ELEMENTAL COMPOSITION OF ENAMEL IN THE TEETH OF ADULT MARBLED SALAMANDERS, *AMBYSTOMA OPACUM*, FROM MIDDLE TENNESSEE

By

Hunter Morrow

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of
Master of Science in Biology

Middle Tennessee State University
May 2018

Thesis Committee:

Chair Dr. Brian T. Miller

Dr. Matthew Klukowski

Dr. Dennis Mullen

ACKNOWLEDGEMENTS

I would like to thank Dr. Brian Miller (Department of Biology, Middle Tennessee State University) for allowing me to use his lab and specimens, as well as his guidance. I would also like to thank Joyce Miller (Supervisor of the Interdisciplinary microanalysis and imaging Center, Middle Tennessee State University) for assistance with both SEM and EDS.

ABSTRACT

Metals are deposited in the enamel of developing teeth in many groups of small vertebrates, including amphibians. For example, iron is a common constituent of enamel in many species of small vertebrates and often causes teeth to appear various shades of red. Although iron deposits in the teeth of small mammals have received considerable attention, several different lineages of salamanders, including ambystomatids, also sequester iron in the enamel of their teeth during amelogenesis. Furthermore, iron is present in the first set of teeth that develop in embryonic salamanders, before the individual is free-living or feeds. Because salamanders are polyphyodont, the amount of metals in their enamel presumably represents the amount accumulated since the previous set of teeth were replaced. Thus, the accumulation of a metal in the enamel of salamanders presumably is associated with the level of metals in the surrounding environment at the time when the most recent set of teeth was being formed. However, too little information is available to adequately address questions about metals, including iron, present in the teeth of salamanders. Here I examined elemental composition of teeth from a population of Marbled Salamanders (*Ambystoma opacum*). Specifically, I examined the types of metals sequestered in the enamel, and distribution and percentage of iron in the teeth of adults. Salamanders used in this study were collected as trap casualties from pits associated with a drift fence constructed at Arnold Air Force Base in Coffee County, Tennessee. The dead salamanders were fixed with 10% buffered formalin, transferred to 70% ETOH, and deposited in the Middle Tennessee State

University Herpetology Collection. To perform elemental analysis, I removed the lower jaw from 10 individuals and separated the left and right halves of the lower jaw at the mental symphysis, removed soft tissue, and mounted each half to an aluminum stub using double stick tape. Furthermore, I used a dental pick to remove the crowns of three teeth from each specimen, and I mounted the excised crowns onto the same stub as the jaw from which they were removed. I used an Oxford INCA Energy 200 Dispersive analyzer synced to a Hitachi S-3400N scanning electron microscope to analyze the extent of Fe, and other trace elements, deposition by ameloblasts throughout the lingual cusp and shaft of the crown during formation of the enamel layer. To ensure that I was analyzing the same amount of area and penetrating the same depth of the enamel in each analysis, I used a magnification of 1,400X and an accelerating voltage of 15 kV for all readings. I analyzed six sites on each tooth, from the apex of the lingual cusp down to the base of the crown. Iron concentration was highest in the apex of the lingual cusp and diminished in a longitudinal fashion towards the base of the crown. In addition to Fe; Al, Cl, Cu, F, K, Mg, Na, S, Si, and W were also present in the enamel, but the mean weight percentage of each element varied among the sites indicating that their incorporation into the enamel was more haphazard than structural.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

Odontogenesis, formation of a fully mature tooth from the tooth germ, is different in the larval and metamorphosed stages of the urodele (salamander) life cycle (Smith and Miles, 1971; Wistuba et al., 2002). The teeth of larval urodeles are monocuspid and non-pedicellate; whereas, the teeth of metamorphosed urodeles consist of a pedicel that connects with the jaw or palate, a bicuspid crown (with labial and lingual cusps), and a fibrous zone of division that connects the pedicel and crown (Beneski and Larson, 1989; Gregory et al., 2016). The teeth of larval urodeles are composed of an enameloid layer and a thin enamel cap; whereas, the teeth of postmetamorphic urodeles have a thick enamel cap but lack an enameloid layer (Davit-Béal et al., 2007a, b). The enameloid matrix is composed of a loose collagenous network that is derived from mesenchymal cells and deposited by odontoblasts; whereas, true enamel is composed of proteins derived from epithelial cells and deposited by ameloblasts (Davit-Béal et al., 2007b; Kogaya, 1999). Furthermore, as enameloid matures, ameloblasts actively harden the collagenous network causing the enameloid to be similar in strength to enamel (Davit-Béal et al., 2007b). Other elements, such as iron (Fe), magnesium (Mg), and fluorine (F), are found in these mineralized tooth layers (Anderson and Miller, 2011; Smith, 1998).

