A New Non-Invasive Technique for Temporarily Tagging Coral Reef Fishes

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The ability to identify individuals is important for the success of many behavioral and ecological studies. In fishes, there is a lot of variation in body size, shape, skin thickness, behavior, and ecology, which means that any given marking/tagging method may not work well for all species. For the Humbug Damselfish *Dascyllus aruanus*, a widely used model species of coral-reef fish, we found that standard tagging methodologies (e.g., phenotypic variation, beads and PVC tags, fluorescent elastomer injections) were ineffective as they could not be applied to the fish or easily detected by observers or from videos. In response, we developed a new method to temporarily tag *D. aruanus* using colored plastic films and topical surgical glue. These films were easily detectable both directly by human observers and indirectly by video/photo-cameras. We tested the efficacy of this new method by examining the survival time of the tags at various positions on the body. Our results showed that the optimal tag locations were dorsal anterior (with a median attachment time of 53 hours) and dorsal middle (with a median attachment time of 49 hours). Total length of fish was not a significant predictor of tag life. In sum, we demonstrate an effective new method for temporarily tagging a widely studied coral reef fish. This method could be applied to other fishes and aquatic organisms (e.g., amphibians) in both marine and freshwater ecosystems.

MANY studies in population ecology and behavioral ecology require individuals to be identifiable (Krebs and Davies, 1997). Generally, animal identification occurs using a means of marking or tagging (Gavin and Haas, 1989). By developing and using multiple tagging methods, scientists have been able to track individuals through time, generate estimates of mortality, and demonstrate variation in behavior amongst individuals within a population (Randall, 1962; Frusher and Hoenig, 2001; Coker et al., 2012). However, the practical difficulties of working with some taxa, notably aquatic organisms, have made acquisition of data on their biology and behavior through individual identification more challenging.

For fishes, external and internal marks and tags have been used for centuries (Rounsefell and Kask, 1945). Nevertheless, the great variation in body size, shape, skin thickness, behavior, and ecology means that the same tagging methods cannot be used for all species. Additionally, some marking/tagging systems that are easily visible to the human eye in one context may not be as readily observable in different contexts (e.g., different light conditions and depths), and they may not work as well once photographed or filmed. Therefore, to optimize studies involving fish identification, it is essential to select the tagging system that works for the species and context under investigation.

In coral reef fishes, there are many different techniques for individual identification: phenotypic variation (Nelson et al., 1994; Booth, 1995; Buston, 2003), genotypic variation (Puebla et al., 2007; Salles et al., 2016), anchor tags such as dart, T-bar and spaghetti (Randall, 1962; Parker, 1990), fluorescent elastomer injection (Beukers et al., 1995; Frederick, 1997; Malone et al., 1999; Hoey and McCormick, 2006), passive integrated transponder (PIT; Holm et al., 2007; Topping and Szedlmayer, 2011), coded wire tags (Beukers et al., 1995; Verweij and Nagelkerken, 2007), bead tags (Verweij and Nagelkerken, 2007), and parasites (Grutter and Poulin, 1998; Cribb et al., 2000). These techniques vary in terms of invasiveness of procedures, duration of effectiveness, visibility, and practical application. However, for some species these methods are not appropriate.

For the widely studied coral-dwelling damselfish, *Dascyllus aruanus*, for example, there is not sufficient phenotypic variation between individuals (besides relative body size, occasional variances in lip coloration and dorsal fin pattern) and hence tagging is required (Branconi, pers. obs.). However, we found that standard methods of tagging were unsuitable for *Dascyllus aruanus* due to their specific ecology and morphology: beads and PVC tags get caught when they swim through the narrow inter-branch spaces in their host corals; fluorescent elastomer injections/tags placed in areas other than the caudal peduncle proved difficult to apply due to their thick scales and ease of bruising (Branconi, pers. obs.). Further, other attempts to tag *D. aruanus* (fluorescent elastomer injections: Booth, 2004; Coker et al., 2012; Kuwamura et al., 2016; mutilation: Sale, 1971; alcian blue dye: Mizushima et al., 2000; tetracycline staining: Forrester, 1990; liquid latex: Forrester, 1991) do not result in marks that are visible from a distance or via video recordings.

