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ABSTRACT

We assessed mortality and tag retention for the Visible Implant Fluorescent Elastomer (VIE) tagging technique in four species of darters. Redline darters (*Etheostoma rufilineatum*) VIE-tagged in the laboratory experienced no mortalities and exhibited 100% tag retention after 125 d. A subset of these *E. rufilineatum* was released in the wild and VIE-tagged individuals were recaptured up to a year after their release with identifiable tags. Gilt (*Percina evides*), blueside (*E. jessiae*), and bluebreast darters (*E. camurum*) were also tagged with VIE in the field. Of the 1,917 darters VIE-tagged and immediately released, only 1.2 % died from the tagging procedure. Subsequent surveys revealed that recaptured *P. evides* retained VIE tags for as long as 915 d (2.5 yrs). Also, one *E. camurum* that had been VIE-tagged in 2003 was recaptured in 2007, representing a tag retention time of approximately four years (1,449 d). While tagging mortality was low and tag retention time high, there were some limitations in tag visibility and discriminating different VIE colors (e.g., green versus yellow).

INTRODUCTION

Marking small fishes for research and conservation purposes has always been problematic. There is a need to develop an effective marking method for small (< 100 mm) fishes that is inexpensive, biocompatible with the organism, permanent, and can be easily used in the field. To conduct precise studies of population dynamics and life histories, a marking method must have minimal effect on fish behavior, reproduction, life span, growth, feeding, movement, and vulnerability to predation. Physical tags are especially cumbersome for small fishes and chemical marking has evolved as a possible alternative method. Fishes have been marked using various chemicals including metallic compounds, fluorescent compounds, radioactive isotopes, latex, plastic, inks, paints, dyes, and stains (Arnold, 1966). Application techniques include immersing fishes in chemical markers, spraying or tattooing fishes, and injecting markers into fishes either with needles or through pressure (Murphy and Willis, 1996). Injection involves embedding inert materials or pigments in or under the epidermis of a fish, thereby creating an internal mark. Marking fishes with injected pigment tags is relatively inexpensive, easy, and can be considered the precursor to the visible implant tag (Murphy and Willis, 1996). Other chemical marking methods are typically used for batch tagging fishes, which does not allow for the identification of individual organisms. Pigment tagging, though, can be applied to a fish using a combination of tag locations and colors, thus creating an individually recognizable subject. Another advantage of pigment tagging is that it also allows for long-term tag retention in small fishes.

In 1991, Northwest Marine Technology, Inc. of Shaw Island, WA, developed a pigment tagging technique which used visible implant fluorescent elastomer (VIE). This two-part material consists of medical grade fluorescent silicone and a curing agent. While it has been used successfully to tag small fishes in several studies (Bonneau et al., 1995; Dewey and Zigler, 1996; Haines and Modde, 1996; Frederick, 1997; Bailey et al., 1998; Olsen and Vollestad, 2001), the utility of VIE for tagging darters (Teleostei: Percidae) has not been extensively tested. The first VIE-tagging of a darter was conducted by a team of researchers from Conservation Fisheries, Inc. (CFI) of Knoxville, TN. The CFI team used the technique beginning in 2000 while propagating, tagging, and monitoring populations of the boulder darter, *Etheostoma wapiti*, in the Elk River system, TN (Rakes and Shute, 2002). Similar fish propagation and monitoring efforts are being made in the Pigeon River in western North Carolina and eastern Tennessee. Although this river has in the past been impacted by pollution and hydrological alteration, recent improvements in water quality have led state, federal, and private agencies to attempt the reintroduction of several fish species. As an offshoot of fish propagation and monitoring efforts in the Pigeon River, we wanted to test the effectiveness of the VIE-tagging technique on darters in terms of fish survival and tag retention. Our hope was that information generated by this research would have implications for our broader studies on accurately determining the survival of reintroduced fishes.

