Avoidance of copper by fathead minnows (*Pimephales promelas*) requires an intact olfactory system

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Fish can detect and respond to a wide variety of cations in their environment, including copper. Most often fish will avoid copper during behavioural trials; however, fish may also show no response or an attraction response, depending on the concentration(s) used. While it may seem intuitive that the response to copper requires olfaction, there is little direct evidence to support this, and what evidence there is remains incomplete. In order to test if olfaction is required for avoidance of copper by fathead minnows (*Pimephales promelas*) copper-induced movement was compared between fish with an intact olfactory system and fish with induced anosmia. Fish in a control group or a mock-anosmic group avoided copper (approximately 10 μg/L or 62.7 nM copper sulphate) while anosmic fish did not. The evidence demonstrates that an intact olfactory system is required for copper sensing in fish.

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12 **Abstract**

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14 Most often fish will avoid copper during behavioural trials; however, fish may also show no

15 response or an attraction response, depending on the concentration(s) used. While it may seem

16 intuitive that the response to copper requires olfaction, there is little direct evidence to support

17 this, and what evidence there is remains incomplete. In order to test if olfaction is required for

18 avoidance of copper by fathead minnows (*Pimephales promelas*) copper-induced movement was

19 compared between fish with an intact olfactory system and fish with induced anosmia. Fish in a

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21 copper sulphate) while anosmic fish did not. The evidence demonstrates that an intact olfactory

22 system is required for copper sensing in fish.

23

Introduction

24

25   The olfactory system in fish is able to recognize a wide array of chemical cues within the

26 environment, including cues to find food, identify kin, evade predators, and mating (Zielinski &

27 Hara, 2006; Tierney et al., 2010; Tierney, 2016). Behavioural responses of fish to cations has

28 been shown to a variety of cations including but not limited to calcium (Bodznick, 1978; Dew &

29 Pyle, 2014; Poulsen et al., 2014), zinc (Sprague, 1964), nickel (Giattina, Garton & Stevens,

30   1982), cadmium (Giattina, Garton & Stevens, 1982), chromium (Hartwell et al., 1989;

31 Svecevičius, 2007), arsenic (Hartwell et al., 1989), cobalt (Hansen et al., 1999a), as well as

32 complex metal mixtures (Sprague 1964; Hansen et al. 1999a). The most studied cation in terms

33 of behavioural responses is copper.

34   Copper induces avoidance behaviours in a wide range of species including the ten-spined

35 stickleback (*Pygosteus pungitius*) (Jones, 1947), goldfish (*Carassius auratus*) (Kleerekoper et

36 al., 1972; Westlake, Kleerekoper & Matis, 1974), golden shiners (*Notemigonus crysoleucas*)

37 (Hartwell et al., 1989), smelt (*Retropinna retopinna*) (Richardson, Williams & Hickey, 2001),

38 inanga (*Galazias maculatus*) (Richardson, Williams & Hickey, 2001), the common bully

39 (*Gobiomorphus cotidianus*) (Richardson, Williams & Hickey, 2001), and a variety of salmonids

40 (Sprague, 1964; Hansen et al., 1999b; Sommers et al., 2016; Van Genderen et al., 2016). While

41 most studies show avoidance of copper, some also demonstrate preference and no response.

42 Collectively the papers cited above demonstrate avoidance to copper at a wide range of

43 concentrations, with preference for copper only occurring at the highest concentrations tested.

44 For example, rainbow trout (*Oncorhynchus mykiss*) avoid copper at a concentration less than 300

45 μg/L and seem to prefer waters containing 330-390 μg/L (Giattina, Garton & Stevens, 1982),

46 while lake whitefish (*Coregonus clupeaformis*) avoid copper at concentrations of 100 – 5,000

47   µg/L and spend more time in waters containing copper in excess of 10,000 µg/L copper (Hara,

48   1981). While most attraction responses occur to high concentrations of copper, attraction to

49   copper has been demonstrated at a concentration as low as 11 µg/L in goldfish (Kleerekoper et

50   al., 1972).

