AGE STUDY OF RED EFTS (*Notophthalmus viridescens viridescens*) FROM A CANNON COUNTY, TENNESSEE METAPOPULATION

by

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A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Master of Science in Biology

Middle Tennessee State University

August, 2015

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ACKNOWLEDGEMENTS

I would like to thank Dr. Brian Miller, thesis advisor, for his instruction in vertebrate biology, his mentoring, and his patience. I would like to recognize Dr. George W. Benz (now deceased) for his passion for the process of science, which strongly resonated with me, and his participation as a member of my graduate committee. I would like to thank Dr. Matthew Klukowski for his instruction in matters related to teaching laboratory human anatomy and physiology, for his willingness to replace Dr. George Benz on my committee, and his participation as a member of my graduate committee. I would like to thank Dr. Dennis M. Mullen for introducing me to the world of ectothermic vertebrates and his participation as a member of my graduate committee. I would like to thank Ms. Joyce Miller for her instruction in microscopic technique, specimen preparation, and laboratory histological procedure. And finally, I would like to thank Dr. Gore Ervin, graduate program advisor, who worked diligently to keep me on track, was an advocate for me, and was always responsive to any concern or issue I had.

ABSTRACT

Many populations of *Notophthalmus viridescens viridescens* have aquatic larvae that metamorphose into a sexually immature Red Efts that leave the water and are land-dwelling for up to seven years. In 2007 and 2008, fifty-six Red Efts were collected as road kill on Burt-Bergen Road, near the Bradyville Hill Road intersection (latitude, longitude coordinates: 35.795048, - 86.150466) in Cannon County, Tennessee. Snout-vent length of each specimen was measured. The right femur was removed from each specimen and the length of some femurs was measured. All 56 specimens were prepared for skeletochronological analysis; however, femur cross-sections from only 28 specimens were useable. Skeletochronological analysis indicated the most frequent age for this sample of Red Efts was three years; age estimates ranged from two to seven years, and mean age was 3.5 years. Age estimates yielded from this skeletochronology study are consistent with the findings of previous skeletochronology studies of Red Efts. This is the lowest latitude Red Eft sample to date in which skeletochronological age estimates are reported. Pearson product-moment correlations were computed for the following pairs of variables: age and snout-vent length, age and femur length, and snout-vent length and femur length. The resulting correlation coefficients were not significant; however, method of collection (road kill) and precision of measurement (to the nearest millimeter) are believed to account for the lack of significance. Red Eft age and snout-vent length were found to be significantly correlated in previous skeletochronology studies.

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CHAPTER ONE: INTRODUCTION

The Eastern Newt, *Notophthalmus viridescens* is the second most widely distributed salamander in North America, and can be found from eastern Texas northward to Canada and eastward to the Atlantic Coast (Petranka, 1998). Within this species, four subspecies are currently recognized (Sever, 2006). A vast amount of literature exists on the life history of *N. viridescens* (e.g., Petranka, 1998), but much of it concerns the northeastern variety, the Red-spotted Newt (*Notophthalmus viridescens viridescens*) (Sever, 2006). The Red-spotted Newt is the largest of the four subspecies and is readily recognizable by its red dorsal spots encircled in black (Petranka, 1998). Much of the interest in *N. v. viridescens* resulted from the discovery that many populations have aquatic larvae that metamorphose into a sexually immature Red Eft (Gibbons and Semlitsch, 1991), which leaves the water and is land-dwelling for up to seven years (Healy, 1974).

The Red Eft's juvenile body form is distinct from that of the aquatic adult newt. Red efts, especially younger ones, can be bright vermillion, have granular skin, and a cylindrical tail; whereas, adults are dorsally a shade of olive green with compressed tails (Petranka, 1998). The aquatic adult males develop especially prominent tail fins during the breeding season (Sever, 2006). In *N. v. viridescens*, both the Red Eft and the adult have a lateral row of red spots bordered by black, thus their common name – the Red-Spotted Newt.

The Red-Spotted Newt was originally described by Constantine Samuel Rafinesque (Rafinesque, 1820). In Rafinesque's description, he placed the adult form and the red eft form in separate subgenera *Triturus (Diemictylus) viridescens* and *Triturus (Notophthalmus) miniatus*, respectively. It was not until 1889 that Edward Drinker Cope in *The Batrachia of North America* concluded the two forms were two different stages of the same subspecies and were described as 'seasonal forms.'

Noble (1929) described the complex life cycle of *N. v. viridescens* as an evolutionary strategy to cope with harsh environmental circumstances. Gill (1978) interpreted the Red Eft stage within the framework of metapopulation theory as an important dispersal stage that served to refresh reproductively static or diminishing demes (i.e., reproductive sinks, for example ponds).

The life cycle of *N. v. viridescens* and the length of the Red Eft stage are variable and impacted the geography, latitude, and immediate environmental circumstance of a specific population (Petranka, 1998). The terrestrial juvenile (Red Eft) stage lasts up to seven years, the average number of breeding seasons for the adult newt appears to be limited to only two or three seasons (Gill, 1985) with a very low rate of female insemination occurring per breeding season (Massey, 1988). However, the length of the adult stage has been estimated to last as long as 15 years based on survivorship data (Gill, 1978, 1985).

Historically, age determination of individual *N. v. viridescens* has been achieved using mark-release-recapture studies or size-class frequency analysis (Forester and Lykens, 1991). However, mark-release-recapture studies suffer from logistical

constraints arising from long-term investment that has to be made, because of the long life of the amphibian; and size-class frequency analysis allows relatively accurate ages to be assigned to smaller individuals but not to larger individuals due to overlap in age classes.

A vast literature began to emerge in the 1960s concerning age determination in extinct and extant species, including extant Urodele species (e.g., Griffiths, 1962), using skeletochronology. Early skeletochronology studies of Salamandridae species included *Salamandra salamandra* (Castanet, 1973) and *Triturus alpestris* (Smirina and Rocek, 1975). Although there have been many more recent skeletochronology studies of other Salamandridae species, there have been only two skeletochronology studies of *N. v. viridescens*: a Maryland sample (latitude of ~ 39º N) with the maximum Red Eft age equaling seven years and a Quebec sample (latitude of ~ 49º N) with the maximum Red Eft age equaling five years. A South Carolina (latitude of ~ 33º N) study included Red Efts along with *N. v. viridescens* adults using humeral and femoral cross-sections to characterize the histology of bone annuli and to determine the regularity of annulus formation (Kazmer, 1986), but did not include age estimates of the individuals efts and adults in the study.

The variation in bone growth patterns in salamanders as a result of a climatic gradient has been well-documented (e.g., Miaud et al., 2000, on *Triturus alpestris*). The emergent literature describes skeletochronology's assumptions as well as the potential threats to the method's accuracy - noncyclical (annual) growth marks and endosteal reabsorption (e.g., Castanet et al., 1991).