To achieve this objective, we conducted both laboratory and field studies to assess the survival of relocated tagged fishes and tag retention using the VIE-tagging technique. For the laboratory studies, we used a common surrogate species, the redline darter (*E. rufilineatum*), to evaluate possible health impacts on these darters and VIE tag retention. For the first set of field studies, we released a subset of these laboratory-tagged *E. rufilineatum* at a site on the Little Pigeon River. This site was subsequently sampled in an attempt to recapture the tagged *E. rufilineatum* and to collect additional darter species for tagging. During this sampling event we found some of our tagged *E. rufilineatum* and collected and VIE-tagged gilt darters (*Percina evides*) and blueside darters (*Etheostoma jessiae*). For the second set of field studies, we released these tagged gilt and blueside darters into one of two Pigeon River sites along with gilt and bluebreast (*E. camurum*) darters collected from a site on the Nolichucky River. Subsequent surveys were then conducted at the Pigeon River release sites to monitor tagged darter survival, assess long-term tag retention, and to determine whether VIE tags could be recognized when marked darters were recaptured in the field.

MATERIALS AND METHODS

Laboratory Studies

The laboratory studies evaluated possible mortality in *E. rufilineatum* due to VIE-tagging and also assessed VIE tag retention over time. This surrogate species was chosen because it is common at our collection and release sites and typically co-occurs with the other three Pigeon River darters we studied. All *E. rufilineatum* used in the laboratory studies were collected on 2 October 2001 from one site on the Little Pigeon River just above its confluence with the West Prong of the Little Pigeon River (latitude 35°52'25"N, longitude 83°23'21"W). Fish were collected by kick seining using a 4.6-m seine (5-mm mesh). The darters were placed in oxygenated insulated holding tanks (coolers) containing ambient river water until ready for transport to the laboratory. Dissolved oxygen concentration and temperature were monitored continuously. Fish were placed in plastic transport bags (55 cm x 39 cm x 37 cm) containing approximately 8-12 L of river water. These bags were filled with oxygen gas and sealed for transport. Bags containing *E. rufilineatum* were placed in sealed coolers, which helped minimize transport stress (no light) and temperature change (Williams et al., 1988). They were transported to the University of Tennessee fisheries laboratory, Johnson Animal Research and Teaching Unit, where they were held overnight. Subsequently, *E. rufilineatum* were taken to CFI where they were acclimated to the new setting and then distributed among three 189-L aquaria that were part of a larger recirculating aquaria system. No mortalities occurred during the acclimation period. All laboratory studies were conducted at the CFI facility.

After four weeks of acclimation, *E. rufilineatum* were removed from the three aquaria and placed in aerated holding buckets in preparation for laboratory study. Water temperature was monitored throughout the procedure and remained between 12-13° C. Immediately prior to tagging, three to four fish were placed in a small aquarium with 100 mg of anesthetic (MS-222) in 1.0 L of ambient water. Once anesthetized, each fish was removed from the aquarium and tagged with VIE using a 0.3-ml insulin syringe with an ultra-fine 29-gauge needle. These same methods were used successfully by CFI to tag and reintroduce endangered *E. wapiti* in the Elk River, TN (Rakes and Shute, 2002).

The tagging material, VIE, is a bio-compatible silicone that when injected as a liquid cures to a pliable solid. VIE comes in five fluorescent colors: red, green, yellow, orange, and pink. These colors are easily seen in ordinary light but in reduced light conditions, a blue LED (light emitting diode) light can be used to enhance tag visibility. Because the curing time of VIE is temperature dependent, it may take 24 hours for the tag to cure if water temperature during tagging < 10°C (Northwest Marine Technology, Inc.).

Initial observations revealed that needle technique was very critical to the visibility and retention of the mark. The technique involved the needle piercing the skin of the fish, bevel up, and then being inserted 5-6 mm just beneath the skin (Figure 1). The goal was to place the VIE between the skin and the muscle layer to maximize tag visibility through the skin layer. Ideally, each tag was 3-5 mm in length. VIE was injected as the needle was withdrawn, filling the cavity made by the needle. Injection was halted just prior to removal of the needle and any excess VIE exuding from the entry wound was wiped off to minimize tag loss after curing.

