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Analysis of persistent marking techniques using passive integrated transponders and visible im-
plant elastomer through metamorphosis in *Ambystoma mavortium*

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Thesis Advisor: Name: Dennis Ferraro Signature: _____

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Abstract

Salamander species are a key component of monitoring ecosystem integrity and health as indicator species. Being able to monitor growth and development from larval to adult forms can provide important descriptive data such as growth, development, and survivorship. Passive integrated transponders (PIT) and visible implant elastomer (VIE) are two methods of permanent tagging through metamorphosis in salamander species. We were interested in what the retention rate of tags using PIT versus VIE through metamorphosis in Ambystoma mavortium. We hypothesized intramuscular PIT tags would be most successful compared to sub-cutaneous PIT tags and VIE injection. In our experiment, PIT tags retention was significantly greater than VIE retention. PIT tag injection resulted in less stress on the individual and held no statistically significant impact on growth (SVL and weight). We recommend using intramuscular PIT tags as a marking technique through metamorphosis in Ambystoma mavortium. This tagging method provides a high retention probability, but is limited by sample size and funding. Large scale experiments with greater sample size may choose to avoid costly PIT tags.

Introduction

Similar to canaries in coal mines, amphibians play an important role as indicator species in aquatic ecosystems due to their high susceptibility to environmental changes such as water quality degradation, human construction acting as barriers, habitat fragmentation, or climatic alterations (Polich et al, 2013; Wake, 2009; Ghioca & Smith, 2008; Ashpole et al, 2011). By monitoring the health and stability of amphibian populations researchers are better able to evaluate the quality of the environment and its conditions. Current data suggests that the Barred Tiger salamander populations are in decline in 25% of eastern Nebraska (Ferraro, unpublished). The most likely cause of this population decline would be either habitat loss and fragmentation or pollution.

The comparison of data collected from recaptured individuals allows researchers and managers to understand population dynamics and health by examining survivorship, growth, development or habitat use and preference. In order to collect data for these comparisons researchers must employ tagging procedures that are reliable, long lasting, easily recognized and cause minimal stress to the specimen. Finding tagging procedures that persists through the physiological changes salamanders undergo during metamorphosis from an aquatic larval to terrestrial adult form (including gill loss, muscle development, and tail shape alteration) is critically important for advancing the understanding of conservation needs. Previous methods of marking salamanders have included pattern mapping, toe-clipping, tattooing, branding, tail-clipping, passive integrated transponders (PIT tags) or visible implant elastomer (VIE) (Davis & Ovaska, 2001; Arntzen et al, 2004; Ireland, 1973; Polich et al, 2013; Seale & Boraas, 1974). Tagging larval amphibians over adults may have advantages such as reduced materials and expenses (Ghioca & Smith, 2007). Larval amphibians have a greater plasticity to their environment (Newman, 1992) and have the greatest amount of factors that influence their development. Life history studies allow better management decisions for both amphibian species and biodiversity dependent on them.

In order to examine the feasibility and retention rates of passive integrated transponders versus visible implant elastomer, we compared growth and retention through metamorphosis in barred tiger salamanders (*Ambystoma mavortium*) with each tag.

Study species

Ambystoma mavortium are a native salamander to the state of Nebraska. This species can range throughout Arizona, California, Colorado, Idaho, Kansas, Minnesota, Montana, North Dakota, Nebraska, New Mexico, Nevada, Oklahoma, Oregon, South Dakota, Texas, Utah, Washington, and Wyoming. Despite its wide range, little information is known about specific populations. . *Ambystoma* salamanders are amongst the largest salamanders in the northern hemisphere, up to eight inches snout-vent length (Petranka, 1998), allowing the most variability in tagging methods in regards to tag size and bodily location for easy identification. Mark recapture studies often require distinctive tagging methods to collect population data. Unlike many other species of invertebrates, the movement patterns and behaviors of ambystomatid salamanders are poorly documented. Emerging only briefly at aquatic breeding sites, barred tiger salamanders spend the majority of their time buried underground (Madison & Ferrand, 1998).