Therefore, our goal was to develop a new method for temporarily tagging this species that is easily detectable by both human observers and indirectly by video/photo-cameras. Specifically, we addressed the following three questions: i) What is the attachment time of the tags? ii) How does fish body size influence the attachment time of the tags? and iii) How does position on the body influence attachment time of the tags? In sum, we developed a new non-invasive technique for temporarily tagging coral reef fishes that is potentially broadly applicable to other aquatic species.

MATERIALS AND METHODS

Natural history.—*Dascyllus aruanus* is a tropical coral-reef fish that is widespread throughout the Indo-Pacific region and lives in social groups in close association with coral colonies of certain branching corals (Sale, 1971; Holbrook et al., 2000). Within each discrete coral patch there is a single group of mostly non-relatives (Sale, 1971; Fricke, 1980; Buston et al., 2009) with average group size of eight individuals (Sale,

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1972; Holbrook et al., 2000) organized into size-based dominance hierarchies (Coates, 1980; Wong et al., 2012).

Collection and housing.—For this study, we used a population of *D. aruanus* at Lizard Island Lagoon on the northern Great Barrier Reef, Australia (14°40′S, 145°27′E). The study was conducted during January 2016. Two collection sites were selected: Palfrey Island (14°41.764′S, 145°26.890′E) and Trawler Beach (14°41.060′S, 145°27.662′E). At each of these sites, we located and mapped 11 corals occupied by a single group of *D. aruanus*. Group sizes ranged from three to four fish and were determined by counting the number of resident fish on the focal host coral. Fish from each group were anesthetized using a clove oil solution (Munday and Wilson, 1997), captured with a slurp gun or hand net, and placed into zip-lock plastic bags to be transferred to the boat. Once onboard, air was put in the bags, the bags were put in a cooler, and the fish were transported to laboratory facilities at Lizard Island Research Station (the time from catching to release the fish into the aquaria was less than one hour).

Each group of fish was placed into a rectangular plastic aquarium (68 liters; external 645x413x397 mm, internal 597x362x381 mm; Nally IH078) containing dead coral heads (coral heads of multiple species with the same morphology as the host corals commonly used by *D. aruanus*) positioned under direct natural sunlight conditions shaded by a plastic screen (11 groups of fish; 36 individuals). A flow-through system provided a continuous supply of fresh seawater (directly pumped from the reef in front of the Research Station) guaranteeing reasonable water quality, temperature, and some natural food within the aquaria. Additionally, fish were fed twice a day with dry fish pellet and living brine shrimp larvae, *Artemia salina*. All fish were held for ten days for the duration of the procedures (see below) before being returned to their original collection site.

Tagging procedure.—The plastic tags consisted of white or black plastic films (60/80 micron continuous polymeric material) cut into approximately 3x3 mm squares (Fig. 1A). The color of the tags (black and white) was chosen to be consistent with the natural coloration of the fish, so as not to increase the visibility of marked individuals to predators but making marked individuals easily observable under different light conditions to the human eye or to video/photo-cameras.

To tag the fish, each individual was removed from the aquaria and placed on its side on a plastic slate positioned on a wet bench with low water flow so that a thin and constant layer of water was in contact with the fish. Fish were not re-anesthetized prior to tagging. Using a cotton tip, a few scales of the fish were dried at the appropriate tag positions (Fig. 1A). Subsequently, a small drop of the topical tissue adhesive "GLUture" (an Octyl/Butyl cyanoacrylate blend that offers a flexible seal; Abbott Laboratories, Chicago, IL) was placed onto the dried scales and a plastic tag placed on the drop of adhesive using tweezers (Fig. 1A).

To ensure that the entire surface of the tag was touching the fish's scale (i.e., there were no air bubbles between the tag and fish scales), the tag was pressed onto the scale for few seconds using a wet cotton tip (Fig. 1A). A small amount of topical tissue adhesive was then placed on top of the right and left extremities of the tag (Fig. 1A). Six tags were placed at various defined positions on the body, three on each side (Fig. 1B). The placement of each tag required approximately 60 seconds. Fish were returned to the aquarium between the placement of each tag. Finally, fish total length was measured to the nearest 0.1 mm using calipers.