The validity of the skeletochronology approach has been demonstrated by capture- mark-recapture studies, captive population studies, and florescent marking studies (Castanet, et al., 1991). Skeletochronology is based on the observation that skeletal growth has periodicity, that a growth zone and a line of arrested growth (LAG) correspond to one growth cycle, and (for temperate species) a growth zone/LAG occurs on an annual basis. However, in a species with unknown biology, the periodicity of a growth zone/LAG has to be determined as the periodicity can vary from species to species (Castanet, et al., 1991). Kazmer (1986), in his study of *N. v. viridescens*, concluded the periodicity of growth zone/LAG formation was annual for both Red Efts and adults.

For extant vertebrates, skeletochronology involves the transverse sectioning of a paraffin-embedded long bone with sections being stained using hematoxylin (Castanet, et al., 1991). Annuli are readily recognizable in a properly stained cross section, because they are hematoxyphillic due to their higher protein content as compared to adjacent osteogenic tissue. The stained section is then examined under a microscope by at least two independent observers, each of which determines the number of annual growth rings (i.e., LAGs). Differences in counts between trained observers must be resolved, and if not resolved, the section (specimen) is removed from the study. While LAGs are easily recognized, as indicated above, not all annuli are annual LAGs. However, with proper training the researcher/observer can learn to identify incomplete (false) annuli, metamorphic annuli, double annuli, etc. and can determine if reabsorption of an annual

annuli or LAG has occurred using histological criterion and/or mathematical methods (Castanet, et al., 1991).

This will be the first skeletochronological study of Red Efts from a Tennessee metapopulation. To date, the Tennessee sample will be the lowest latitude (~ 35ºN) sample in the literature for which age estimates are reported. The goals of this research project are twofold: (a) to estimate the age of a sample of at least 20 Red Efts using skeletochronology and (b) to statistically examine the relationship between estimated age and two growth measures: snout-vent length and femur length. Skeletochronology represents a reliable alternative to other age estimation methodologies for studying populations of *N. v. viridescens*. If properly employed, the skeletochronology method is a valuable tool in assessing population structure and examining a variety of other interesting ecological relationships (Forester and Lykens, 1991).

CHAPTER TWO: LITERATURE REVIEW

Life History of *Notophthalmus viridescens viridescens*

Notophthalmus viridescens has one of the most variable and complex life cycles of any North American salamander (Petranka, 1998). Most populations of *N. v. viridescens* exhibit four distinct life history stages: the egg, the aquatic larva, terrestrial red eft, and aquatic adult (Petranka, 1998). The existence of a terrestrial stage and aquatic stage is not limited to *N. viridescens* but is a characteristic of all salamanders in the family Salamandridae (Sever, 2006), to which *N. v. viridescens* belongs.

The Red Eft stage occurs when the animal metamorphoses from an aquatic larva into an immature form that assumes a terrestrial existence prior to maturing into an aquatic adult form. After the time spent on land, Red Efts migrate back to breeding sites and metamorphose into aquatic adults with lungs (Healy, 1974; Forester and Lykens, 1991; and Caetano and LeClair, 1996). Depending on the geographic population, this migration may begin in the spring or autumn, or occur during both seasons (Bishop, 1941; Chadwick, 1944; Gill, 1978; and Healy, 1975). The lunged adults may remain in water for the remainder of their lives, but there are circumstances which will cause them to leave the water and temporarily live on land; for example, if the water level drops or the temperature rises in the pond (Noble, 1926; Gill, 1978, Hurlbert, 1969; Massey, 1990). They will also leave the water if they are plagued by parasites (e.g., leeches) and temporarily take to land to remove the parasites (Gill, 1978). Adults that remain on land for a prolonged period of time will show morphological change, that is, a reduction in the dorsal tail fin and development of a more granular skin (Petranka, 1998). In some

populations, such as those that reside in sandy coastal areas, the Red Eft stage is rare (Noble, 1926, 1929; Brandon and Bremer, 1966; Healy, 1974). Aquatic larvae either metamorphose into aquatic juveniles, which remain in the pond and mature into adults with lungs, or larvae will partially metamorphose and become sexually mature newts that retain their larval gills (i.e., undergo paedomorphsis) (Brandon and Bremer, 1966; Healy, 1974; Noble, 1926, 1929; Reilly 1986, 1987). However, in most populations of *N. v. viridescens*, Red Efts are present and require a longer time to attain sexual maturity than those that skip the Red Eft stage to become lunged adults or those that undergo partial metamorphosis to become gilled adults (Petranka, 1998).

The duration of the Red Eft stage is variable (Forester and Lykens, 1991) and may vary according to latitude of the population; however, a predictable relationship between latitude and years spent in the Red Eft stage has not been firmly established. Two factors have obfuscated the establishment of a clear latitude-age relationship: (a) the lack of age studies of Red Eft populations from different latitudes and (b) and the accuracy of age estimates used in existing studies. Representative age studies of *N. v. viridescens* illustrate the lack of predictable relationship between latitude and duration of the Red Eft stage. In high latitude Canadian populations, the Red Eft stage has been estimated to last from two to seven years (Hurlbert, 1969; Pough, 1971; Healy, 1975; Gill, 1978, 1985). In a lower latitude (New York) population, the Red Eft stage was estimated to last only two to three years (Bishop, 1941). However, in an even lower latitude (western North Carolina) population, the Red Eft stage was estimated to last four years (Chadwick, 1944).

Notophthalmus. viridescens hatchlings have pond-type morphology and within one to two weeks the larvae metamorphose into Red Efts and individuals will take on a bright red color (Petranka, 1998). However, Red Efts do vary in color from bright vermillion (when small) to dull red or greenish brown (as they near sexual maturity), but always have conspicuously coarse, granular skin (Petranka, 1998). Their bright coloration functions as a warning coloration, which is important because Red Efts are more active during the daytime than are most other salamanders (Petranka, 1998). *Notophthalmus viridescens* is thought to be one of a group of salamanders that belong to a Müllerian mimicry complex (Petranka, 1998).

Aquatic adults are light yellow below and olive green above and the skin is only slightly granular (Petranka, 1998). Snout-vent length (SVL) of aquatic adults measures 31 – 54 mm and tail length measures 65 – 112 mm; the tail is narrowly keeled above and below and is approximately 50% of the total body length (Petranka, 1998).

The Reproductive Importance of the Red Eft Stage

Red Eft Dispersal: A Means of Maintaining Reproductive Sinks

Noble (1929) explained the complex life history of *N. viridescens* (i.e., a variable stage life history, involving both terrestrial and aquatic habitats, and occurring over a long span of time per life history stage) as an evolutionary strategy allowing this newt to endure fluctuations in seasonal weather patterns as well as habitat succession. However, Gill (1978) highlighted the evolutionary importance of the Red Eft stage as a 'dispersal stage' within the life cycle of the Red-Spotted Newt. In his model, a metapopulation of *N. viridescens* is viewed as a grouping of subpopulations (demes), most of which

function as reproductive sinks. The various demes are connected together by juveniles (Red Efts) emigrating from a central, highly productive pond to colonize other demes. The location of the reproductive center may shift with time. Gill (1978) notes the evolutionary advantages of this model are twofold: (a) a deme can quickly change from a state of no reproduction to become a reproductive center because of Red Eft immigration; and (b) within the deme, adult newts are not totally eliminated from reproductive potential as they would be if the entire deme went extinct due to reproductive failure. Supporting Gill's model, Petranka (1998) observed the emigration of Red Efts in a Virginia population, from breeding centers having high juvenile production to breeding centers where larval survival rates were very low. Such Red Eft emigration is important because *N. viridescens* adults exhibit philopatry (i.e., fidelity to their home ponds) and have homing ability that allows them to repeatedly return to their home ponds after winter hibernation, limiting or excluding adult emigration (Petranka, 1998).