Classification of subspecies can be different throughout the US. The subspecies examined here may be defined as *Ambystoma mavortium mavortium*. The family of *Ambystoma* salamanders can have adaptive polymorphisms including cannibalistic morphs of Tiger Salamander (*Ambystoma tigrinum*) (Wake, 2009; Larson et al., 1999) a subspecies very closely related to the barred tiger salamander. Small populations of barred tiger salamanders may remain as paedotypic specimens, and even small populations are classified as cannibal morphs. Paedomorphic salamanders, in which the adult animal remains fully aquatic, can be found in the life history of tiger salamander species (Fitzpatrick & Shaffer, 2004). Cannibal morphs were first described in *A. mavortium* and are known to occur regularly in the species (Larson et al., 1999). Relying on a tagging method that is persistent in the aquatic paedotypic specimen is imperative to monitoring their drawn out metamorphosis period and in order to monitor larvae which may present the cannibal morph.

Due to a complex breeding ritual, researchers have been unable to reproduce *A. mavortium* in a lab setting, thus emphasizing the need for a permanent marking technique to gather information about reproduction. This species is hatched into an aquatic larval stage that may persist for a single season before metamorphose into a terrestrial adult specimen when triggered by environmental stimuli (Dempster, 1930). Larvae may take several months before metamorphosing, after which they disperse overland (Petranka, 1998). The larval form is entirely aquatic with external gills and a large dorsal fin from the base of the head to the tail that will dissolve as metamorphosis is undertaken (Figure 1). Most salamanders have a short (few weeks to months) aquatic larval stage and a long (several years) terrestrial adult stage life span (Wake, 2009). While ambystomatidae salamanders typically have rather active larval stage specimens, post-metamorphic animals are much more secretive and choose to spend much of their time buried in the landscape (Polich et al., 2013). This makes tagging at the aquatic level much easier in a field setting, with large numbers of larvae available in the same location. Amphibian larvae are the most plastic in terms of feedback systems, so tagging at this stage may provide the best data in monitoring populations.

Methods

Marking Methods

There are three main types of identification marking as defined by Kinkead et al. (2006): generic marking – animal has been previously captured and little information is available upon recapture; bath or cohort marks – assigns a group mark based upon time and location of capture; and individual marks – exact capture history of each animal and the most information is availa-

ble upon recapture. Investigators should be considerate of life stage of interest when determining marking techniques (Regester & Woosley, 2005).

Although toe-clipping has been classified as a humane method of marking (Kinkead et al, 2006), it may affect the ability of an individual to take full advantage of optimal foraging conditions in the wild and recapture rates are significantly lower than VIE or PIT marked individuals (Davis & Ovaska, 2001). Salamanders may regenerate lost toes completely within one year and often up to seven months after removal (Heatwole, 1961) and long term studies may mean that individuals may be subjected to multiple toe-clippings in life. Toe-clipping marking procedures in amphibians can often lead to ambiguous results as these specimens are able to regenerate lost digits and/or may commonly lose digits in the wild (Davis & Ovaska, 2001; Ott & Scott, 1999; Kinkead et al 2006).

Passive integrated transponders and visible implant elastomer types of marking procedures are being pursued as an alternative to toe-clipping and color mapping techniques. Passive integrated transponders (PIT tags) are microchips that are injected under the skin of an animal. Each tag contains a unique identification number that can be scanned and recorded to allow individual data collection. This individual data is used to obtain information on growth rates, age of sexual maturity, frequency of reproduction, survivorship, life-history parameters and other aspects of behavioral ecology (Davis & Ovaska, 2001). PIT tags are commonly used in a range of organisms, from fish to large reptiles for marking purposes. Radio-implant tags have been used successfully on larger caudate species with little effect on adult survival and behavior (Madison & Farrand, 1998), but have not been tested through the metamorphose process. However, due to the unique physiological changes that salamanders undergo and their thin, delicate skin, larger PIT tags have a higher likelihood of ejection. Recently a new size of PIT tag has been developed which is 8.4mm in length (Figure 2). This smaller tag is used in the research procedure. We predict that by using the smallest available size of PIT tag and injecting the tag so that it rests beneath the musculature of the dorsal posterior limb region, ejection of the tag will not occur.