Experimental design.—Each individual within each group of fish (n = 36 individuals; n = 11 groups) was randomly assigned a different combination of tags, of which there were six possible combinations (Fig. 1B). Each combination was defined by the position of three tags on both the left and right side of the fish along i) horizontal positions—subdivided into posterior, middle, and anterior and ii) vertical positions—subdivided into dorsal, central, and ventral (Fig. 1B). Following standard operating procedures approved by the animal ethics committee of the University of Wollongong (UOW), after application of tags, a qualitative assessment of fish behavior and health was conducted checking for any sign of stress (e.g., abnormal breathing rate, irregular movements and buoyancy, or anomalous feeding activity).

Subsequently, the presence–absence of the tags was monitored every two hours for the first three days and then every four hours for the following four days (total of seven days). After tag loss, fish were observed for three days to assess scale replacement. To ensure that tags were not missed, each check was performed by an observer (RB) watching the fish in their aquaria, and by analyzing five minutes of video recordings taken by HERO3+ GoPro™ cameras (GoPro Inc., San Mateo, CA) fixed on tripods placed inside the aquaria.

Statistical analyses.—To generate a preliminary visual understanding of whether attachment time of tags differed based on tag position, an Exploratory Data Analysis (EDA) was performed by producing and examining three figures: i) box plots of attachment time of tag by tag position for both fish sides; ii) color gradient plot of mean attachment time for each tag location; and iii) box plots of attachment time of tag by fish size ordered according to fish size.

Subsequently, we used the observations from the EDA to inform more rigorous statistical testing. More specifically, we fitted a linear mixed-effects model to the data using the R package lme4 (R Core Team, 2014; Bates et al., 2015) to assess whether tag attachment time was influenced by position and/or fish total length. To normalize the data (based on visual inspection of QQ plots), tag attachment time was log transformed. A linear mixed-effects model was fitted with tag position and fish total length as predictors and log tag attachment time as the response. Fish ID was included as a random effect to account for lack of independence among multiple tags on the same fish. *P*-values were calculated using the Kenward Rogers approximation for the degrees of freedom.

RESULTS

In this study, we used a total of 36 individuals (average total length = 45.2 mm, ranging from 72.6 mm to 20.2 mm) and attached a total of 216 tags. Following UOW Standard Operating Procedures, no signs of stress were visible in the fish. Within five minutes of the tagging procedure, the behavior of the marked individuals appeared normal (i.e., regular breathing rate, movements, and buoyancy). By the end of our experiment, all fish appeared healthy (i.e., normal activity and regular feeding), and the majority of fish possessed new scales as replacements for scales lost with the tags. In sum, the tagging procedure caused no apparent harm to the fish within the timeframe of the experiment.