Several different lineages of urodeles, including ambystomatids, sequester Fe in the enamel of their teeth (Anderson and Miller, 2011; Sato et al., 1992, 1993; Shimada et al., 1993). Furthermore, Fe is present in the first set of teeth that develop in the embryo (Anderson and Miller, 2011; Smith and Miles, 1971). Urodeles are polyphyodont (Graver, 1973) and have many sets of teeth during their life, and Fe and other metals in

the environment are potentially ingested in food and are incorporated into enamel of urodele teeth (Kara, 1994; Sato et al., 1992), similar to that described for shrews and voles (Dumont et al., 2014; Appleton et al., 2000; Gdula-Argasińska et al., 2004).

The amount of metals, such as Fe, in the teeth of small mammals represents accumulation during their formation. In rodents, such as Bank Voles, incisors grow continuously during their lives and can give an accurate representation of amounts of metals in the environment at the time a section is formed (Appleton et al., 2000; Dumont et al., 2014; Gdula-Argasińska et al., 2004). In contrast, the amount of metals in the teeth of urodeles presumably represents only that amount accumulated since the previous set of teeth were replaced. Possibly, Fe is sequestered in crowns to stabilize distal apatite crystals and prevent demineralization of the enamel (Sato et al., 1991, 1992; Shimada et al., 1993), strengthen the apical surface of the crown as occurs in rodents (Wen and Paine, 2013), or eliminate excessive Fe via tooth loss (Suga, 1984; Anderson and Miller, 2011).

Here, I use energy dispersive X-ray spectroscopy (EDS), coupled with a scanning electron microscope, to examine elemental composition of teeth in adult Marbled Salamanders, *Ambystoma opacum*, from Arnold Air Force Base (AAFB) in Coffee County, Tennessee, USA. The primary purposes of this study are to determine (1) the distribution of Fe in the enamel, (2) percentage of Fe make-up of the enamel, and (3) which, if any, other elements are sequestered into the enamel. I chose to study this population of salamanders because of the potential for environmental contamination at the site, which could increase the likelihood of metal sequestration in the enamel. For example, AAFB has a history of water contamination, and since 1987 catfish in Woods

Reservoir have shown high levels of PCBs, and more recent studies have shown that there is a metal contamination of surface and groundwater (Agency for Toxic Substances and Disease Registry, 2000; Haugh and Mahoney, 1994). Thus, accumulation of a metal in enamel of *A. opacum* presumably is associated with the level of metals in the surrounding environment at the time when the most recent set of teeth was being formed.

CHAPTER II: METHODS

A series of Marbled Salamanders (*Ambystoma opacum*) were collected on 25 September 1997 at a small pond located at the northern end of Arnold Air Force Base (AAFB) in Coffee County, Tennessee. The urodeles were collected as trap casualties from pits associated with a drift fence that encircled the pond. The dead salamanders were fixed with 10% buffered formalin, transferred to 70% ETOH, and deposited in the Middle Tennessee State University Herpetology Collection.

Specimen Preparation — I removed the lower jaw from 10 *A. opacum* and separated the left and right halves of the lower jaw at the mental symphysis. I used a scalpel to remove most of the soft tissue from the jaws and then placed each half into a 95% ETOH solution to dry the remaining soft tissue. I used double stick tape to mount each half of the jaw to an aluminum stub. I mounted the two halves at different angles to better visualize all regions of the teeth in an individual specimen. Furthermore, I used forceps, a dental pick, and an eyelash brush to remove the crowns of three teeth from each specimen, and I mounted the excised crowns onto the same stub as the jaw from which they were removed.

Energy Dispersive X-Ray Spectroscopy (EDS) — I used an Oxford INCA Energy 200 Dispersive analyzer synced to a Hitachi S-3400N scanning electron microscope to analyze the elemental composition of enamel. I used an accelerating voltage of 15 kV on each specimen to ensure that the depth of penetration into the enamel was similar in all teeth. Furthermore, to ensure that I was analyzing the same amount of area in each analysis, I used a magnification of 1,400X on all readings. I used a point analysis in EDS

(Greven, 2010) to determine the elemental composition at six sites along the crown. To examine potential regional variation, I took three spectroscopy readings from the surface of the lingual cusp: the apical region of the cusp (ac), the medial region of the cusp (mc), and the basal region of the cusp (bc). Below the cusp, I analyzed three regions of the surface of the shaft: the apical region of the shaft (as), the medial region of the shaft (ms), and the basal region of the shaft (bs) (Figure 1). I analyzed three teeth from each of the ten specimens for a total of 180 readings.