Visible implant elastomer (VIE) is a two part mixture of fluorescent dye and hardening agent that when mixed creates a solid but flexible mass. The mixture can be injected beneath the skin of an organism and is visible through the skin under blue light with amber glasses, ultraviolet light, or deep-violet (405nm) light, but can also be seen with the bare eye (Figure 3). VIE may be best suited to small sized animals as each tag requires very little elastomer to be visible (Binkley et al., n.d.) and large marks are more likely to be lost or migrate within the body cavity than small marks (Grant, 2008). VIE has been used to identify large egg clusters with easy identification, but marks do not adhere to individuals (Regester & Woosley, 2005). VIE does require immediate tagging procedures to be undertaken as the mixture hardens quickly and once solidifies can be impossible to inject or risk injury to the animal being marked. However VIE is recognized as relatively non-invasive and long lasting (Davis & Ovaska, 2001) and a low level of mortality is observed when using the elastomer (Monti, 2002).

Using reliable tagging procedures may aid researchers in accurately tracking this species. Unlike most other reptiles or amphibians, the thin and semi-permeable skin of salamanders will occasionally eject the passive integrated transponders through the skin after implantation. It is hoped that by placing the tag in the subcutaneous region of the limb in the larvae, it will be unable to be rejected and will simply be accepted into the body of the salamander, allowing for long-term identification of the individual. Salamanders are capable of digital regeneration and have a fast acting caudal nervous system, allowing skin healing to take place over the span of a few hours (Polich et al, 2013). Visible implant elastomer is to be injected in the subdermal space in

the central cranial region. We predict that this location will provide the clearest location for visibility and will reduce the chance of mark migration (Grant, 2008).

Experimental Procedure

Forty-five specimens of *Ambystoma mavortium* were obtained from a bait supplier in Minnesota. All specimens of *A. mavortium* used in trials were rated as at least stage 46 according to Caudata development stages. We ran three trials where specimens spent one week in the intermediate indoor aquatic tank before being transferred to the outdoor trial tanks. The trial tank was divided into four equal holding arenas roughly 15-20 gallons each. Two salamanders were placed in each subdivision. The tank had a mechanical filter in place to provide aeration and waste disposal. All salamanders were fed 2-3 worms every 2-3 days. Once a week approximately 5 gallons of water were removed per holding area to lower water level and promote development. When salamanders exhibited total gill loss, they were transferred to a dry land tank (~50% sand, ~50% dirt, large buried water dish).

Control

Once received in lab, eight salamanders were separated and placed in a holding aquatic tank while remaining salamanders were placed in large filtered water tanks in a walk-in cooler (~10°C) to delay development. Eight salamanders were used to establish a baseline for development. In subsequent trials, two salamanders were left untagged to ensure a proper baseline for each trial. Two salamanders were injected with visible implant elastomer (VIE), two salamanders were injected with 8.4 mm passive integrated transponder (PIT) tags subcutaneously and the remaining two were injected with PIT tags intramuscularly. Tagging procedures for VIE and PIT tags are as follows:

Visible Implant Elastomer

In trial two, a small amount (~0.01cc) of curing agent was dispensed into an injection syringe. Coloring agent (~0.1cc) was added into the injection syringe and mixed thoroughly for exactly one minute. After mixing the tags hardened within minutes, so they had to be placed quickly to avoid hardening. The syringe, needle, and injection site on the salamanders were rinsed with E-pure water as a disinfectant. Salamanders were first weighed and SVL measured before being tagged. Salamanders were injected with the VIE directly under the skin of the center dorsal cranial region. The specimens received 2-3 injections in different areas within the vicinity to ensure dye would be highly visible. After tagging, salamanders were quickly returned to their aquatic tank.