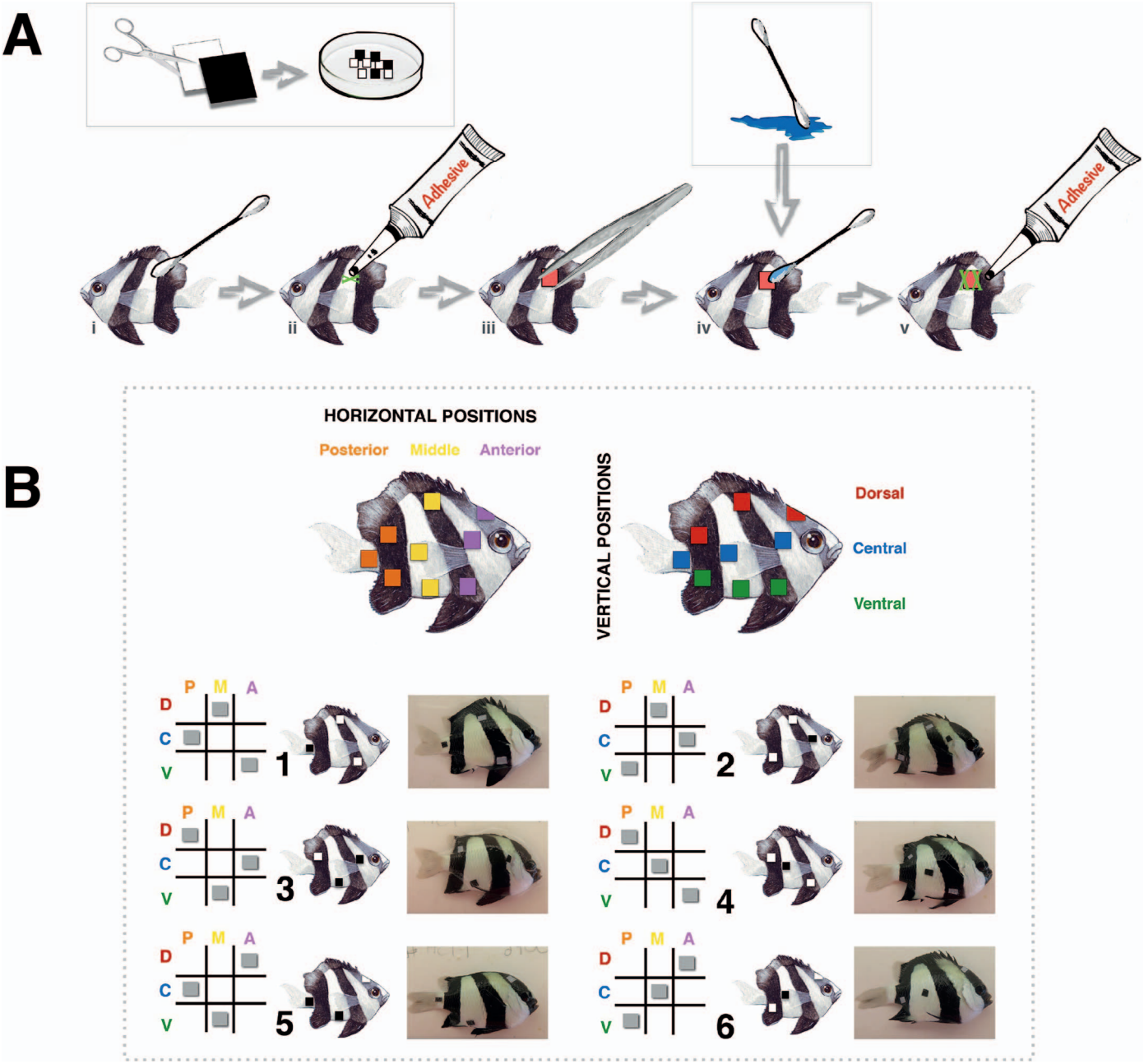

Fig. 1. (A) Graphic scheme of the tagging procedure: i) Dry the scales of the fish, scraping gently with a clean cotton tip (moving the top of the cotton tip in the same direction as the fish scales); ii) Apply on the dried area a very small drop of topical tissue adhesive; iii) Place the tag on the spot of adhesive using tweezers; iv) Using a wet cotton tip, press on the tag for few seconds (ensuring that there are no bubbles of air between the tag and the fish scales); v) Finally, apply a small amount of adhesive on the right and left extremities of the tag. This procedure requires less than a minute for each tag. (B) Graphic scheme and photo examples of the six possible combinations of the tags. Positions of the tags along the fish body (horizontal—posterior in orange, middle in yellow, and anterior in purple; and vertical—dorsal in red, central in blue, and ventral in green). Illustrations by RB.

Initial EDAs revealed that median attachment time of tags ranged widely with tag position, from a minimum of 15 hours for ventral anterior (VA) positions to a maximum of 145 hours for central middle (CM) positions (see Table 1 for the values of median, minimum, and maximum tag attachment time for each position). More specifically, the box plots displaying tag attachment time by tag position (Fig. 2A) and the color gradient plot displaying the mean attachment time of each tag location (Fig. 2B) suggested that optimal tag locations are dorsal anterior (DA), with a median attachment time of 53 hours (mean attachment time = 62 hours), and dorsal middle (DM), with a median attachment time of 49 hours (mean attachment time = 50 hours). On the other hand, central anterior (CA) locations and ventral posterior (VP) locations seemed to be the worst tag locations, presenting a median attachment time of 22 hours (mean attachment time = 39 hours) and 25 hours, respectively (mean attachment time = 40 hours; Fig. 2A, B). The box plots also indicated that the pattern of tag life was similar on the right and left sides of the fish (Fig. 2A), suggesting that fish side does not influence tag attachment time. The box plots displaying tag attachment time by individual ordered by size (Fig. 3) were not suggestive of any effect of body size on tag attachment time.

Table 1. Median, minimum, and maximum tag attachment time (hr) for each position: dorsal anterior, middle, and posterior (DA, DM, DP); central anterior, middle, and posterior (CA, CM, CP); ventral anterior, middle, and posterior (VA, VM, VP).