Ninety *E. rufilineatum* (36 - 83 mm TL) were double-tagged, meaning VIE was injected on each side of the second dorsal fin just adjacent to the midline and parallel to the dorsal fin. This double-tagging approach was done to make it easier for surveyors to identify a tagged fish upon recapture. Each of three groups of 30 *E. rufilineatum* was marked with either red, green, or yellow VIE. After tagging, fish were placed in an aerated recovery tank. Upon recovery, 10 *E. rufilineatum* of each color were placed in one of three aquaria for a total of 30 fish per aquarium.

Fish were observed at 24- and 48-hours post-tagging to assess mortality. No fish were handled in the first 14-d post-tagging to minimize stress, allow the VIE to cure, and ensure that the tissue injection site had time to heal. For observations conducted at 14, 30, 60, 90, and 125 d post-tagging, *E. rufilineatum* were netted from the aquaria and observed individually for general health and tag retention. The parameters used to determine the health of the fish included clarity of eyes, condition of the fins, and activity level. These same parameters were used for all subsequent observations. VIE tags were initially checked for

visibility with the naked eye on day 14. All subsequent observations were checked for tag visibility with the eye, and if not readily visible, a LED blue light flashlight and amber glasses were used to locate the tag. A color reference block of latex containing samples of all VIE colors was provided by CFI for the 90-d and final observations. When used in conjunction with the blue light and amber lenses, tag color identification became much more reliable.

Field Methods

For the first set of field studies, 60 of the 90 VIE-tagged *E. rufilineatum* from the laboratory studies were released at the Little Pigeon River collection site on 15 March 2002. Three sets of fish with different color VIE tags were released: 20 red, 20 green, and 20 yellow. A total of 16 seine samples each covering an area 91.4 m^2 per effort was conducted 7 d later at the release site and in areas just upstream and downstream in an attempt to recapture tagged *E. rufilineatum*. All captured darters were observed in an attempt to detect VIE tags.

For the second set of field studies, we collected individuals of *P. evides*, *E. jessiae*, and *E. camurum* from two sites: the Little Pigeon River from below the Highway 66 Bridge to just upstream of its confluence with the West Prong of the Little Pigeon River in Blount County, near Sevierville (latitude 35°52'24"N, longitude 83°34'20"W) near the *E. rufilineatum* collection site and the Nolichucky River (at river mile 28) just downstream of Hale Bridge at Bewley Island in Greene County (latitude 36°05'58"N, longitude 83°03'17"W). All collections and releases were made between 14 March 2001 and 13 March 2003. After tagging, all darters were released at a reintroduction site on the Pigeon River located at Tannery Island, Cocke County (latitude 35°56'39"N, longitude 83°10'44"W) or, for later reintroductions of *E. jessiae*, at McSween Memorial Bridge in Newport, TN (latitude 35°56'39"N, longitude 83°10'44"W). All four sites are within Tennessee.

To avoid heat stress on the fishes, collection and tagging operations were conducted during cooler, non-summer months. Collection methods followed those for the laboratory-tagged *E. rufilineatum.* On warm days, the temperature of the MS-222 solution was maintained by placing ice in sealed plastic bags which were floated in the holding container. Temperature and dissolved oxygen were monitored in all fish containers. We used five different VIE colors (red, green, yellow, orange, and pink) in each batch tagging effort so that river source, timing of release (spring or fall), and year of collection could be discerned based on tag color.

Monitoring for the tagged reintroduced species began 1 October 2001 and was accomplished, in part, by qualitative and quantitative underwater visual surveys. Other occurrence data were obtained from the annual Index of Biotic Integrity survey (IBI) conducted at the Tannery Island reintroduction site by a multi-agency effort by Tennessee Department of Environment and Conservation, Tennessee Wildlife Resources Agency, and the Tennessee Valley Authority. A qualitative survey consisted of a snorkel surveyor following an arbitrary zig-zag pattern while moving in a downstream direction. The quantitative snorkel survey was conducted on 26 July 2002 and involved using the strip transect method of Watson et al. (1995). The survey consisted of five parallel line transects at the Tannery Island site and covered a downstream distance of 100 m. The width of each transect lane was set at 1.20 m because, during previous qualitative surveys (1 October 2001, 10 June 2002, and 1 July 2002) at the release site, it was determined that neither the tagging site nor tag color of a tagged darter could be confirmed at a distance > 0.60 m in full sunlight. This recognition distance was typically > 0.45 m in the shade.