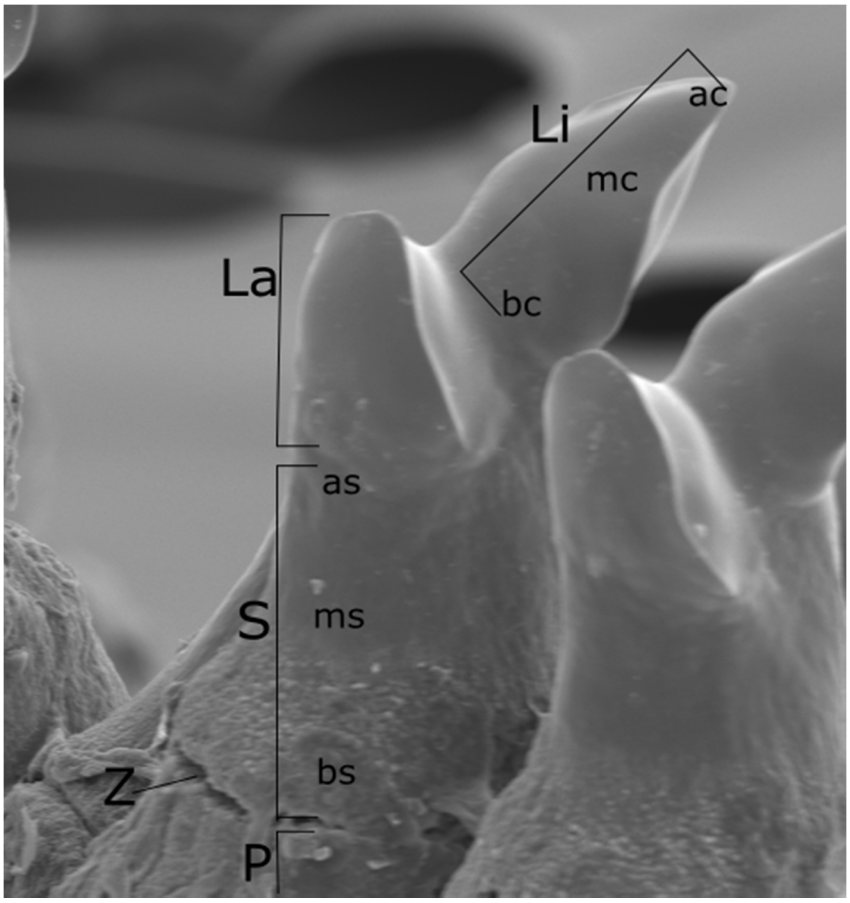

Figure 1. SEM micrograph of a tooth from the lower jaw of a Marbled Salamander, *Ambystoma opacum*. The tooth is composed of a basal pedicel (P) and distal crown (S, La, and Li), which is composed of a Lingual (Li) cusp that projects towards the tongue and a labial (La) cusp that projects towards the lip. An unmineralized zone of division (Z) separates the pedicel from the crown. The Shaft (S) supports the cusps and links the crown to the zone of division. I used energy dispersive X-ray spectroscopy to determine elemental composition of the tooth surface at six locations on each tooth analyzed: apical (ac), medial (mc), and basal (bc) sites of the lingual cusp, and apical (as), medial (ms), and basal (bs) sites of the shaft.

I used a Kruskal-Wallis rank sum test to test for differences in Fe weight percentage among the six areas tested on each crown. Following Anderson and Miller (2011), I used Dunn's post hoc test to determine if Fe concentration differed among the basal, medial, and apical portions of the lingual cusp and shaft of the crown. I calculated mean weight percentage for all elements at each of the six sites analyzed. I excluded calcium (Ca) and phosphorus (P) from the statistical analyses because of their overwhelming presence in the EDS readings.

CHAPTER III: RESULTS

Elemental composition of the enamel was similar among all teeth analyzed, both within and among individuals. Calcium and phosphorous are major components of the enamel layer (Clemen et al., 1980) and, therefore, were omitted from the statistical analyses to better determine the concentrations of other elements. Iron was the third most abundant element within the enamel and was present in each tooth analyzed, but it was unequally distributed throughout the crown. For example, Fe was detected in 92% of the sites of the lingual cusp analyzed (83 of 90 sites; three per tooth, per specimen), but was less frequently detected in the shaft (31% of sites; 28 of 90) (Table 1). Furthermore, the concentration of Fe was highest in the apex of the lingual cusp (7.95 ± 0.75 weight %), and concentrations diminished from the tip of the cusp towards the zone of division, with the lowest concentrations in the medial (0.20 ± 0.19 weight %) and basal (0.05 ± 0.04 weight %) sites of the crown shaft (Table 2).

Table 1. Summary of energy dispersive X-ray spectroscopy (EDS) analyses in the enamel of the teeth from the lower jaw of the Marbled Salamander, *Ambystoma opacum* from middle Tennessee.

