle. D) Make a loop in the line and tie the first half of a square knot. Adjust 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.