RESULTS

Laboratory Results

There were no observed mortalities or abnormal behaviors of VIE-tagged fishes 24 - 48 h after tagging and there were no mortalities after 125 d. All fishes exhibited 100% tag retention through the end of the 125 d study period. Although fish health was good and behavior appeared normal on subsequent checks, tag visibility varied. Thirty days after tagging, VIE tags on darkly pigmented *E. rufilineatum* were difficult to see, especially those consisting of green and yellow VIE. While these two VIE colors appeared to be the same under the blue light, the use of amber glasses removed the blue tint of the blue LED light, allowing the true color to be recognized. The combination of using the blue light in conjunction with the amber glasses helped differentiate between larger green and yellow tags but did not help in identifying smaller yellow and green tags. Smaller VIE tags were the result of inexperienced tagging personnel who did not inject uniform amounts of VIE. The ideal VIE tag was a 3 – 5 mm stripe; however, some of the smaller tags appeared as blotches or pinpoints. At 60 d post-tagging, more tags became visible only with the blue light while the problem of differentiating between green and yellow tags also remained.

Tag visibility was greatly affected by the depth under the darter's skin at which the tag was placed. It was noted that inexperienced personnel were more likely to inject the VIE too deeply into the tissue instead of just under the skin. This reduced tag visibility. Tag visibility also appeared to be affected by fish coloration and tag color, in addition to the experience level of the tagger and the observer, though an overall pattern of tag loss could not be detected from these observations. The number of tags visible to the naked eye and the number visible with the blue light varied among observations. The yellow VIE was the least visible of the three tag colors used throughout the study. However, on the final observation (125 d post-tagging), 8 of 30 yellow tags and 1 of 30 red tags were not

visible to the naked eye, but all were visible under the blue LED light. At 125 d, all 30 green tags were visible to the naked eye.

Field Results

On 22 March 2002 (7 d after being released), 5 of 60 tagged *E. rufilineatum* were collected at or around the Little Pigeon River release site: 1 red tag, 3 green tags, and 1 yellow tag (Table 1). For all 16 seine samples taken, only one tagged fish was caught in any one effort. All VIE-tagged *E. rufilineatum* were collected downstream of the release site. Tagged *E. rufilineatum* were also caught incidentally during subsequent collections for other target species: one fish was caught on 21 May 2002 (red tag), three fish were caught on 28 May 2002 (red tags), and three more fish were caught on 23 October 2002 (2 green tags, 1 yellow; Table 1). In all recaptured *E. rufilineatum*, the VIE tags were visible without artificial illumination. All recaptured *E. rufilineatum* had been originally tagged for the laboratory studies on 30 October 2001, indicating a possible tag retention time in the field of approximately one year.

Of the 1,867 darters VIE-tagged in the field and released (939 *P. evides*, 619 *E. jessiae*, and 309 *E. camurum*), only 24 (14 *P. evides*, 9 *E. jessiae*, and 1 *E. camurum*) died before being released. This represents a tagging mortality rate of 1.2 %. During the quantitative snorkel survey conducted on 26 July 2002 at the Tannery Island release site, 173 *P. evides* (19 red, 22 green, 11 yellow, and 78 orange tags) and 2 *E. camurum* (yellow tags) were recaptured and had visible VIE tags (Table 1). No VIE-tagged *E. jessiae* were recaptured. Based on these results, we determined that the Tannery Island release site had only marginal habitat for *E. jessiae*. Therefore, later reintroductions of *E. jessiae* were conducted at the McSween Memorial Bridge release site.