Tooth Portion	Number of Analyses	Iron Present	Iron Absent
Apex of Cusp	30	30	0
Medial of Cusp	30	28	2
Basal of Cusp	30	25	5
Apex of Shaft	30	22	8
Medial of Shaft	30	2	28
Basal of Shaft	30	4	26
Total	180	111	69

Table 2. Weight percentage of Fe found in the enamel of teeth from the lower jaw of the Marbled Salamander, *Ambystoma opacum*, using energy dispersive X-ray spectroscopy.

Tooth section	Iron proportion		
	Mean ± SE	Median	Variance
Cusp			
Apical	7.95 ± 0.75	7.86	16.81
Medial	2.38 ± 0.56	1.31	9.40
Basal	1.70 ± 0.52	0.96	8.11
Shaft			
Apical	0.64 ± 0.31	0.04	2.87
Medial	0.20 ± 0.19	0	1.14
Basal	0.05 ± 0.04	0	0.05

Although Fe was the most abundant element (omitting Ca and P), aluminum (Al), chlorine (Cl), copper (Cu), fluorine (F), magnesium (Mg), potassium (K), silicon (Si), sodium (Na), sulfur (S), and tungsten (W) were occasionally present, but in varying

concentrations (Table 3). The concentration of Al, K, and Na did not vary among regions of the crown analyzed; however, concentrations of Cl, F, Mg, and S were greater in the shaft than in the cusps. Furthermore, Cu, Si, and W were found primarily in the cusp, but at relatively low concentrations (Table 3).

Table 3. Mean weight percentages of trace elements found in the enamel of teeth from the lower jaw of the Marbled Salamander, *Ambystoma opacum*, using energy dispersive X-ray spectroscopy. Values given are mean ± S.E.

	Cusp			**Shaft**		
Element	**Apical**	**Medial**	**Basal**	**Apical**	**Medial**	**Basal**
Al	0.03 ± 0.02	0.17 ± 0.14	0.30 ± 0.21	0.16 ± 0.05	0.26 ± 0.12	0.17 ± 0.04
Cl	0.02 ± 0.01	0.07 ± 0.02	0.14 ± 0.06	0.20 ± 0.07	0.31 ± 0.13	0.25 ± 0.18
Cu	0.00 ± 0.00	0.02 ± 0.02	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
F	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.08	0.19 ± 0.10
Mg	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.01	0.24 ± 0.04	0.27 ± 0.04	0.22 ± 0.03
K	0.05 ± 0.01	0.11 ± 0.02	0.13 ± 0.03	0.08 ± 0.02	0.02 ± 0.01	0.16 ± 0.13
Si	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Na	0.64 ± 0.04	1.01 ± 0.09	0.80 ± 0.08	0.79 ± 0.09	0.79 ± 0.10	0.63 ± 0.08
S	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.03 ± 0.02	0.07 ± 0.01	0.09 ± 0.03
W	0.03 ± 0.03	0.02 ± 0.02	0.04 ± 0.03	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00

Based on the Kruskal-Wallis analyses, the mean ranks of Fe weight percentage differed among the six sites analyzed ($n = 30$ readings per site; $H = 119.83$, $df = 5$, $p < 0.001$). I used Dunn's post hoc test to determine differences between the sites analyzed by pooling the 30 readings from each of the six sites. Although there is a reduction of Fe from the apex of the cusp to the basal site of the shaft, some sites did not show a statistically significant difference when the mean weight percentages between the different EDS reading sites were analyzed using Dunn's post hoc test. Using the abbreviations given in figure 1 and moving in the proximal direction; the concentration of ac is greater than mc through bs, mc is greater than as through bs, bc is greater than ms and bs, and as is greater than ms and bs (Table 4). The comparisons of the medial sites of the cusp vs basal sites of the cusp, the medial sites of the shaft vs basal sites of the shaft, and the basal site of the cusp vs apical site of the shaft showed no significant different in mean weight percentage of Fe ($p > 0.05$) (Table 4).

Table 4. Comparison of Fe mean weight percentages of energy dispersive X-ray spectroscopy analysis sites in the enamel of the teeth from the lower jaw of the Marbled Salamander, *Ambystoma opacum* ($\alpha = 0.05$).

Tooth portion comparison	**$p < 0.05$**
Apex of cusp vs medial of shaft	Yes
Apex of cusp vs basal of cusp	Yes
Medial of cusp vs basal of cusp	No
Apex of cusp vs apex of shaft	Yes
Medial of cusp vs apex of shaft	Yes
Basal of cusp vs apex of shaft	No
Apex of cusp vs medial of shaft	Yes
Medial of cusp vs medial of shaft	Yes
Basal of cusp vs medial of shaft	Yes
Apex of shaft vs medial of shaft	Yes
Apex of cusp vs basal of shaft	Yes
Medial of cusp vs basal of shaft	Yes
Basal of cusp vs basal of shaft	Yes
Apex of shaft vs basal of shaft	Yes
Medial of shaft vs basal of shaft	No