Massey (1988) reports the rate of insemination of females by courting males and the rate of insemination of females by a rival male depositing a spermatophore were 6% each out of 131 courtship bouts involving *Notophthalmus viridescens* male-female amplectic pairs from natural populations in New York and Virginia. Gill (1985) estimated the median number of breeding seasons to be three for Red-Spotted Newts living in the Shenandoah Mountains of West Virginia.

There are a limited number of age studies on Red Eft populations. A *N. viridescens* population in Maryland was studied and skeletochronology was used to estimate the ages of adult newts and Red Efts. The mean age for adults was seven years

and for Red Efts, it was 4.4 years. The modal age for the Red Efts was five years and for adults, it was seven years (Forester and Lykens, 1991). For a Quebec population, Caetano and LeClair (1996) estimated the mean adult age to be 6.1 years and the most frequent age of the red efts being 3.5 – 4.0 years (determined by inferring age from the most frequent body sizes: 32-34 mm). Minimum and modal ages (respectively) were four and seven years for aquatic adult newts (Caetano and LeClair, 1996). LeClair et al. (2005) suggest the observed difference of ~ 2.0 – 2.5 years may represent the average number of breeding seasons for a typical adult newt. However, these researchers caution against drawing a definitive conclusion about the local or geographic variation in the mean number of eastern newt breeding seasons, because the age structures of corresponding Red Eft populations remain relatively unknown.

Foraging and Growth: The Importance of Body Size

Noble's original hypothesis (1929) viewed natural selection favoring a complex life history for the red-spotted newt enabling it to survive an unpredictable and harsh environment. Certainly, this is reasonable; however, it does not fully explain why natural selection would favor a terrestrial juvenile stage that lasts up to seven years in some populations, while the number of breeding seasons across populations appears to be limited to only two to three (LeClair et al., 2005) with each of those breeding seasons resulting in a low success rate of female insemination (Massey, 1988).

After the publication of Gill's metapopulation hypothesis (1978), researchers began to look beyond the short-term advantages of the Red Eft stage, that is surviving unfavorable climatic conditions and habitat succession (Noble, 1929), to the long-term

advantages of remaining in the red eft stage for a minimum number of years (e.g., Kaplan and Salthe 1979; Massey, 1988; Verrell 1982, 1985, 1989; Forester and Lykens 1991; Caetano and LeClair 1996; LeClair et. al., 2005). For example, Forester and Lykens (1991), in a Maryland sample, found only a few Red Efts had become sexually mature and returned to an aquatic habitat following their third year of life and an equal portion of efts remained as terrestrial foragers during their seventh year of life. Researchers began to examine the hypothesis that the length of an individual's red eft stage could infer a reproductive advantage for that individual as an adult aquatic newt.

The Red Eft's exploiting of the terrestrial habitat over a number of breeding seasons has been observed to result in a gain in body size reflected by an increase in snout to vent length (SVL) (Forester and Lykens, 1991; LeClair, et. al., 2005). Verrell (1982, 1985) found larger *N. viridescens* adult males had greater reproductive success. Verrell (1989) found rival male newts spend more time trying to dislodge an amplectic male if the female is large (i.e., fecund) or if the male is relatively small. In another Salamandridae species (*Triturus vulgaris*), testis mass has been demonstrated to positively correlate with SVL (Verrell and Francillon, 1986). For amphibians in general, Kaplan and Salthe (1979) found the number and size of eggs produced related to the body size of female; that is, larger females produced more eggs of greater size compared to smaller females. These studies suggest large body size may serve to convey a reproductive advantage in both male and female adults during the breeding season.

In Red Efts, growth rate is directly proportional to the amount of food consumed (Springer, 1909) and, in turn, growth depends not only on food abundance, but also on

foraging activity (Forester and Lykens, 1991). For Red Efts, growth rate is the highest during summer (Petranka, 1998) with Red Eft activity positively correlated to temperature and humidity (Healy, 1973). Roe and Grayson (2008) found a Virginia sample of Red Efts capable of traveling long distances in the terrestrial habitat in the short amount of time (i.e., mean nightly distance traveled = 29.2 m; standard error = +/- 13.9 m) when climatic conditions permit (i.e., temperature on day of movement and rain and humidity on the day prior to movement). Additionally, the distance traveled by Red Efts was not found to significantly differ from the distance traveled by adult newts, but the direction of travel for Red Efts was variable, whereas adults maintained a straighter trajectory. These researchers interpreted the meandering behavior of Red Efts as the result of foraging activity in their home territories. In a Massachusetts population, Red Efts required about a year to migrate to woodlands 800 m from their natal pond. In periods of dry weather, they were found to reside under leaf litter and were most active on the ground surface during rainy weather when temperatures were above 12º C. Resident Red Efts were observed to shift to different microhabitats seasonally and local movement appeared to occur in response to shifts in local food abundance (Healy 1974, 1975).

Fat Bodies: The Relationship between Size Increase and Testes Mass

Fat bodies, discussed by Jørgensen in Feder and Burggren (1992), enlarge in size during periods of growth and for the Red Eft growth is at its maximum during the summer months (Petranka, 1998). The following discussion of fat bodies in amphibians is taken from Jørgensen (1992). Fat bodies are located in close proximity to the gonads

and appear to have a direct physiological relationship with the gonads. Both fat bodies and gonads have a common embryological origin, differentiating from a peritoneal fold at the base of the mesentery with the fat bodies originating from the anterior end and the gonads from the posterior end. In amphibians, fat is stored in the tail as well as in the liver. In temperate amphibian species, this occurs on a cyclic basis according to seasonal fluctuations. An inverse relationship exists between fat bodies and the development of testis and ovaries in anurans and urodeles: in juvenile amphibians fat bodies are large and gonads are immature, whereas in adults the exact opposite exists. While the relationship between fat bodies and gonads is disputed, fat bodies do appear to specifically serve gonads, as opposed to other somatic tissues. One line of evidence supporting this hypothesis is the observation that fat bodies reduce in size before and during breeding; that is, the fat is being used as an energy source for gamete formation in addition to sustenance during hibernation. Adams and Rae (1929) provide experimental evidence supporting the fat in service to gonad hypothesis. They observed a generalized reduction in gametes when fat bodies were excised in *N. viridescens* males and females. They also observed the maximum size of fat bodies occurred before winter hibernation and the minimum size occurred during spawning in adult *N. viridescens*.