Due to complications with hardening time and mixing of VIE components in Trial 2, an injection syringe with a removable needle was used in place for Trial 3. Needle removal prevented curing agent from accumulating in the needle tip and creating an incompletely mixed VIE. No other component of the VIE procedure was changed.

Passive Integrated Transponder

Passive integrated transponder (PIT) tags were used in two conditions per trial. Salamanders were measured and weighed before injection. E-pure water was used to sanitize equipment and injection site of salamander. A Biomark 8.4mm PIT tag was loaded into an injection syringe and injected directly into the region between spine and limb on the right posterior side of the specimen. We used two different placements: one under the skin but above the muscle in the

fatty tissue and the second under the muscle in the fatty tissue. PIT tags were scanned and recorded for each specimen before being returned to the aquatic tank after injection.

Results

For the four conditions, initial average weight and SVL values were roughly equivalent with the exception of VIE specimens who had a slightly greater initial mass. Mean final weights for the four conditions were roughly equivalent. Sub-cutaneous PIT tags displayed the longest mean days to metamorphosis (17.25 days), but intramuscular PIT tags were similar (17 days). Control specimens displayed the shortest mean number of days to complete metamorphosis, but were in the middle ground for mean final weight (Table 1).

Of the four individuals injected with sub-cutaneous PIT tags, all retained the tag. Of the four salamanders injected with an intramuscular PIT tag, one individual ejected the tag due to improper insertion. VIE injections were unsuccessful in all but one case as the elastomer was ejected from the skin fully within two to three days of injection (Table 2). Success rate of tagging methods by percentage was 100% for PIT sub-cutaneous, 84% for PIT intramuscular, 16% for VIE (Figure 4).

Suitability of tag was analyzed using an average difference of mass calculation (Figure 5). ANOVA analysis (p -value = 0.2281) determined a failure to reject the null hypothesis (H_0 = Control = PIT Sub-cutaneous = PIT Intramuscular = VIE) (Table 3).

Discussion

The results from this experiment demonstrate PIT tags are the most efficient method of mark and recapture tagging techniques in *A. mavortium*. Previous studies have shown toe and tail clipping to be ineffective in recapture scenarios due to regrowth of digits. VIE is also the most costly option for tagging specimens. PIT tags individually can cost between \$3-\$7 while a VIE kit for two colors can cost roughly \$250. These price constraints limit the amount of specimens which can be marked. Cost should always be considered when planning methodology for short versus long-term studies. These marking techniques would most likely be more useful in longer term studies when the ratio of cost to pertinent data collection would be highest while other methods, such as toe-clipping, could be utilized for short-term studies (i.e. less than one year).

VIE has been used successfully in fish and anurans, yet due to the difficult nature of injecting this tag in this experiment, we do not recommend VIE as a viable method of tagging for larval caudata Chase et al. (2015) had little difficulty injecting VIE into *A. maculatum* yet discussed difficulty correctly identifying specimens based on pigmentation of skin, fragmentation or migration of tag. In comparison PIT tags allow consistent and complete individual identification of a recaptured specimen.

While sample size in this experiment was low leading to a possible increase in Type II error, these results suggest VIE is not a viable method of permanent marking analysis for *A. mavortium*. The thin and semi-permeable nature of salamander skin may have caused the VIE ejection, but VIE may also not have been successful in this experiment due to methodology – difficulty in mixing elastomer properly and injecting it deep enough beneath the dermis in order to be retained.

I would recommend further research examine marking protocols utilizing intramuscular PIT tag injection and various methods of VIE injection. Although the success rate for sub-cutaneous PIT tag injection was higher than intramuscular, the error associated with intramuscu-

lar stemmed from improper injection, not from the specimen ejecting the tag. Combined with lack of significant impact on growth, intramuscular PIT tags provide the most security in knowing tag has not migrated or been injected while avoiding negative influence on the specimen. Further research should consider the implications of larger tag sizes on smaller salamander species, such as *Plethodontidae*. This experiment should be replicated with larger sample sizes to determine the most suitable placement for PIT tag injection – such as the impact of injection behind pectoral versus pelvic girdle or within the tail. Further study using VIE should include its associated risk of higher predation by strong color-vision predators (i.e. birds) when tag is visible.