CHAPTER IV: DISCUSSION

The morphology of teeth in premetamorphic (larval) and postmetamorphic urodeles has been described for many species (Anderson and Miller, 2011; Beneski and Larsen, 1989; Greven, 1989; Pederson, 1991; Sato et al., 1992, 1993; Shimada et al., 1993; Wistuba et al., 2002). The teeth of free-living larval urodeles invariably are non-pedicellate and monocuspid; whereas, the teeth of postmetamorphic urodeles typically are pedicellate and bicuspid (Beneski and Larsen, 1989; Greven, 1989; Sato et al., 1992, 1993; Shimada et al., 1993; Wistuba et al., 2002). Although the general morphology is similar among postmetamorphic urodeles, subtle differences exist, both within (Pederson, 1991) and among species (Beneski and Larsen, 1989; Miller and Larsen, 1989; Gregory et al., 2016). A growing body of evidence suggests that the basic chemical organization of teeth is similar among urodeles (Sato et al., 1992, 1993; Shimada et al., 1993), although too little information is currently available to determine if subtle differences exist within or among species.

Calcium and phosphorous are the dominant elements involved in the chemical composition of teeth (Clemen et al., 1980), but various other elements, including Fe, are present in lower concentrations in the highly-mineralized enamel and enameloid tooth layers (Smith, 1998). The enameloid matrix covers the dentine shaft of teeth of larval salamanders, and a thin layer of true enamel covers the enameloid layer (Anderson and Miller, 2011; Wistuba et al., 2002; Smith and Miles, 1971). In some species, larval urodeles deposit Fe in their teeth, including the first set of teeth to erupt after hatching (Anderson and Miller, 2011; Smith and Miles, 1971). Larval urodeles typically use a

gape-and-suck feeding mechanism to capture small prey, including zooplankton (Deban and Wake, 2000; Smith and Petranka, 1987). The usefulness of strengthening teeth with Fe or other metals in larvae that use a suction feeding mechanism to capture small prey is questionable, prompting Anderson and Miller (2011) to suggest that Fe in the enameloid and enamel layers potentially serves a purpose other than strengthening the teeth to aide in grasping struggling prey.

Dentine and enamel surround the pulp cavity in the crown of the teeth of adult urodeles (Davit-Béal et al., 2007a, b; Smith, 1998; Wistuba et al., 2002). The enameloid layer present in larval teeth is lost and a thickening of the dentine and enamel layers takes place (Davit-Béal et al., 2007a, b). Metamorphosed urodeles feed on a variety of invertebrate prey (Jaeger, 1979; Jaeger et al., 1981). In contrast to larval urodeles, an Fe-rich enamel layer in postmetamorphic urodeles could be beneficial in strengthening the enamel to allow penetration of the chitinous exoskeleton of the intended prey (small invertebrates). Strengthening of the enamel with Fe to facilitating grasping prey has been suggested for other vertebrates, including the Butterfly Fish (Motta, 1987). However, Fe may also function to stabilize hydroxyapatite crystals arranged aprismatically in the enamel (Koyaga, 1999). If so, then Fe deposition is important for the process of amelogenesis (Sato et al., 1991, 1992; Shimada et al., 1993). Smith (1998) stated that, at least in humans, amelogenesis has a secretion and a maturation phase with the secretion phase consisting of a fluid-filled matrix with protein fibers that slowly gets replaced as hydroxyapatite crystals grow and fill the spaces during the maturation phase. During the maturation phase, Fe replaces some of the calcium located in the apatite crystal lattice as

ferritin being collected within ameloblasts and secreted at the time of enamel formation (Smith, 1998).

Although relatively little information on the distribution and abundance of Fe in teeth of urodeles is available, particularly compared to the availability of similar information for mammals (Appleton et al., 2000; Brudevold and Söremark, 1967; Dumont et al., 2014; Gdula-Argasińska et al., 2004; Wen and Paine, 2013), some generalizations can be made. Shimada et al. (1993) and Wistuba et al. (2002) report that the concentration of Fe is greater in the lingual cusp than in the labial cusp of teeth of the Japanese Giant Salamander (*Andrias japonicus*). Furthermore, they report that the enamel layer of the lingual cusp is thicker than that of the labial cusp, and they suggest that the thicker enamel is associated with the greater concentration of Fe. My data on Fe concentration supports this suggestion. In *Ambystoma opacum,* the concentration of Fe decreases in correlation to the thickness of the enamel layer. In areas where the enamel is thickest, such as near the tips of the cusps (Smith and Miles, 1971), Fe is found in higher levels than near the pedicel where enamel is thinnest (Shimada et al., 1993; Wistuba et al., 2002).