Snout-vent length is positively correlated with Red Eft age (Forester and Lykens, 1991; LeClair Jr, LeClair, and Levasseur, 2005) and fat bodies have been observed to be larger in the juvenile stage compared to the adult stage, presumably because they provide the energy for gonad development in the transforming amphibian (Jørgensen, 1994). In another Salamandridae species (*Triturus vulgaris*), Verrell and Francillon (1986)

demonstrated testis mass also positively correlates with SVL. Given these observations, it is reasonable to hypothesize the increase in Red Eft body size as a result of being a juvenile terrestrial forager for a minimum number of years provides a future physiological advantage; i.e., fat stored during the terrestrial Red Eft stage equates to energy for gonad development during the eft-to-newt transformation. And if the size of fat bodies does play a role in determining the future size of gonad mass, then it becomes reasonable to further hypothesize the amount of fat store indirectly impacts the future amount of gamete production an adult *N. viridescens* is capable of during its reproductive life.

The Importance of Age as Related to Body Size

Red Efts are in the forest-floor habitats until nearly mature, and then migrate back to breeding sites where they transform into aquatic adults. Typically, they reach sexual maturity shortly before or after arriving at the breeding site (Petranka, 1998). In another Salamandridae species, *Triturus vulgaris*, Verrell and Francillon (1986), concluded the onset of sexual maturity was determined by age and not by body size. Hagström (1977, 1980) in his studies of a *T. vulgaris* population using skeletochronology, found the modal age was five years for both sexes; therefore, many newts did not breed for the first time until after spending four or even five winters on land in a sexually immature form. Marnell (1998), also using skeletochronology age estimates, found for his *T. vulgaris* sample, three years to be the minimal age for breeding (i.e., a minimum of two to three winters spent in a sexually immature form). These findings mirror the developmental pattern seen in the red-spotted newt; that is, on the average, the red eft stage lasts four to

five years with most individuals not becoming sexually mature until five to six years old (Petranka, 1998). This developmental pattern appears to be an apomorphy of the Family Salamandridae, given all species in this family exhibit a terrestrial juvenile stage; albeit, it varies in length per Salamandridae species (Sever, 2006).

Longevity is also similar in both species. Hagström (1977), using skeleto-chronology, reported a male *T. vulgaris* of 10 years of age; however, this observation may represent a statistical outlier given the maximum age for the rest of his sample was eight years. Aronsson and Stenson (1995), in their study sample, found seven years to be the maximum adult age for *T. vulgaris*. For a Maryland population of red-spotted newts, Forester and Lykens (1991) found the mean age of adult newts to be seven years with the adult population declining during the eighth year, due to death by senescence. This contrasts to Gill (1985) who reported some adult Red-Spotted Newts in his Maryland sample reproduced as long as 10 years.

Length of time the Red Eft spends in a terrestrial environment (i.e., age), eft body size, and timing of sexual maturity may well all be linked by physiological need. Healy (1974, 1975) believed a minimum body size was needed for reproduction in the red-spotted newt; however, age appears to be the more important variable. A minimum age may be required for fat stores to become large enough to sufficiently power gonad development during eft-to-adult transformation. If this hypothesis is true, the Red Efts' length of stay in a terrestrial environment is likely to be determined by physiological need (i.e., for adequate fat stores) dependent on environmental factors (i.e., seasons, length of seasons, temperature, humidity, and food abundance) that impact foraging behavior. For

Red Efts who opt out of breeding and stay longer in the terrestrial habitat, the advantage gained is not only the increase in size of gonads, but also an increase in body size, both of which (as discussed previously) provide future reproductive advantages to the adult in a challenging reproductive environment, where the number of breeding seasons is limited and female insemination rates are low. If these reproductive advantages significantly increased the number of 'large body' alleles over 'small body' alleles within a deme's genetic pool, the developmental pattern of terrestrial juveniles foraging for a minimum number of years to maximize body size and fat stores would then be selected for and stabilized in geographically local populations. This is a K-selection strategy, whereby development is slower and results in greater competitive ability, resource thresholds are lower, reproduction is delayed, a larger body size is obtained, and iteroparity occurs (Minkoff, 1983).

The minimum number of years a terrestrial juvenile would spend foraging appears variable for Red Eft populations at different latitudes. Latitude determines those environmental factors (i.e., seasons and season length, temperature, humidity, and food abundance) that impact foraging success (i.e., developing necessary fat stores) from year to year. Age estimates of Red Eft samples from two different latitudes (i.e., Leclair et al., 2005: a Quebec sample = mean age of 3.0 years and Forester and Lykens, 1991: a Maryland sample = mean age of 4.4 years) suggest higher latitude *N. viridescens* populations exhibit a shorter red eft stage compared to lower latitude populations. Possibly, this occurs because lower latitudes are more subject to periods of drought than are higher latitudes.

Skeletochronology

Skeletal Growth Marks

Aristotle initially hypothesized the importance of the growth rings or the zone/annulus pattern observed in the shell of a marine gastropod (see Warren, 1963). Castanet et al. (1993) has empirically demonstrated that all living vertebrates (for which data exists) lay down annual growth marks with the exception of those vertebrates that attain full growth in less than one year (e.g., birds and small mammals). The hypothesis is the 'zones' represent relatively fast periods of skeletal growth with the 'annuli' representing periods of arrested skeletal growth (i.e., lines of arrested growth or LAGs) (Castanet et al., 1993). This hypothesis is supported by an abundance of research literature spanning more than 60 years (see Woodward et al., 2013 for representative studies of extant and extinct taxa). Historically, this pattern of skeletal growth has been assumed to be determined by fixed period endogenous and/or exogenous rhythms (e.g., circadian or circannual rhythm) for a fixed period of growth with one zone and annulus forming per annum. Consequently, this pattern of skeletal growth can effectively be used to estimate individual age (Kazmer, 1986).

More recently, Woodward et al. (2013) considers it unclear as to why vertebrates lay down cyclical growth marks and these researchers believe the reason for this pattern of skeletal growth may not be the same for all vertebrates. Woodward et al. (2013) view the zone and annulus pattern of skeletal growth as a plesiomorphic character uniting all vertebrates, including the basal vertebrates. Their rationale is as follows: because basal vertebrates were ectothermic and temperature dependent, when seasonal changes in light

and temperature negatively altered food resources, the basal vertebrate response was to slow metabolism (thus growth) until food resources improved.