51   For all the work that has been done to determine how fish respond to copper, very little

52   has been done to demonstrate how fish detect copper in their environment. Neurophysiological

53   measures of the olfactory system, namely electro-encephalograms (EEGs) at the olfactory bulb

54   and electro-olfactograms (EOGs) at the olfactory epithelium have shown that the olfactory

55   system in fish responds to calcium, sodium, and magnesium, but not potassium (Bodznick, 1978;

56   Hubbard, Barata & Canario, 2000; Dew & Pyle, 2014). A recent study has also demonstrated

57   that rainbow trout show an EOG response when presented with copper, cadmium, or nickel as an

58   olfactory cue (Lari, Bogart & Pyle, 2018). Only one study to date has investigated the responses

59   of the olfactory system to copper via direct neurophysiological measures and behavioural

60   responses, and they concluded that lake whitefish show a depression in background EEG activity

61   to increasing concentrations of copper, which is not a response normally associated with

62   perception of an odour (Hara, 1981). The apparent disconnect between the behavioural response

63   and the direct neurophysiological measurements in lake whitefish raises the question of whether

64   the response of fish to copper is mediated by olfaction or another sensory modality.

65   In this study fathead minnows (*Pimephales promelas*) were used to test if the avoidance

66   response of a cyprinid to copper is dependent on olfaction. The behavioural response of fathead

67   minnows to copper was compared between intact fish, anosmic fish, and fish that had undergone

68   a mock-anosmic treatment. We hypothesize that fathead minnows will avoid copper when

69   olfaction is intact and will not avoid copper when anosmic. This study design mirrors a study

70 previously performed with calcium that demonstrated calcium is an odorant for fathead minnows

71 (Dew & Pyle, 2014).

72 **Materials & Methods**

73   Adult fathead minnows were obtained from Laurier University's Center for Cold Region

74 and Water Science facility from a breeding colony and housed at the aquatic facility at Trent

75 University. Minnows were held in static tanks filled with ozonated and sand-filtered Otonabee

76 river water held at 20°C with supplemental aeration. All water was allowed to age for 24 hours to

77 allow excess ozone and oxygen to off-gas to ensure oxygen concentrations were appropriate for

78 use with fish. A 12 h:12 h light:dark cycle was maintained in the holding room and fish were fed

79 twice a day on a diet of live brine shrimp and fish flake. Enrichment was provided in the form of

80 spawning tiles and other structures. Prior to being used for any experiments, fish acclimated to

81 lab conditions for 2 weeks. Fish were monitored daily for disease, distress, or an inability to right

82 themselves (i.e., maintain balance), all of which were criteria for euthanasia (no animals were

83 euthanized in the holding tanks). All procedures were approved by the Trent University Animal

84 Care Committee (IACUC number 24420) in accordance with guidelines from the Canadian

85 Council on Animal Care.

86   All solutions, including water used for all behavioural trials, were made using filtered and

87 ozonated Otonabee River water (i.e., the same source as the holding water). Behavioural trials

88 were conducted using I-mazes (55 cm x 11.5 cm x 14 cm) (length x width x height) that had a

89 piece of white corrugated plastic (coroplast) affixed to the bottom of the maze sectioned into

90 three equally sized zones. Each maze was filled with 3 L of Otonabee River water and movement

91 of fish in the chamber was monitored using a webcam suspended above the behavioural maze. A

92 copper sulphate solution (1.5 mg/L or 9.4 mM) was prepared in Otonabee River water so that

93   when a 10 mL aliquot was administered at one end of the behavioural maze it would have

94   diffused to make one half of the water, a volume of 1.5 L, a concentration of approximately 10

95   $\mu g/L$ (62.7 nM). One fish was randomly selected from the appropriate holding tank and placed

96   into a maze. A white curtain was then drawn around the mazes to prevent any movement by the

97   researchers from affecting fish behavior. The fish were allowed to acclimate for 5 minutes. After

98   the acclimation period 10 mL of the copper solution was administered to one randomly selected

99   (via coin flip) end of the maze (i.e., the copper-containing end) and 10 mL of aged ozonated

100 Otonabee river water was administered to the other end (i.e., the blank end). Preliminary dye

101 trials demonstrated that it took 3 minutes for the dye to reach the center of the maze, therefore

102 the copper and blank solutions were allowed 3 minutes to diffuse after being added. After the

103 diffusion period, the position of the fish in the maze was then recorded every 10 s for 5 minutes.