Tag atttachment time (hr)			
Position	Min	Median	Max
DA	18.25	53.00	143.00
DM	19.00	49.25	116.25
DP	17.50	29.00	96.75
CA	18.00	22.12	116.25
CM	16.50	29.75	144.75
CP	17.00	44.00	92.25
VA	15.00	28.00	120.75
VM	16.50	27.12	96.75
VP	18.00	25.50	140.25

The linear mixed-effects model revealed that the dorsal anterior (DA) and dorsal middle (DM) positions had significant and large positive coefficients (0.504 and 0.284, respectively), indicating that these tag positions resulted in the longest tag life, confirming the results of our initial EDA (see Table 2 for the values of the model coefficients and relative estimate, standard error, 95% confidence intervals, and t- and *P*-values of all positions). Finally, the linear mixed-effects model revealed that fish total length was not a significant predictor of tag attachment time (Table 2), which is also consistent with the results of our initial EDA.

DISCUSSION

Here we developed a new non-invasive method to temporarily tag *Dascyllus aruanus* that is easily detectable both directly by human observers and indirectly by video/photo-cameras.

Firstly, we found that tags lasted longer at specific body positions, namely on the dorsal anterior and dorsal middle locations. This could be due to the fact that fish are more likely to scrape the rounded areas of their body (i.e., central and ventral positions) against the corals while swimming within the tight spaces between the coral branches. Similarly, the motion of the caudal fin could possibly have reduced tag attachment times on the posterior relative to anterior and middle positions of the fish.

Secondly, our results suggest that some individual characteristics other than total fish length were affecting tag life given the variation observed between individual fish (Fig. 3). A possible methodological explanation is that we were more or less successful at tagging some fish compared to others, generating inter-individual variability in tag life. This is unlikely to be the case, however, given that only one investigator applied the tags (RB) and all fish were tagged within the same amount of time. Another explanation could relate to variation in behavior among individuals, such as variation in activity or social interactions that may have had an effect on tag attachment times (e.g., Wong et al., 2013).

Importantly, there were no observable negative effects of tagging on the behavior, health, and survival of the fish. Within five minutes (and always within the lifetime of the tag), the behavior of the marked individuals appeared normal (i.e., regular breathing rate and movements), suggesting that this tagging method can be used for studies that involve behavioral observations. By the end of our experiment, all fish were healthy (i.e., normal activity and regular feeding), and the majority of them possessed new scales as replacements for the ones lost with the tags. In some cases, we observed exposed skin prior to scale replacement, but scale replacement happened within three to four days. Thus, for studies in which it is necessary to temporarily tag the same individuals multiple times, we suggest waiting a reasonable amount of time before marking the fish again using this method (or alternating tag position along the fish's body).

Lastly, one of the interesting features of our new technique is its temporary nature, which may be useful for some types of studies but not others. More specifically, for short-term