How Fe is accumulated for sequestration into the enamel is poorly understood. As a new tooth is being formed in urodeles, the existing tooth that is being replaced is broken down and resorbed (Davit-Béal et al., 2007a); furthermore, the pedicel, and part of the crown, are attacked by osteoclasts when the tip of the newly formed tooth touches the existing tooth. I am unaware of any studies indicating that the enamel cap of the crown is broken down and resorbed in this fashion, but resorption would allow for reuse of minerals and organic matrix in later tooth formation (Davit-Béal et al., 2007a). The

skin of *A. opacum* is permeable, which allows water and some ions to diffuse from a moist environment into the body (Whitford and Hutchison, 1963; Stiffler, 1988); however, studies indicating Fe absorption from the environment through the skin are lacking. Brudevold and Söremark (1967) suggest that ingested trace elements are used in place of similar sized and charged elements commonly found in the hydroxyapatite crystals. These trace elements, at least in humans, can diffuse into enamel and incorporate into new complexes as Ca and P dissociate.

Trace elements are not uncommon in the enamel of urodeles (Davit-Béal et al., 2007; Sato et al., 1992, 1993; Smith and Miles, 1971; Wistuba et al., 2002). Sato et al. (1993) found that the elemental composition of enamel is influenced by the amount of trace elements at the time of tooth formation; some trace elements, such as F, Fe, and Mg (Sato et al., 1993; Shimada et al., 1993), are present in many of the EDS, and electron microprobe studies performed on urodele tooth enamel. This may be associated with greater background levels of these elements in the environment, or more involvement in enamel formation. The elemental composition of, and the sequestration of trace elements in, enamel differs from species to species, but individuals within species should show similar compositions (Sato et al., 1992; Wistuba et al., 2002). This presumably is associated with inherent differences in sequestration between species or differences in the quantity of trace elements present in the environment.

In addition to Fe, other elements are occasionally incorporated into the enamel of teeth of *A. opacum*, including Al, Cl, Cu, F, K, Mg, Na, S, Si, and W. Aluminum (Kang et al., 2004; Koletsi-Kounari et al., 2012; Shashikiran et al., 2007), Cl (Dykes and Elliot, 1971; Kang et al., 2004), Cu (Shashikiran et al., 2007), F (Kang et al., 2004; Randall,

1966; Sato et al., 1993; Shashikiran et al., 2007; Shimada, et al., 1993), K (Kang et al., 2004; Shashikiran et al., 2007), Mg (Cuy et al., 2002; Kang et al., 2004; Shashikiran et al., 2007), Na (Kang et al., 2004), and Si (Shashikiran et al., 2007) have been reported as constituents of enamel in previous studies using either EDS and electron microprobe analyses. Thus, the typical elemental constituents of the apatite crystals can be replaced by various elements during tooth formation (Humphrey et al., 2008). Aluminum (Koletsi-Kounari et al., 2012), Cu (Brookes et al., 2003), and F (Koletsi-Kounari et al., 2012), at least in humans, show cariostatic properties that inhibit tooth decay through the formation of enamel that is more acid resistant. Nanci and Smith (1992) describe considerable uptake of sulfate in enamel during the secretory phase of amelogenesis. Tungsten has been found in the enamel and dentin of human teeth with an increase in concentration in areas with higher natural background levels (Iyengar et al., 1978; Nanci and Smith, 1992; Syracuse Research Corporation, 2005). Higher concentrations have also been found in soldiers with wounds from shrapnel that contains W (Iyengar et al., 1978).

Although W is known to be found in small arms ammunition (Selstrum, 2005) and shrapnel (Iyengar et al., 1978), I was unable to find any evidence of W contamination at AAFB. Furthermore, I interpret the low concentration of W in tooth enamel of *A. opacum* to indicate that it is not an important component of enamel. Every trace element found in enamel of the teeth of *A. opacum*, apart from W and Cu, is present in the list of inorganic substances found in the surface water at AAFB (Haugh and Mahoney, 1994). Although present, some elements were found in much lower concentrations that others. The low concentrations may indicate lower background levels of these elements in the

environment available for sequestration into the enamel, a reduced role in enamel formation compared to elements found in higher concentrations, or a method to rid the unwanted elements from the body. I am uncertain why some elements were sequestered into the enamel of some of the teeth in *A. opacum* but not sequestered in others. An EDS study of Marbled Salamanders teeth from other regions, with different background levels of elements, would be beneficial for better understanding sequestration of elements into the enamel.

LITERATURE CITED

Agency for Toxic Substances and Disease Registry. 2000. Public Health Assessment for Arnold Engineering Development Center. Available via the Internet (http://www.atsdr.cdc.gov/HAC/pha/PHA.asp?docid=1348&pg=0)

Anderson, M. A., and B. T. Miller. 2011. Early iron deposition in teeth of the Streamside Salamander, *Ambystoma barbouri*. Journal of Herpetology 45:336-338.