The multi-agency annual IBI survey conducted at the same release site on 10-11 July 2002, yielded five tagged *P. evides* (1 red, 1 green, and 3 orange tags) at the Tannery Island site, which was the same area of the reintroductions. Three VIE-tagged *P. evides* (1 red and 2 orange tags) were also collected during this survey in the riffle area above the reintroduction site. The qualitative snorkel surveys conducted at riffle areas upstream and downstream of Tannery Island (in an attempt to locate darters which may have moved from the reintroduction site) also produced VIE-tagged darters. For example, 21 tagged *P. evides* (5 red, 1 green, and 15 orange tags) and 1 tagged *E. camurum* (yellow tag) were identified on 13 August 2002 at the riffle above Tannery Island. Further qualitative snorkel surveys and IBI surveys conducted from 2003 - 2007 yielded more observations of tagged *P. evides* in 2003 and 2004 (Table 1). Recaptured *P. evides* were shown to retain VIE tags for as long as 915 d (2.5 yrs). Also, as recently as 2007, one *E. camurum* was collected at Tannery Island that had been tagged in 2003. This represents a retention time of approximately four years (1,449 d).

DISCUSSION

We have shown through both laboratory and field studies that VIE-tagging can be used successfully on darter species being reintroduced into natural habitats. While tagging mortality was low (0 - 1.2 %) and tag retention time high (up to four years), there were some limitations in tag visibility and discriminating different VIE colors (e.g., yellow versus green). Our studies suggest that low mortality can be added to the advantages that VIE-tagging has over other methods for marking small fishes. Not only are the VIE tags externally visible, they are entirely internal and biocompatible with the fish's tissue. After the injection site wound has healed, there are no long-term openings in the tissue that may lead to infection as with external tags. This small internal tag is less vulnerable to environmental damage and is less likely to alter fish behavior compared with typical marking methods (Hale and Gray, 1998). Other benefits are that the expense of extracting and reading of other types of tags is eliminated and the VIE tags can be identified without sacrificing the fish. Also VIE-tagged fishes can be identified without handling or removing the fish from its environment which will also improve survivorship. The tag mortality we observed in the field studies was most likely a result of multiple environmental stressors. Stress results when fish experience fright, discomfort, or pain (Schreck, 1981). Loss of mucus or scales, breaks in the skin, or damage to internal organs can lead to shock, increased susceptibility to infection, suppressed immune system, and delayed mortality (Schreck, 1981). The fishes tagged for the field studies were trapped in a net, carried in small buckets, confined in a cooler, dropped into an anesthetizing bath, and stuck with a needle. Some were confined in an unfamiliar environment for as many as 6 d. Also, six of the darters that died were in peak spawning condition and were likely already in duress from reproductive activities.

Our results indicate that another advantage of VIE-tagging is successful long-term tag retention. In our laboratory studies, VIE tags in *E. rufilineatum* were retained for 125 d with no mortalities. Even more impressive are those VIE-tagged fishes we recollected 2 to 4 years after tagging. Although these field tag retention times are somewhat anecdotal, they have value for the broader study to determine the survival of transplanted darters. Having tags that are retained over 2 to 4 years is especially helpful considering that the reported life span for some of the darter species we used is not much greater than this (Etnier and Starnes, 1993). It is also interesting to note that the number of recaptures did not appear to be biased toward any one color of VIE tag or the timing of the release (Table 1). We also demonstrated one basic benefit of such tagged reintroductions in that we could roughly assess initial reintroduction success. Based on the lack of *E. jessiae* recaptures at the Tannery Island release site, we were able to take action against making more introductions of *E. jessiae* in marginal habitats and continue reintroductions else-

where. Future attempts at darter reintroduction can use VIE-tagging and expect to accurately assess whether released individuals survived in the new habitat.

The two disadvantages of VIE-tagging we experienced were reduced tag visibility and discriminating between yellow and green tags. In some cases differentiation was difficult without using the blue LED light and amber glasses which may limit the usefulness of the technique in field settings. Tag visibility was affected most by two factors: subcutaneous depth of the injected VIE, and darter skin pigmentation at the injection site. With practice and experience, though, these problems can be avoided. Inexperienced taggers affected tag visibility in other studies (Frederick, 1997; Bailey et al., 1998; Close and Jones, 2002). For example, Kelly (1967) stated that the placement of the tagging material at the proper intra-cutaneous level is likely the most critical factor in subsequent recognition of tags. He further suggested that the needle insertion may be judged as being at the correct depth if the needle shows as a dark line under the skin. Based on the difficulty of differentiating between yellow and green tags in this study, it is not recommended to use both colors in a study using a single species. Tagging with red, orange, and pink VIE in a single species with the same tag location may also pose a similar problem. Tag location and VIE color should be assessed on a species-specific basis. Before using VIE in a research study requiring marked fish, investigators should be trained and experienced in tagging fish to assure maximum tag retention and visibility.