104 Five mazes were monitored at the same time, with each maze being washed and thoroughly

105 rinsed after each trial to ensure no carry-over between trials. Preliminary trials were performed to

106 measure the response of intact fish taken from the holding tank to copper. All experimental

107 groups (control, mock anosmic and anosmic) were interspersed so that at least one fish per group

108 were done in a group of 5. No fish demonstrated a typical fright response of remaining still

109 during the study. The identity of the group a fish belonged to was known to researchers at all

110 times, but given the nature of the measurements (instance in a section of the maze), it is unlikely

111 this influenced results.

112   Anosmia was induced using the technique described in Dew and Pyle (2014). Fish were

113 anesthetized in 1 L of aged ozonated Otonabee River water containing 120 mg of tricaine

114 methanesulfonate, or MS-222, (anesthetic agent) and 240 mg of sodium bicarbonate ($NaHCO_3$)

115 to bring the final solution to pH 7.0. Once the fish began to lose its ability to swim upright in the

116 tank, it was removed from the tank, each naris cleared of water using a small piece of paper

117 towel, and a small amount of tissue glue (1-2 µL) (3M Vetbond, Milton, ON) was inserted into

118 each nasal cavity using a micropipette. This was just enough tissue glue to cover the olfactory

119 epithelium without covering any other structures such as the eyes or gills. This procedure was

120 done as quickly and efficiently as possible with the fish being placed into a labeled recovery

121 tank. A mock anosmic treatment was performed on a second group of fish in which fish were

122 handled in the same manner as the anosmic group (i.e., anesthetized with MS-222), with 1-2 µL

123 of water added to each olfactory chamber instead of tissue glue. The only difference between the

124 anosmic and mock anosmic treatments was the presence of tissue glue in the anosmic group. All

125 fish that underwent anesthesia were then allowed to recover in a tank of clean water for a

126 minimum of 24 hours. The behavioural method described above was performed on anosmic and

127 mock anosmic fish, as well as a control group consisting of fish taken directly from a holding

128 tank. An n = 14 per group (the experimental unit was an individual fish) was used for the

129 behavioural trials, which is supported by a previous study using calcium as an avoidance cue

130 (Dew & Pyle, 2014). All fish in the anosmic group were euthanized with an overdose of MS-222

131 at the end of the experiment, all other fish were transferred to other protocols. A schematic

132 detailing the experimental design is found in Figure 1.

133   The proportion of time spent in the copper-containing arm was calculated for each fish as per

134 equation (1) (Lari et al., 2015; Fischer & Dew, 2021):

135 $$(1)\ Preference = \frac{(time\ in\ cue\ arm\ -\ time\ in\ control\ arm)}{(time\ in\ cue\ arm\ +\ time\ in\ control\ arm)}$$

136   A value at or near 0 indicates fish spent an equal amount of time in either end, a value

137 significantly less than 0 would indicate that fish spent less time in the copper-containing arm

138 than the blank arm (i.e., they avoided the cue), and a value above 0 indicates attraction to the

139 cue. Parametric assumptions held for each trial and a one-sample t-test against 0 was performed

140 for each of the three treatments to determine if any treatment resulted in more time being spent in

141 either end of the maze. A Holm-Bonferroni sequential correction was applied to produce

142 corrected p-values due to the use of multiple t-tests, with a p-value of <0.05 being considered

143 significant. Cohen's d was calculated for effect size using the effsize package (Torchiano, 2020).

144 An ANOVA followed by a Tukey's Honest Significant Difference test as a post-hoc was

145 performed to compare among and between the different groups. An η^2 value was calculated for

146 the effect size for the ANOVA using the lsr package in R (Navarro, 2015).All analyses were

147 done using R (R Core Development Team, 2021) with graphs produced in R using the ggpubr

148 package (Kassambara, 2020). Raw data have been provided as supplementary material.