Lack of impairment on growth is important for life-development stages in amphibians. Tagging methods should aim to prevent risk of increased predation or lowered survivorship. Between the two tagging methods, growth and development of all specimens were within normal parameters for each tagging method. Overlapping confidence intervals in average difference of mass analysis suggest tagging method had little to no impact on growth in this study.

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Figures and Tables

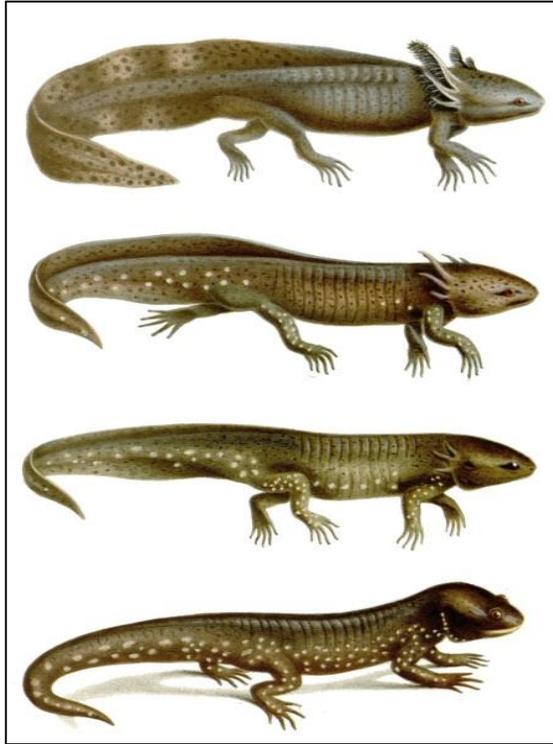


Figure 1: Major stages of development from larval to adult form in *Ambystoma mavortium*.

Photo courtesy of Josephe Huet.



Figure 2: 12mm and 8mm PIT tag comparison



Figure 3: Visible implant elastomer in anuran; Brannelly et al., 2014

Table 1: Values for comparison of three tagging methods

Tagging method	Mean Initial weight (g)	Mean Initial snout-vent length (cm)	Mean Final weight (g)	Mean Final snout-vent length (cm)	Mean Days to complete metamorphose
Control	78.75	10.81	90.0	11.38	15.6
VIE	92	12.625	96.5	12.625	15.75
PIT sub-cutaneous	66.25	11.5	84.5	11.75	17.25
PIT intramuscular	75.5	11.375	86	11.625	17

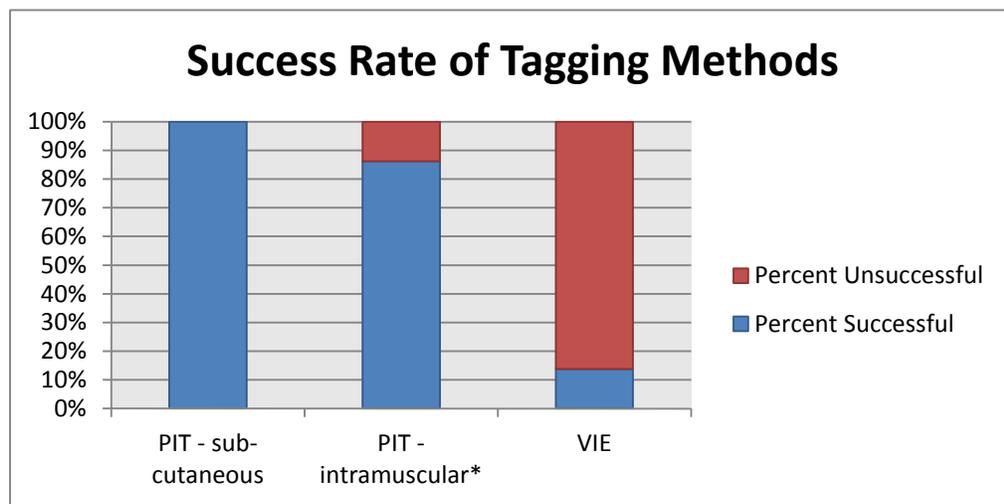


Figure 4: Graphical illustration for success rate of tested tagging methods.