Appleton, J., K. M. Lee, K. S. Kapusta, M. Damek, and M. Cooke. 2000. The heavy metal content of the teeth of the bank vole (*Clethrionomys glareolus*) as an exposure marker of environmental pollution in Poland. Environmental Pollution 110:441-449.

Beneski, J. T., and J. H. Larsen. 1989. Interspecific, ontogenetic, and life history variation in the tooth morphology of mole salamanders (Amphibia, Urodela, and Ambystomatidae). Journal of Morphology 199:53-69.

Brookes, S. J., R. C. Shore, C. Robinson, S. R. Wood, and J. Kirkham. 2003. Copper ions inhibit the demineralisation of human enamel. Archives of oral biology 48:25-30.

Brudevold, F., and R. Soremark. 1967. Chemistry of the mineral phase of enamel. Structural and Chemical Organization of Teeth 2:247-277.

Clemen, G. M., H. Greven, and B. Schroder. 1980. Ca and P concentrations of a urodele tooth as revealed by electron microprobe analysis. Anatomischer Anzeiger 148:422-427.

Cuy, J. L., A. B. Mann, K. J. Livi, M. F. Teaford, and T. P. Weihs. 2002. Nanoindentation mapping of the mechanical properties of human molar tooth enamel. Archives of Oral Biology 47:281-291.

Davit-Béal, T., H. Chisaka, S. Delgado, and J. Y. Sire. 2007a. Amphibian teeth: current knowledge, unanswered questions, and some directions for future research. Biological Reviews 82:49-81.

Davit-Béal, T., F. Allizard, and J. Y. Sire. 2007b. Enameloid/enamel transition through successive tooth replacements in *Pleurodeles waltl* (Lissamphibia, Caudata). Cell and Tissue Research 328:167-183.

Deban, S. M., and D. B. Wake. 2000. Aquatic feeding in salamanders. In K. Schwenk, Feeding: Form, Function and Evolution in Tetrapod Vertebrates pp. 65–94. Academic Press, San Diego, CA.

Dumont, M., T. Tütken, A. Kostka, M. J. Duarte, and S. Borodin. 2014. Structural and functional characterization of enamel pigmentation in shrews. Journal of Structural Biology186:38-48.

Dykes, E., and J. C. Elliott. 1971. The occurrence of chloride ions in the apatite lattice of Holly Springs hydroxyapatite and dental enamel. Calcified Tissue Research 7:241-248.

Gdula-Argasińska, J., J. Appleton, K. Sawicka-Kapusta, and B. Spence. 2004. Further investigation of the heavy metal content of the teeth of the Bank Vole as an exposure indicator of environmental pollution in Poland. Environmental Pollution 131:71-79.

Graver, H.T. 1973. The polarity of the dental lamina in the regenerating salamander jaw. Development 30:635-646.

Gregory, A. L., B. R. Sears, J. A. Wooten, C. D. Camp, A. Falk, K. O'Quin, and T. K. Pauley. 2016. Evolution of dentition in salamanders: relative roles of phylogeny and diet. Biological Journal of the Linnean Society 119:960-973.

Greven, H. 1989. Teeth of extant Amphibia: morphology and some implications. Fortschritte der Zoologie 35:451-455.

Greven, H. 2010. A microscopic and microanalytical study (Fe, Ca) of the teeth of the larval juvenile *Ambystoma mexicanum* (Amphibia: Urodela: Ambystomatidae). Vertebrate Zoology 60:27-35.

Haugh, C. J., and E. N. Mahoney. 1994. Hydrogeology and simulation of ground-water flow at Arnold Air Force Base, Coffee and Franklin counties, Tennessee (No. 93-4207). US Geological Survey; Earth Science Information Center Open-File Reports Section.

Humphrey, L. T., M. C. Dean, T. E. Jeffries, and M. Penn. 2008. Unlocking evidence of early diet from tooth enamel. Proceedings of the National Academy of Sciences 105:6834-6839.

Iyengar, G. V., W. E. Kollmer, and H. J. M. Bowen. 1978. The elemental composition of human tissues and body fluids: a compilation of values for adults. Weinheim, NY: Verlag Chemie.

Jaeger, R. G. 1979. Fluctuations in prey availability and food limitation for a terrestrial salamander. Oecologia 44:335-341.

Jaeger, R. G., R. G. Joseph, and D. E. Barnard. 1981. Foraging tactics of a terrestrial salamander: sustained yield in territories. Animal Behaviour 29:1100-1105.

Kang, D., D. Amarasiriwardena, and A. H. Goodman. 2004. Application of laser ablation–inductively coupled plasma-mass spectrometry (LA–ICP–MS) to investigate trace metal spatial distributions in human tooth enamel and dentine growth layers and pulp. Analytical and Bioanalytical Chemistry 378:1608-1615.