While this logic is certainly plausible for ectothermic vertebrates, Woodward et al. (2013) realized it does not explain why endothermic and tachymetabolic vertebrates lay down annual growth rings. They discuss this issue and review several pertinent studies of mammals (citations given within text) and draw the following conclusions: (a) cyclical growth marks observed in some mammals are not the result of environmental stresses, but appear to be a response to environmental cues (Castanet et al., 2004); (b) LAG formation is triggered by genetically controlled hormonal cues in homeothermic endotherms and not by environmental stresses (Köhler et al., 2012); and (c) within a single mammal taxon, a single biological rhythm governs lamellar bone formation (e.g., rats – seven days, macaque and pata monkeys – 28 days, sheep – 35 days, and humans – 56 days) (Bromage et al., 2009). Woodward et al. (2013) conclude their discussion noting the unresolved issues appear to be when and how the production of LAGs changed from representing an environmental constraint on growth (i.e., the ectothermic vertebrate response: slowing metabolism (thus growth) until food resources improve) to representing environmental cues that affect growth (i.e., as seen in mammals). They believe part of the answer may lie in the phylogenetic shift from bone that has alternating lamellar and avascular zones (i.e., lamellar-zonal bone) to dynamic bone that consistently experiences active growth (i.e., fibrolamellar bone).

The Accuracy of Age Determination Using Skeletochronology

Accurate age determination using skeletochronology methodology is dependent on three factors: (a) the vertebrate lays down zone/annuli on an annual basis, (b) the annual LAG can be distinguished from other skeletal growth marks (e.g., metamorphic annuli), and (c) endosteal reabsorption of LAGs has not occurred or the skeleto-chronology analysis is corrected for LAG loss.

Annual Zones and Annuli (LAGs)

As discussed above amphibians and reptiles demonstrate growth marks in their bones with broader zones reflecting episodes of osteogenesis (zones) and lines of arrested growth (LAGs) corresponding to periods of muted osteogenesis (Hemelaar, 1981, 1983; Castanet and Smirina, 1990; Smirina, 1994). LAGs have a very low thickness (a few micrometers) and are most chromophilic with dyes such as hematoxylin (Castanet et al., 1993) staining darker purple than other osteogenic tissue. In most amphibian species studied, each LAG represents an annual bone growth cycle and can be used to estimate the age and longevity of the animal (Castanet and Smirina, 1990; Smirina, 1994). Bone growth cycles occur in geographic regions where growth is punctuated and stopped by seasonal fluctuation (i.e., in temperate climates) (Hemelaar 1981, 1983; Castanet and Smirina, 1990; Smirina, 1994). Kazmer (1986) studied a South Carolina population of larval, eft, and adult *N. v. viridescens* using mark-release-recapture technique and an *in vivo* alizarin bone-labeling procedure in combination with skeletochronological analyses. His studies supported the hypothesis that *N. v. viridescens* produce only one LAG per annum.

Other Skeletal Growth Marks

Kazmer (1986) discusses the origin of the two types of bone observed and problematic growth marks that he observed in his study of larval, eft, and adult forms of *N. v. viridescens*. Consistent with Francillon's (1979) long-term bone-labeling study on *Tritus cristatus cristatus* (also a member of the Salamandridae Family), Kazmer observed that in the larval form of *N. v. viridescens* a cartilage template is still present with primary bone growth being characterized by periosteal apposition of parallel-fibered bone; whereas, the secondary bone growth that occurs on the endosteal surface in efts and adults is characterized by lamellar bone, which can only form after the removal of the cartilaginous template. Kazmer notes that there are hematoxylinophillic lines of varying description present in both primary and secondary bone tissue; however only those lines (i.e., LAGs) that reside within the primary bone tissue are of value in estimating the age of the animal. He characterizes the other hematoxylinophillic lines seen in the secondary bone as forming a much smoother arc, having a higher stain density, being much closer together, not forming complete rings, and not following the contours of the developing bone surface - all of which make them readily distinguishable when using just a light microscope for examination.

Kazmer (1986) also discusses the problematic nature of false annuli, multiple annuli, and the metamorphic annulus. False annuli are characterized by incomplete rings that are weakly hematoxylinophillic and are often ubiquitous. He notes it is conjectured that each line represents a short-lived environmental stress, but this interpretation remains unsubstantiated. Kazmer views multiple annuli (i.e., doublets, triplets, etc.) as being

more problematic than false annuli in that each group of multiple annuli is composed of individual annuli that are not structurally distinct from one another. He gives an example of a 'doublet' that in one part of a cross-section two closely adjacent annuli are clearly visible and in another part of the cross-section the two annuli fuse to form a single annulus with a high stain density. Castanet (1978) and Francillon (1979) also provide examples of doublets that they count as one true annulus.

Kazmer (1986) characterizes the metamorphic annuli in *N. v. viridescens* as follows: the two innermost annuli are very close together, the first annulus in the pair is the metamorphic annulus, the width of the zone between the metamorphic annulus and the outermost (true) annulus is much less than the width of zones observed between LAGs. He also notes the first true annulus (LAG) is recognizable because it has a higher stain density as compared to other subsequent LAGs.

Endosteal Reabsorption

The validity of age estimation by skeletochronology was first demonstrated on 42 bullfrogs (*Rana catesbeiana*) of known age by Schroeder and Baskett (1968) and, more recently, with the California Tiger Salamander (*Ambystoma californiense*) by Trenham et al. (2000). However, Eden et al. (2007) found skeletochronology to underestimate the age in adult Tiger Salamanders (*Ambystoma tigrinum nebulosum*) due to endosteal resorption coupled with rapprochement of the most peripheral LAG. Kazmer (1986) observed only two out of 37 (~ 5%) adult *N. v. viridescens* had completely reabsorbed the first LAG, whereas partial LAG reabsorption was the more common occurrence (i.e., 51% or 19 adult newts). When he measured the bone and annulus cross-section areas

of a sample of Red Efts, he did not find any evidence indicating reabsorption of the first LAG had occurred.

Kazmer (1986) provides useful, qualitative criteria for the determination of the presence of the first true LAG either in full or partial form. He defines three histological characteristics that are present only in or near the first true LAG and relate to events that are known only to occur prior to or during the formation of the first true LAG. One of these criteria, the dark staining of first true LAG, has been previously discussed and the other two criteria are discussed below.

Substantial change, both morphological and physiological, occurs during transformation from the larval stage to the Red Eft stage, which includes the cessation of feeding during metamorphic climax (see Kazmer, 1986). Growth trajectories of larval amphibians asymptotically approach a maximum body size during the later stages of metamorphosis suggesting a period of arrested growth accompanies metamorphosis (Wilbur and Collins, 1973). The presence of a metamorphic annulus followed by the first true LAG reflects a period of arrested growth. The metamorphic annulus may indicate an interval during which significant growth occurred between metamorphic climax and the time during which the first true LAG formed. The first true LAG represents a prolonged period of arrested growth presumably during the winter months (Kazmer, 1986). However, if metamorphosis occurs late in the season the metamorphic annulus will be absent, but the first true annulus or LAG will be evident. This occurs because of the lack of time for growth to occur (Kazmer, 1986).