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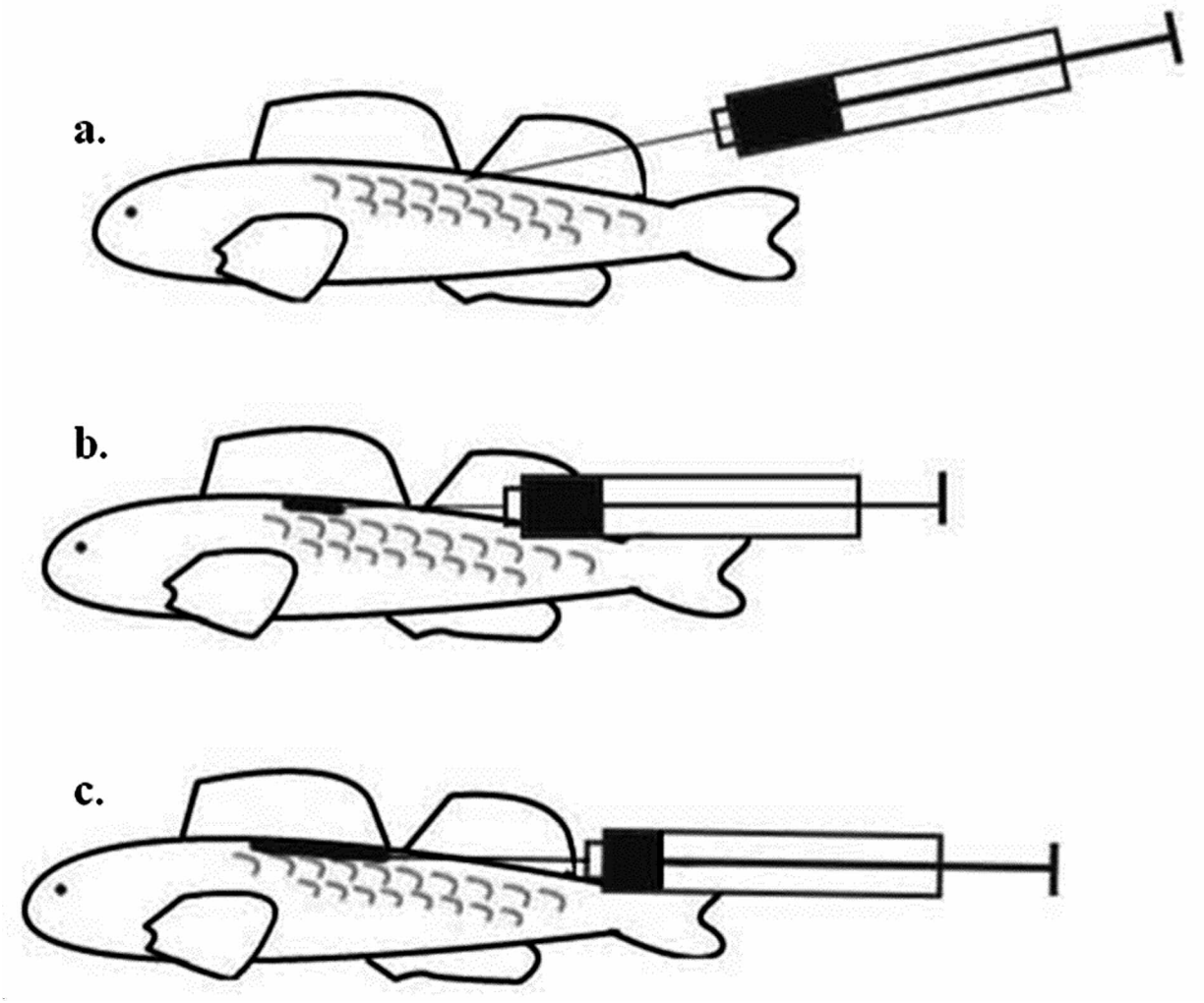