Table 2: Determination of mark retention and description of time elapsed until tag was rejected per salamander per trial per condition.

Trial	Individual	Condition	Injection Successful	Mark Retained	Days Tag Retained
2	1	VIE	Yes	No	3
	2		Yes	No	2
	3	PIT –	Yes	Yes	180+
	4	Sub-cutaneous	Yes	Yes	180+
	5	PIT -	Yes	Yes	180+
	6	Intramuscular	Yes	Yes	180+
3	1	VIE	Yes	No	2
	2		Yes	Yes	180+
	3	PIT –	Yes	Yes	180+
	4	Sub-cutaneous	Yes	Yes	180+
	5	PIT –	Yes	Yes	180+
	6	Intramuscular	No	No	2

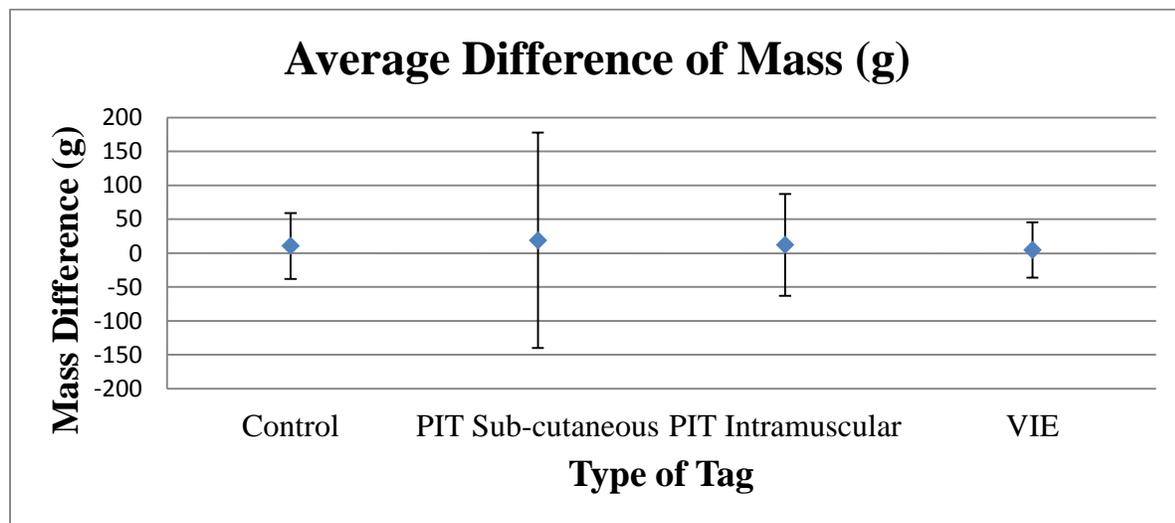


Figure 5: Graphical variation with confidence interval in average difference of mass from initial to final for each tagging method.

Table 3a: ANOVA analysis of average weight difference between initial and final result for the experiment

Groups	Count	Sum	Average	Variance	Standard Deviation	Standard Error	Confidence Interval
Control	4	42	10.5	49.67	7.05	24.83	48.67
PIT Sub-cutaneous	4	75	18.75	162.25	12.74	81.13	159.01
PIT Intra-muscular	4	48	12	76.67	8.76	38.33	75.13
VIE	4	18	4.5	41.67	6.45	20.83	40.83

Table 3b: ANOVA Single Factor Summary

Source of Variation	SS	df	MS	F	P-value	Fcrit
Between Groups	411.1875	3	137.06	1.66	0.2281	3.49
Within Groups	990.75	12	82.56			
Total	1401.94	15				