The third histological indicator characterizes only the first true LAG: one or more

small-scale invaginations of the LAG. These invaginations appear as the bone surface enlarges in well-developed larvae and in the first LAG of postmetamorphic individuals. The significance of these invaginations is unknown. Kazmer (1986) notes these invaginations were not present in all diaphyseal cross-sections from the same individual. Therefore, the absence of this characteristic in a particular cross-section does not necessarily indicate the complete reabsorption of the first true LAG. The procedural implication here is to obtain serial sections of the diaphysis to increase the likelihood of observing first LAG margin invaginations. Kazmer (1986) notes scoring the presence or absence of the first true LAG and likewise for small-scale invaginations is subjective and human error can occur. Additionally, he warns that partial reabsorption of the first true LAG can occur, obfuscating accurate scoring. Nevertheless, the three histological indicators provide a reasonable, albeit not perfect, qualitative index useful in identifying the presence or absence of the first true LAG.

CHAPTER THREE: METHODS AND MATERIALS

Study Species: *Notophthalmus viridescens viridescens*

Notophthalmus viridescens viridescens, the Eastern Red-Spotted Newt has a broad distribution across eastern North America. The species range extends down from southern Ontario, southern Quebec, and the maritime provinces of Canada to central Georgia and Alabama. From the Atlantic coast, the species range extends to central Indiana, western Kentucky, and Tennessee but excludes the southeastern coastal plain (Meacham, 1967). Specimens used in this study were collected as road kill on Burt-Bergen Road, near the Bradyville Hill Road intersection (latitude, longitude coordinates: 35.795048, - 86.150466) in Cannon County, Tennessee.

Although there can be considerable variability, the life cycle of *N. v. viridescens* typically consists of three morphologically distinct stages: aquatic larval stage; sexually immature, terrestrial Red Eft stage; and sexually mature adult stage, which may either be permanently aquatic or only seasonally aquatic (Hulbert, 1969, 1970; Gill, 1978). Collected specimens consisted of both Red Efts and sexually mature adults. For this study, 28 terrestrial juveniles (Red Efts) were selected for skeletochronological analysis.

Mark-release-recapture studies, the most accurate means of obtaining a specimen's age, indicate the length of the Red Eft stage can range from three to seven years (Healy, 1974). For sexually mature males and females, Gill (1978) estimated their mean life expectancies were 1.89 and 1.31 breeding seasons respectively. The sexual maturity of collected specimens was determined by non-invasive, visual examination based on color and color pattern and, to a lesser degree, size. Specimens were

categorized as either sexually immature (Red Eft), transitioning to sexually maturity, or sexually mature. Only those individuals categorized as sexually immature were included in this skeletochronology study.

Histological Procedures

I prepared one femur from each specimen following the procedure of McCreary et al. (2008) which represents a refinement of the traditional procedure described by Hemelaar and Van Gelder (1980) and others (e.g., Caetano and Leclair, 1996, 1999). Although designed for anuran phalanges, and not femurs of salamanders, the authors note that one of their intentions in writing and publishing this manual was to make available a histological procedure that could be adapted to fit the needs of different researchers using skeletochronology to estimate age of amphibian species. Modification to this histological procedure for processing *N. v. viridescens* femurs was limited to the use of 10% buffered formalin for long-term storage of specimen femurs, increasing the amount of time in the formic acid bath for bone decalcification as needed, and the use of a paraffin hot pot and slide warmer in addition to a paraffin embedding unit for block preparation.

Measurement, Fixation, and Decalcification

I rinsed the specimens in tap water for five to ten minutes, then I fixed them in 10% buffered formalin (pH: 7.20 – 7.30) for 24 hours. After rinsing and prior to fixation, I measured and recorded the snout-vent length of each specimen to the nearest millimeter using a centimeter ruler. For mangled specimens, I measured snout-vent length but recorded the measurement as an estimate. After fixation, I removed the right femur of each specimen. I manually removed soft tissue with a pair of jeweler tweezers while

viewing the femur under a dissection microscope. For most specimens, total femur length was measured to the nearest millimeter using a centimeter ruler. I stored the body of each specimen in 10% buffered formalin in a plastic centrifuge tube labeled with the specimen number written on the tube with a permanent marker.

After measurement, I placed the right femur in a small paper envelop folded from a sheet of paper used in the cosmetology industry for setting hair permanents. I then placed the enveloped femur in a Fischer Scientific Omnisette Tissue Cassette. The specimen number was written on each respective cassette in permanent marker. I stored the cassettes in 95% ethanol (ETOH) until the femurs were to be decalcified. To decalcify the femurs, the cassettes were placed in a large jar containing Cal – Ex II (formic acid 2.4 – 3.0 M; formaldehyde 1.0 M) for a minimum of eight hours. After eight hours, I opened each cassette and checked the femur to determine if further decalcification was necessary (i.e., if bone remained rigid). For femurs requiring no further decalcification, I rewrapped them and placed them back in their respective cassettes. I rinsed them for a minimum of 12 hours in tap water, followed by a soaking in deionized water for one hour, followed by a final rinse in fresh deionized water for five minutes. I drained the cassettes and stored them back in 95% ETOH. If I determined any femur was in need of further decalcification, I placed that femur back in the formic acid solution for an additional hour and then checked it. This procedure was continued until I judged decalcification was adequate (i.e., the bone had become flexible).

Dehydration, Clearing, and Infiltration

In preparation for paraffin infiltration and embedding, I removed cassettes containing the femurs from the fixing jar and sequentially placed them in the following solutions to dehydrate: fresh 95% ETOH for one hour and then 100% ETOH overnight. The next day, I removed the cassettes from 100% ETOH and placed them sequentially in the following solutions to clear: 100% Shandon Xylene Substitute (Isoalkanes C9 – 12) for one hour followed by one hour in fresh 100% xylene substitute.

While cassettes were clearing, I filled two dishes with embedding paraffin chips (Paraplast-xtra) and warmed them to 58 - 60º C. When the cassettes were finished clearing, I removed them from the xylene substitute, towel dried them, and placed them in one of the infiltrating dishes containing molten paraffin and placed the dish in the warming compartment of a Lecia EG 1160 compact paraffin embedding unit or on a Chicago Surgical & Electrical Company bench-top slide warming tray. I checked the cassettes to ensure that all of them were completely immersed in paraffin. The cassettes were left uncovered in the warming units for two hours for initial infiltration. I then removed the cassettes from the first infiltration dish and placed each of them in a second infiltration dish. Again, I checked the cassettes to ensure they were fully immersed in the paraffin, left them uncovered, and infiltrated them for either two hours or overnight.

Embedding

I used a Lecia EG 1160 compact bench-top paraffin embedding unit to heat, dispense, and cool the paraffin; alternatively, I used a paraffin pot to melt paraffin and

hand-poured the paraffin into the Fischer Metal Histo Base Molds (24 x 24 x 5 mm). I then warmed the molds either in the embedding unit or on a slide warmer.