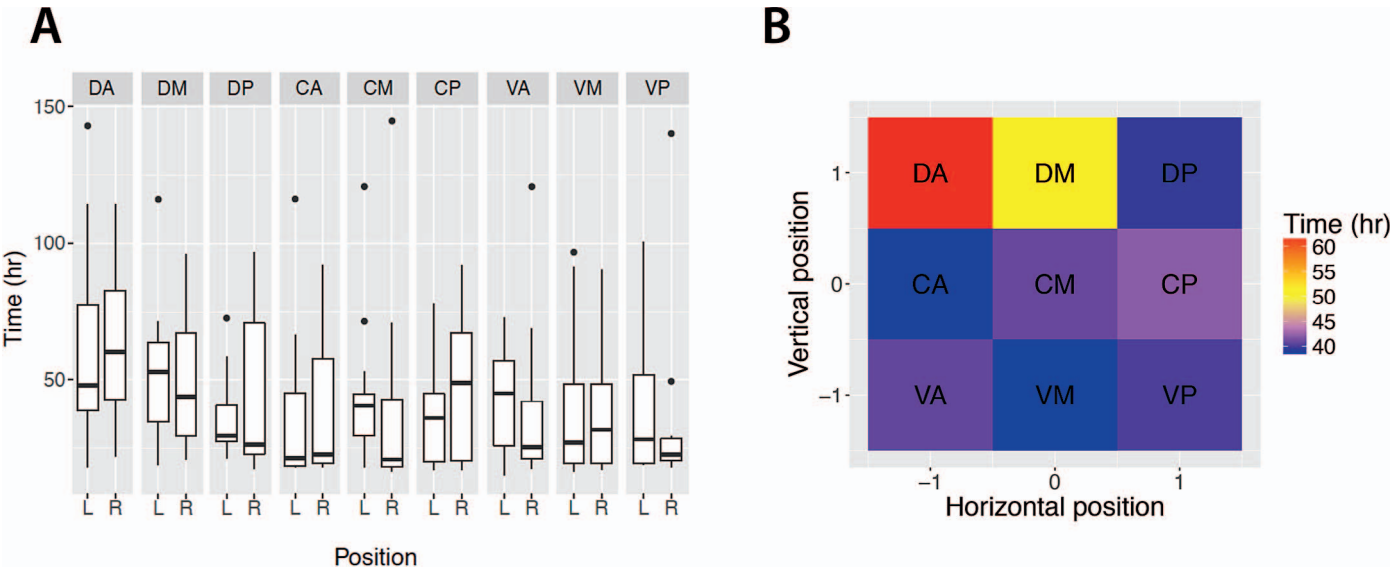

Fig. 2. (A) Comparison of attachment time of tags by position (horizontal positions—posterior, middle, and anterior; vertical positions—dorsal, central, and ventral) for left (L) and right (R) side of the fish; box plots show medians, 25th, and 75th percentiles. Black dots represent outliers. (B) Mean attachment time grid by position. Each square represents one of the nine possible tag positions with the relative mean attachment time represented by different color shades (blue-dark purple for mean attachment time between 40–45 hours; light purple-yellow for mean attachment time between 45–50 hours; yellow-orange for mean attachment time between 50–55 hours; orange-red for mean attachment time between 55–60 hours).