FIGURE 1. Schematic of VIE tagging technique as used on darters. A hypodermic needle is inserted 5-6 mm beneath the skin (a) with the intent of injecting VIE between the skin and the muscle. VIE is injected as the needle is withdrawn (b) filling the cavity created by the needle. Injection of VIE is halted just prior to removal of the needle (c) and any excess VIE exuding from the injection point is wiped away to minimize tag loss after curing.

TABLE 1. Tagging data for VIE-tagged darters, including maximum tag retention time for different VIE tag colors based on recapture data. Laboratory-tagged *E. rufilineatum* were tagged and held in the laboratory for a 125 d observation period after which they were held an extra 301 d prior to being released in the river on 15 March 2002. The other three species (*P. evides*, *E. camurum*, and *E. jessiae*) were VIE-tagged in the field and released immediately into the natural habitats on the indicated date. Collection and release site localities are: Little Pigeon River just upstream of its confluence with the West Prong of the Little Pigeon River (LPR), Little River at Coulters Bridge (LRCB), Pigeon River at Tannery Island (TI), Nolichucky River just downstream of Hale Bridge at Bewley Island (NOL), and Pigeon River at McSween Memorial Bridge in Newport (MMB).

Species	Number Initially Tagged	Collection Site	Tag Color	Tag Date	Release Site	Recapture Dates (number of tagged darters)	Maximum Tag Retention Time (days)
E. rufilineatum (laboratory-tagged)	20	LPR	red	30 Oct 2001	LPR	22 March 2002 (1); 21 May 2002 (1); 28 May 2002 (3)	206
	20	LPR	green	30 Oct 2001	LPR	22 March 2002 (3); 23 Oct 2002 (2)	474
	20	LPR	yellow	30 Oct 2001	LPR	22 March 2002 (1); 23 Oct 2002 (1)	474
P. evides	120	LPR	red	23 May 2001	TI	10-11 July 2002 (2); 26 July 2002 (19);	497
	61	LPR	red	13 Mar 2003	TI	13 Aug 2002 (5); 2 Oct 2002 (1)	
	132	LPR	green	2 Oct 2001	TI	10-11 July 2002 (1); 26 July 2002 (22); 13 Aug 2002 (1); 24 Jun 2003 (1)	630
	52	NOL	yellow	6 Nov 2001	TI	26 July 2002 (11); 2 Oct 2003 (1)	696
	41	NOL	yellow	9 Oct 2001	TI		
	51	LPR	orange	8 Feb 2002	TI	10-11 July 2002 (5); 26 July 2002 (78);	915
	43	LPR	orange	15 Feb 2002	TI	13 Aug 2002 (15); 9 Aug 2004 (1)	
	157	LPR	orange	21 May 2002	TI		
	136	LPR	orange	28 May 2002	TI		
	28	NOL	pink	28 Aug 2002	TI	2 Oct 2003 (4)	401
	126	LPR	pink	23 Oct 2002	TI		
E. camurum	121	NOL	yellow	9 Oct 2001	TI	13 Aug 2002 (1)	308
	122	NOL	yellow	6 Nov 2001	TI		
	86	NOL	pink	28 Aug 2002	TI	no recaptures	-
	5	LRCB	red	21 Jul 2003	TI	12 Jul 2007 (1)	1,449
E. jessiae	128	LPR	red	14 Mar 2001	TI	no recaptures	
	1	LPR	red	23 May 2001	TI		
	4	LPR	green	2 Oct 2001	TI	no recaptures	-
	6	NOL	yellow	6 Nov 2001	TI	no recaptures	-
	113	LPR	orange	8 Feb 2002	MMB	no recaptures	-
	107	LPR	orange	15 Feb 2002	MMB		
	145	LPR	orange	15 Mar 2002	MMB		
	115	LPR	red	13 Mar 2003	MMB		