When using the embedding unit, I filled the paraffin reservoir with wax pellets and heated it to 60ºC to ensure an easy flow of wax. When the wax was heated, I removed four infiltrated cassettes from the warming compartment and placed them in a recessed surface-heated area filled with molten wax for temporary storage to prevent solidification of infiltrated specimens. I then placed a heated metal embedding mold under the dispenser outlet nozzle and filled it with paraffin using the dispenser handle. I removed an infiltrated cassette from the heated recess area and labeled a Fischer Scientific Tissue Path Embedding Ring with the specimen number on the cassette, removed the femur, and discarded the cassette and paper envelope. I used forceps to place the femur in a half-paraffin-filled metal embedding mold with the femoral head pointing downward in the mold. I then placed the mold on the refrigeration spot of the embedding unit for a moment while the femur was vertically-oriented in the embedding mold. When the femur appeared to be in a vertical position, I moved the half-paraffin-filled embedding mold back to the paraffin dispenser and fully filled it with molten paraffin. After this, I fitted the labeled embedding ring within and on top of the metal embedding mold and topped it off with liquid paraffin. I then moved the embedding mold and attached ring to the refrigeration unit on the embedding unit. After a few minutes the paraffin was completely solidified and I then removed the paraffin block from the embedding mold with the embedding ring attached. I removed any residual wax on the embedding mold using xylene substitute and subsequently cleaned the mold using

warm water and liquid detergent, rinsed it using deionized water, and dried it overnight. I stored embedded femurs in paraffin blocks in a lab refrigerator until faced and sectioned.

Alternatively, I followed the same basic procedure when using a paraffin pot (i.e., a Lipshaw Electric Paraffin Picture) in conjunction with a slide warmer. When necessary, I cooled the paraffin by either allowing the mold to cool at room temperature or by placing the mold in a laboratory refrigeration unit for a short time. Although this approach slowed the embedding process, the same end result was achieved. One or the other procedures was followed until all femurs for this study were embedded and prepared for trimming, facing, and sectioning.

Trimming, Facing, and Sectioning

I trimmed mounted paraffin blocks manually using a dissection scalpel to form an approximate 0.5 cm square surface with each side having an equal depth of approximately 0.3 – 0.4 cm measured from the top of the femoral head. This allowed enough depth to face the trimmed paraffin block down to 0.2 to 0.4 cm, which is the general vicinity of the beginning of the femur shaft in an average-sized specimen.

I used an American Optical Spencer Rotary Microtome (Model 820) to face the trimmed block down to the general location of the femur shaft and to produce thin sections 15 *um* thick. Serial sections were checked using a Bausch & Lomb dissection microscope to determine the presence of sectioned bone (i.e., appears glassy and highly reflective compared to surrounding paraffin). When sections were obtained, usually as a ribbon, I removed the ribbon from the microtome using jeweler forceps and placed it in a heated water bath (60º C) located on the slide warmer until the ribbon relaxed. I labeled

a Thermo Scientific Superfrost Plus slide with the specimen number and floated the ribbon onto the slide and the slide was then placed on the slide warmer until the paraffin ribbon melted and turned clear. I then stored the slide in an enclosed slide case, at least overnight, until stained.

Alternatively, I dissolved powdered albumin into deionized water (until a slight gel consistency was obtained) and applied a line of this mixture long enough and wide enough to encompass the ribbon to a Red Label Microslide. In this procedure, I removed the ribbon from the microtome and laid it on top of the albumin mixture and then left it to dry overnight. The next day, I heated the slide on the slide warmer at 60º C for two hours. I then removed the slide from the slide warmer and left it overnight.

Slide Preparation: Staining and Mounting

I prepared and performed slide baths in a fume hood. The first two baths consisted of placing the slides in xylene substitute until the paraffin loosened and fell from the slide. The xylene substitute for the second bath was changed after ten slides had been bathed. Each xylene substitute bath lasted ten minutes. After the baths, I checked each slide for the presence of residual wax. If wax was present, I returned the slide to the first xylene substitute bath and gently dipped it up and down in the bath until the paraffin loosened and was removed. If necessary, I removed resistant wax with a dissection needle in combination with dipping. I then placed the slide in the first xylene substitute bath for one minute and afterwards I reexamined it under a dissection microscope for the presence or absence of wax. If wax was absent, I dipped the slide into the fresh xylene substitute bath for one minute.

After all visible paraffin was removed I cleared the slide using the following baths: 100% ETOH for two minutes, 95% ETOH for 2 minutes, 70% ETOH for 2 minutes, and de-ionized water for 2 minutes. I stained serial cross-sections by placing slides in Harris' Hematoxylin stain (Fischer Scientific) for eight minutes and then rinsing them in deionized water for two minutes. I examined each slide under an Olympus CX41 light microscope at 100X to determine if a proper staining had occurred. If the slide was too lightly stained, I repeated the stain/rinse procedure and then reexamined the slide using the light microscope.

I dehydrated the stained serial cross-sections using a series of ETOH baths (i.e., 70%, 95%, 100%) lasting two minutes each. Finally, slides were cleared of ETOH by bathing them in xylene substitute for three to five minutes and they were kept moist with xylene substitute until mounted. I used Shandon Xylene Substitute Mountant to secure a Corning (22 x 50 mm) glass cover slip over the serial sections. After the cover slip was in place, I allowed the slide to dry for 24 hours in an enclosed slide case, after which the slide was cleaned of excess mountant and was then restored in the slide case until examined for LAGS.

Skeletochronological Analysis: Reading Cross-Sections

I examined all prepared slides to identify those slides containing cross sections of the femur shaft that would be included in the study. After those slides were identified, I examined each slide under 40X, 100X, and 400X magnifications to count the number of LAGs present. I recorded the number of LAGs, relevant histomorphological

features of cross-section(s) (e.g., double lines), and if there was any uncertainty as to the exact number of LAGs for a particular specimen.

Afterwards, Brian Miller, Ph.D. (thesis advisor) independently examined the same set of specimen slides and recorded the number of LAGs for each specimen on a separate data form and also noted relevant histomorphological features of cross-section(s). Dr. Miller and I then met and viewed each specimen slide again, discussed the LAG count for each specimen, and resolved any difference between our LAG counts. We agreed to count the outer border of the cross-section as a LAG, making the number of LAGs equivalent to the specimen's age. The LAG closest to the endosteum was not included in our count and was considered the metamorphic LAG. The following criteria were used to identify the metamorphic LAG: 1) the first annulus is the metamorphic annulus, 2) the width of the zone between the metamorphic annulus and the outermost (true) annulus is much less than the width of zones observed between subsequent LAGs, 3) and the first true LAG has a higher stain density as compared to other subsequent LAGs.

Statistical Analysis

I reported the number of specimens included in this skeletochronology study (sample number), computed the lowest to highest age estimate (range), the most frequent age estimate (mode), and the average specimen age (mean). I also computed the Pearson product-moment correlation (r) for age and femur length, for age and snout-vent length, and for femur length and snout-vent length. Data was analyzed using Minitab statistical software package version 17. 1.

CHAPTER FOUR: RESULTS

Although slides of cross-sections from 56 specimen femurs were prepared for skeletochronological analysis, useable cross-section slides were yielded from only 28 specimens. This occurred due to the failure of the Scientific SuperFrost Plus (charged) slides to retain femur cross-sections during the course of the histochemical bath series.