Kara, T. C. 1994. Ageing in amphibians. Gerontology 40:161-173.

Kogaya, Y. 1999. Immunohistochemical localisation of amelogenin-like proteins and type I collagen and histochemical demonstration of sulphated glycoconjugates in developing enameloid and enamel matrices of the larval urodele (*Triturus pyrrhogaster*) teeth. Journal of Anatomy 195:455-464.

Koletsi-Kounari, H., E. Mamai-Homata, and I. Diamanti. 2012. An in vitro study of the effect of aluminum and the combined effect of strontium, aluminum, and fluoride elements on early enamel carious lesions. Biological Trace Element Research 147:418-427.

Miller, B. T., and J. H. Larsen Jr. 1989. Feeding performance in aquatic postmetamorphic newts (Urodela: Salamandridae): are bidirectional flow systems necessarily inefficient? Canadian Journal of Zoology 67:2414-2421.

Motta, P. J. 1987. A quantitative analysis of ferric iron in Butterfly Fish teeth (Chaetodontidae, Perciformes) and the relationship to feeding ecology. Canadian Journal of Zoology 65:106-112.

Nanci, A., and C. E. Smith. 1992. Development and calcification of enamel. In Ermanno Bonucci, Calcification in Biological Systems pp. 313-343. CRC Press, Boca Raton, FL.

Pedersen, S. C. 1991. Dental morphology of the cannibal morph in the Tiger Salamander, *Ambystoma tigrinum*. Amphibia-Reptilia, 12:1-14.

Randall, M. 1966. Electron microscopical demonstration of ferritin in the dental epithelial cells of urodeles. Nature 210:1325–1326.

Sato, I., K. Shimada, A. Yokoi, T. Sato, and T. Kitagawa. 1991. Fine structure and elemental analysis of the enamel in *Andrius davidianus* (Cryptobranchidae) and *Onychodactylus japonicus* (Hynobiidae). Journal of Herpetology 25:141–146.

Sato, I., K. Shimada, and T. Sato. 1992. Morphology of the teeth of adult Caudata and Apoda: fine structure and chemistry of enamel. Journal of Morphology 214:341-350.

Sato, I., K. Shimada, T. Sato, and R. Etherige. 1993. Morphological study of the teeth of *Ambystoma maculatum*, *Salamandra salamandra* and *Aneides lugubris*: fine structure and chemistry of enamel. Okajimas Folia Anatomica Japonica 70:157-163.

Shashikiran, N. D., V. S. Reddy, and M. C. Hiremath. 2007. Estimation of trace elements in sound and carious enamel of primary and permanent teeth by atomic absorption spectrophotometry: an in vitro study. Indian Journal of Dental Research 18:157.

Selstrum, C. J. 2005. The defense science board provides DoD a roadmap for the future on unexploded ordnance. Gsystems 10:18-19.

Shimada, K., I. Sato, G. Murakami, and T. Ikeda. 1993. Fine structure and elemental analysis of the enamel in the cryptobranchid salamander *Megalobatrachus japonicus*. Okajimas Folia Anatomica Japonica 69:345-351.

Smith, M. M., and A. E. W. Miles. 1971. The ultrastructure of odontogenesis in larval and adult urodeles; differentiation of the dental epithelial cells. Zeitschrift für Zellforschung und Mikroskopische Anatomie 121:470-498.

Smith, C. K., and J. W. Petranka. 1987. Prey size-distributions and size-specific foraging success of *Ambystoma* larvae. Oecologia 71:239-244.

Smith, C. E. 1998. Cellular and chemical events during enamel maturation. Critical Reviews in Oral Biology & Medicine 9:128-161.

Stiffler, D. F. 1988. Cutaneous exchange of ions in lower vertebrates. American Zoologist 28:1019-1029.

Syracuse Research Corporation, and S. Keith. 2005. Toxicology profile for tungsten. Agency for Toxic Substances and Disease Registry pp. 109-111. Agency for Toxic Substances and Disease Registry, Atlanta, Ga.

Suga, S. 1984. The role of fluoride and iron in mineralization of fish enameloid. In R. W. and S. Suga, Tooth Enamel pp. 472-477. Elsevier Science Publishers BV, Amsterdam, Netherlands.

Wen, X., and M. L. Paine. 2013. Iron deposition and ferritin heavy chain (Fth) localization in rodent teeth. BMC research notes 6:1.

Wistuba, J., H. Greven, and G. Clemen. 2002. Development of larval and transformed teeth in *Ambystoma mexicanum* (Urodela, Amphibia): an ultrastructural study. Tissue and Cell 34:14-27.

Whitford, W. G., and V. H. Hutchison. 1963. Cutaneous and pulmonary gas exchange in the Spotted Salamander, *Ambystoma maculatum*. The Biological Bulletin 124:344.