149 **Results**

150   In preliminary trials, fish taken from the holding tanks avoided copper (t(8) = -2.55, p =

151 0.034, n = 9, df = 8, d = -0.85; Figure 2). For the experimental groups, fish avoided the copper

152 containing arm in the control (t(13) = -3.66, p = 0.006, n = 14, df = 13, d = -0.97; Figure 3) and

153 mock anosmic (t = -5.71, p<0.001, n = 14, df = 13, d = -1.52) treatment groups, but not in the

154 anosmic group (t(13) = 1.23, p = 0.24, n = 14, df = 13, d = 0.33). Direct comparison of the

155 groups showed that the mock anomsic and control groups were not significantly different;

156 however. the anosmic group was significantly different from both the mock anosmic and control

157 group (F(2,39) = 1.78, p < 0.001, n = 42 (14 per group), df =(2,39), η^2 = 0.36).

158 **Discussion**

159   The data demonstrate that without an intact olfactory system, fathead minnows do not

160 avoid copper in the environment. This is supported by a study that used chemical ablation of the

161 olfactory epithelium with nitric acid to demonstrate impairment of some, but not all, behavioural

162 responses to copper (Hara, 1981). There is lack of clarity in the literature concerning the

163   response of fish to copper as measured using neurophysiological methods. Rainbow trout show

164   an EOG response to copper while whitefish show a decrease in background electrical activity

165   levels due to increasing copper concentration, something not normally associated with an

166   olfactory response (Hara, 1981; Lari, Bogart & Pyle, 2018). The apparent discrepancy between

167   the EOG and EEG results could be due to several reasons. An EOG measures the bulk response

168   of olfactory sensory neurons (i.e., EOG measures the generator potential of many neurons),

169   while EEG measures electrical activity at the olfactory bulb. It may be that olfactory sensory

170   neurons are responding but the signal is not being propagated to the olfactory bulb, or it could be

171   that a depression in electrical activity is due to impairment of the olfactory system by copper. In

172   the Hara (1981) study they used concentrations of copper from 25 µg/L - 2.5 mg/L, which is

173   greater than what has been demonstrated to impair olfaction in short-term exposures (Green et

174   al., 2010; Dew, Wood & Pyle, 2012). It is possible, then, that when the copper was perfused over

175   the olfactory epithelium in the Hara (1981) study there was an immediate impairment of the

176   olfactory system that prevented any response to the cue. That being said, it is unclear why EOG

177   responses would not have been impaired in rainbow trout as both studies used similar

178   concentrations. Despite a possible impairment of olfaction as measured by EEG, whitefish

179   demonstrated an avoidance to low concentrations of copper. This avoidance was most likely due

180   to the fact they were exposed to a concentration gradient of copper in the behavioral maze,

181   meaning they were likely avoiding copper and concentrations far below what was used for the

182   EEG experiments. It is also possible that the response to copper as measured by EEG, EOG, or

183   behaviour is not due to copper being an odorant, but instead as a response to damage to the

184   olfactory epithelium caused by copper. Regardless of the reason for the discrepancy, our study

185 clearly demonstrates that avoidance of copper requires an intact olfactory system, regardless of

186 questions raised by the neurophysiological results of previous studies.

187   A variety of reasons have been proposed as to why fish respond to copper in their

188 environment. The attraction response seen at low concentrations of copper in goldfish may be

189 due to the fact that copper is an essential element for fish and that by moving into an area with a

190 low concentration of copper the fish can absorb needed copper through the gills (Kleerekoper et

191 al., 1972). At higher concentrations copper can cause a variety of toxic endpoints in fish,

192 therefore avoidance of areas with higher concentrations of copper may be an adaptive response

193 to prevent toxicity. A bigger question is why do fish demonstrate an attraction response to copper

194 at concentrations well above those demonstrated to show olfactory impairment? It has been

195 suggested that when fish swim into an area of high copper they become stupefied and unable to

196 swim out of the copper due to a toxic effect, therefore, the preference seen at high concentrations

197 may not truly be a preference for copper but the result of a toxic endpoint (Jones, 1947;

198 Kleerekoper et al., 1972). Fish may also avoid areas with a differing concentration of cations to

199 avoid osmotic pressure that would result from moving into an area with a drastically different

200 cation concentration. Regardless of why fish respond to copper, this study demonstrates that

201 without an intact olfactory system, there is no behavioural response.