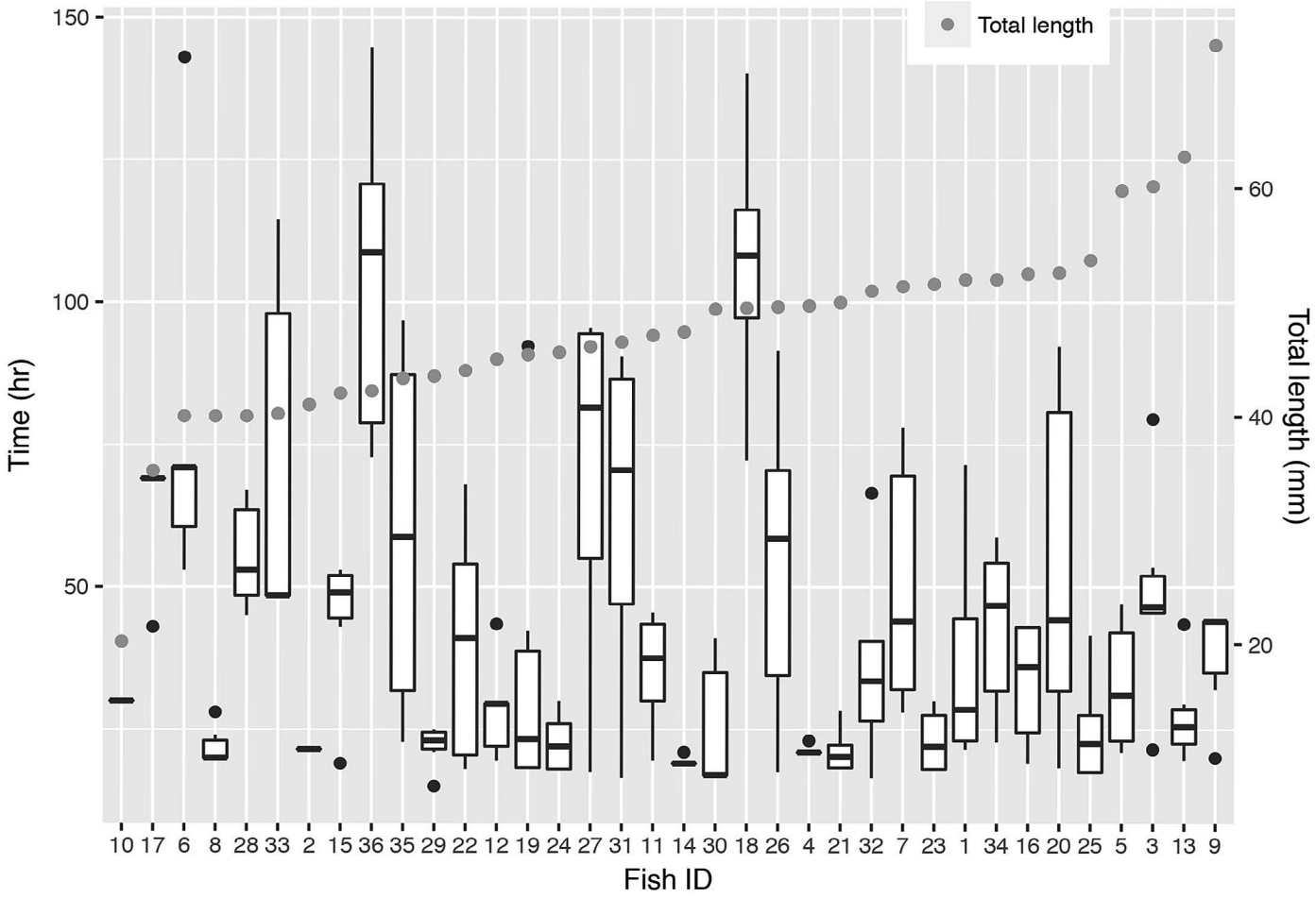

Fig. 3. Comparison of attachment time (hr) of tags by fish (Fish Identity) ordered by fish size (total length in mm). Box plots show medians, 25th, and 75th percentiles. Black dots represent outliers; gray dots represent total length of fish.

studies in which it is necessary to observe fish for just a few days, temporary markers may be preferable over permanent ones because they are more cost effective, quick to apply, and naturally wear off over time, hence minimizing the need to permanently alter animals and limiting the negative impacts from trapping and recapturing them to remove tags. Therefore, our new technique could be used widely from ethological studies (e.g., characterizing temporary internal structure of social groups) to ecological studies (e.g., identifying factors influencing movements between different areas or groups). On the other hand, for long-term studies in which the same individuals need to be observed for a prolonged amount of time (e.g., months or years), permanent markers still remain the best choice.

In conclusion, we believe that this new tagging method could be used in future behavioral to ecological studies of this species both in the field and in the lab. Indeed, we were able to use this method, both in the field and in the laboratory, to facilitate a Social Network Analysis study in this species (Branconi et al., unpubl. data). Our study demonstrates the importance of considering tag position rather than just fish body size for the placement of tags to maximize tag attachment. Future work will include the quantification of how behavioral variation between individuals might also influence tag attachment time, the examination of any long-

Table 2. Summary of the model coefficients with estimate, standard error along with their 95% confidence intervals, relative t-values, and *P*-values for each position: dorsal anterior, middle, and posterior (DA, DM, DP); central anterior, middle, and posterior (CA, CM, CP); ventral anterior, middle, and posterior (VA, VM, VP). CA is absent as it is the reference tag position.

Position	Estimate	St. error	2.5% C.I.	97.5% C.I.	t-value	*P*-value
DA	0.504	0.139	0.236	0.773	3.620	8.46e–04
DM	0.284	0.131	0.032	0.538	2.168	3.64e–02
DP	0.090	0.125	–0.150	0.330	0.725	4.73e–01
CM	0.092	0.138	–0.173	0.358	0.671	5.06e–01
CP	0.045	0.139	–0.222	0.315	0.326	7.46e–01
VA	0.060	0.139	–0.207	0.329	0.432	6.68e–01
VM	–0.005	0.126	–0.247	0.238	–0.041	9.68e–01
VP	0.064	0.132	–0.189	0.318	0.486	6.29e–01
Total length	–0.011	0.009	–0.028	0.007	–1.198	2.38e–01

term effects of tagging both in the laboratory and in the field, as well as the comparison of tag attachment times in the laboratory versus field conditions.

The technique could also have different applications in other aquatic organisms (e.g., other fishes and amphibians) in both marine and freshwater environments. We encourage future studies aiming for easy visual detection by observers or video analysis to improve this new non-invasive tagging method, using biodegradable films or other eco-friendly alternatives.

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