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207

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291

Figure 1

Schematic of experimental design

There were three groups of fish used for this experiment, fish taken directly from holding, fish made anosmic, or fish that went through the anosmia-inducing procedure but were not made anosmic (i.e., Mock-Anosmic). The response of each group of fish to copper was assessed using a trough maze.
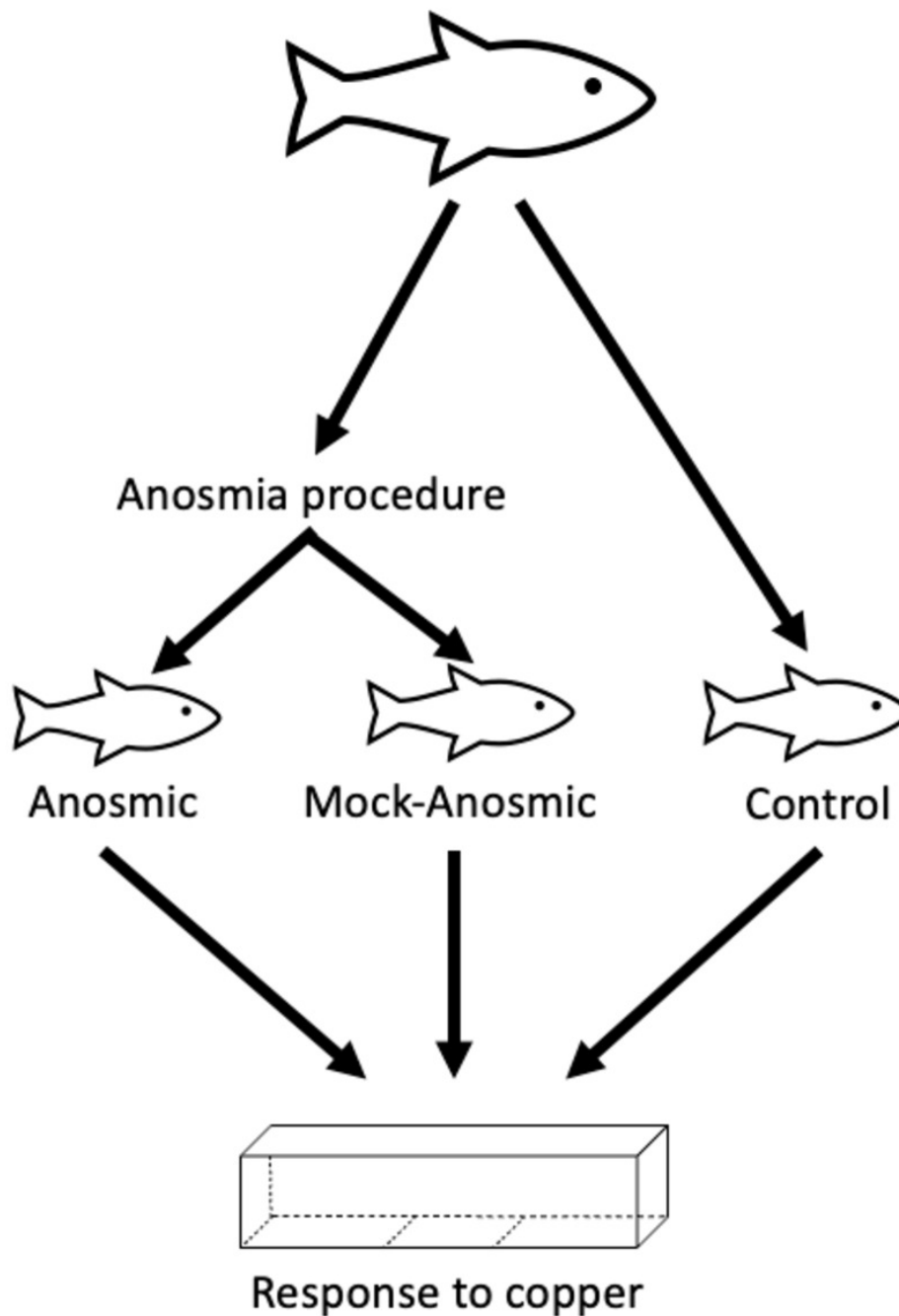
Figure 2

Average proportion of time spent in the copper-containing arm – preliminary study

The mean +/- SEM for the proportion of time spent in the copper-containing arm of the maze (i.e., value for copper arm preference) for animals removed from the holding tank.An asterisk denotes significant difference from 0, n = 9.
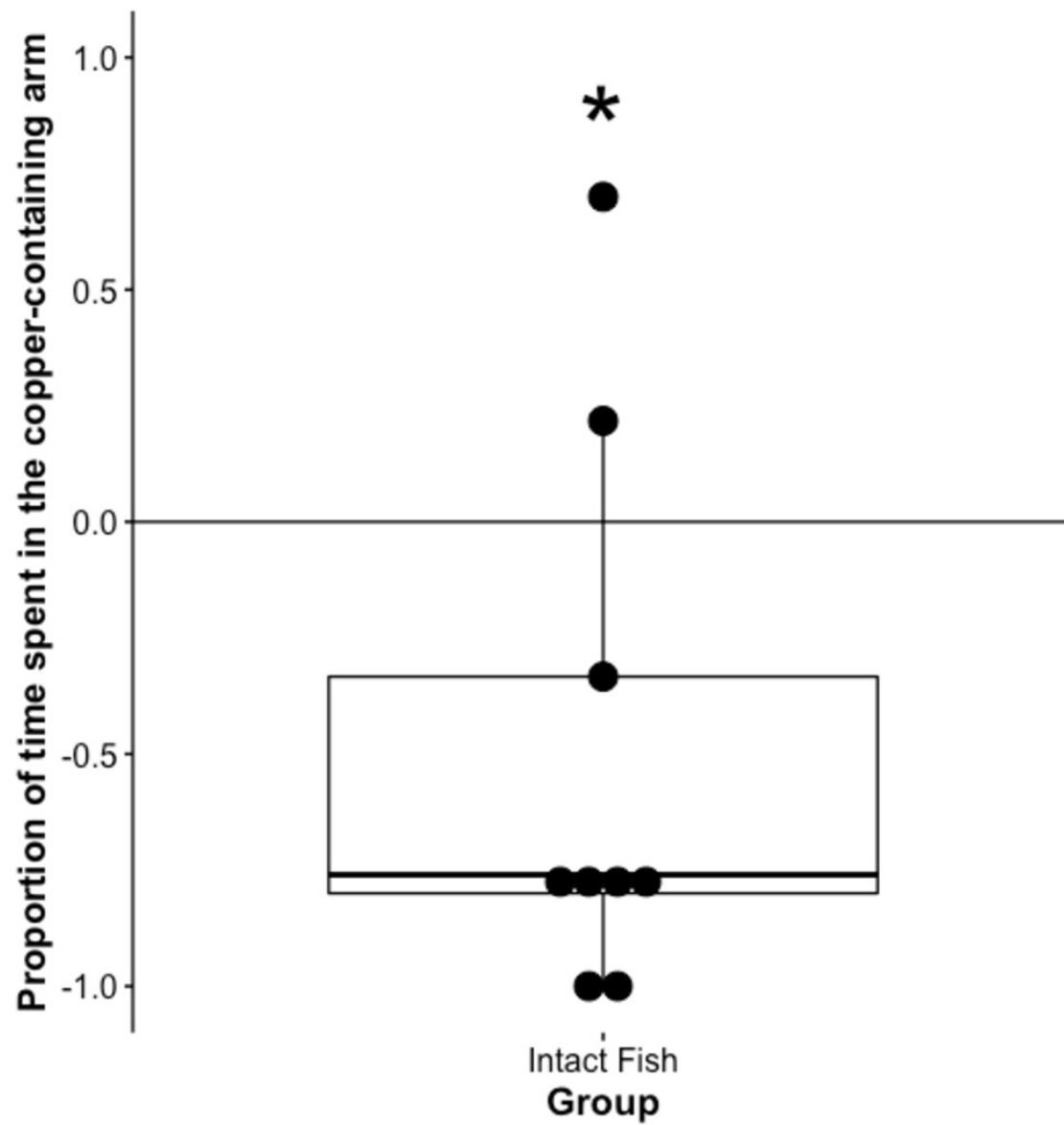
Figure 3

Average proportion of time spent in the copper-containing arm - anosmia trials

The mean +/- SEM for the proportion of time spent in the copper-containing arm of the maze (i.e., value for copper arm preference).An asterisk denotes significant difference from 0, dissimilar letters denote a significant difference, n = 14 for each trial.