LAGs were counted and age estimates were determined for all 28 specimens (Table 1). Femur length was measured for only 19 of the 28 specimens. This occurred because measurement of femur length was not part of the original research protocol and was initiated after some femurs had already been embedded. Snout-vent length (SVL) was estimated for four specimens and measured for 19 specimens. Snout-vent length could not be determined for five specimens because their bodies were severely mangled.

Table 1

Skeletochronology Data for N. v. viridescens Sample (dashes = data absent)

Specimen Number	Age Estimate	Femur Length	Measured SVL	Estimated SVL
1	5	0.6	3.8	-
2	7	0.6	-	3.5
3	4	0.7	-	-
4	3	0.6	3.9	-
5	4	-	3.5	-
6	5	0.6	3.6	-

Table 1 (continued)

Skeletochronology Data for N. v. viridescens Sample (dashes = data absent)

Specimen Number	Age Estimate	Femur Length	Measured SVL	Estimated SVL
7	2	-	3.7	-
8	4	0.8	4.2	-
9	3	-	3.8	-
10	3	-	3.1	-
11	4	-	3.6	-
12	4	-	-	4.0
13	2	0.4	-	-
14	2	0.7	3.7	-
15	3	0.6	3.8	-
16	3	-	-	4.1
17	2	0.4	3.4	-
18	2	0.6	4.3	-
19	3	-	4.2	-
20	2	0.7	4.0	-
21	3	-	-	4.0
22	3	0.6	-	-
23	5	0.6	4.4	-

Table 1 (continued)

Skeletochronology Data for N. v. viridescens Sample (dashes = data absent)

Specimen Number	Age Estimate	Femur Length	Measured SVL	Estimated SVL
24	5	0.5	4.2	-
25	5	-	-	-
26	4	0.5	-	-
27	3	0.6	3.9	-
28	2	0.5	3.9	-

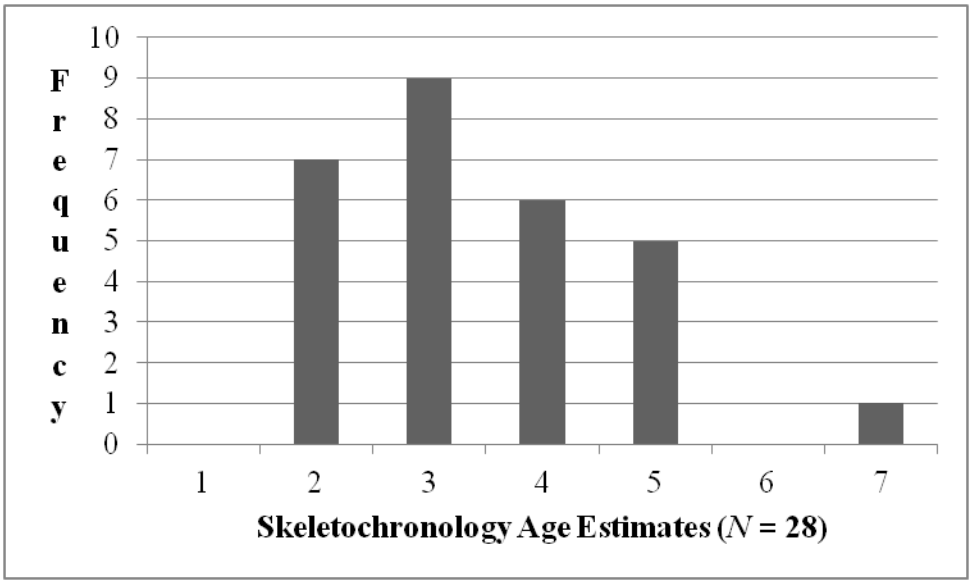

Figure 1. Frequency of skeletochronology ages.

Specimen age estimates ranged from two years old to seven years old with the most frequent age estimate being three years (Figure 1). Mean specimen age was 3.5 years. Pearson Product Moment Correlations were computed for age and femur length, for age and measured snout-vent length (MSVL), and for femur length and snout-vent length to determine the presence of linear relationship amongst paired variables (Table 2). Based on yielded correlation coefficients and their P-values, a stepwise regression analysis using femur length and snout-vent length as predictor variables for specimen age was deemed unnecessary.

Table 2

Variable Correlation Coefficients with P-values (n = 19)

Variables	Correlation Coefficient	Significance (P-value)
Age/Femur Length	0.157	0.534
Age/MSVL	0.208	0.408
Femur Length/MSVL	0.349	0.243

CHAPTER FIVE: DISCUSSION

Age estimates were determined for 28 specimens. Annuli were visible and LAGs were easily counted for most specimens. Difficulty was encountered with two slides that had a limited number of cross-sections per slide and slide preparation was generally poor. However, Dr. Miller and I were able to obtain agreement on the number of LAGs present. As described by Forester and Lykens (1991), endosteal bone reabsorption and multiple annuli (doublets) were observed. Although endosteal bone reabsorption was frequently observed, incomplete and double LAGs (i.e., two LAGs in such close proximity to each other that there can be points of contact between the two LAGs) were infrequently observed. The reason for incomplete and double LAGs is not clear; however, it is thought they are a reflection of metabolic inactivity during periods of severe drought (Forest and Lykens, 1991).

Bone is a dynamic tissue in which internal remodeling occurs (Woodward et al., 2013). Growth in amphibians continues throughout their life time; however, it is interrupted by winter torpor, which results in the deposition of a line of arrested growth (LAG) (Kazmer, 1986). As bone is being laid down by the periosteum, calcium salts and other minerals are being recycled (i.e., reabsorbed) resulting in the bone being reorganized at the endosteal surface, the surface adjacent to the central lumen of the long bone (Forest and Lykens, 1991). In cross-section, the endosteal bone ring appears wide, dense, and deeply stained. Endosteal reabsorption is possible and could obliterate one or more of the inner LAGs. In this sample of newts, the endosteal ring was readily identifiable and following it, in whole or in part (due to reabsorption), there was a

very narrow first periosteal growth ring (i.e., the metamorphic LAG). The width of the zone between the metamorphic annulus and the outermost (true) annulus was much less than the width of zones observed between subsequent LAGs. Also, the first true LAG had a higher stain density as compared to other subsequent LAGs and often showed invaginations. Because these characteristics were identifiable in cross-sections, I believe accurate counts of LAGs were obtained in spite of the presence of some endosteal bone reabsorption (Figure 2). In this sample, the observed presence of the metamorphic LAG outside and close to the endosteal surface and with limited endosteal reabsorption occurring is consistent with the observations of Red Efts in the studies of both Kazmer (1986) using humerus cross-sections from a South Carolina sample, Forester and Lykens (1991) using femur cross-sections from a Maryland sample, and Leclair et al. (2005) using humerus cross-sections from a Quebec sample. Collectively, this study and others support the hypothesis that endosteal bone is eroded over time, but that it is not a significant factor that obfuscates the accurate age determination of Red Efts using the skeletochronology method.

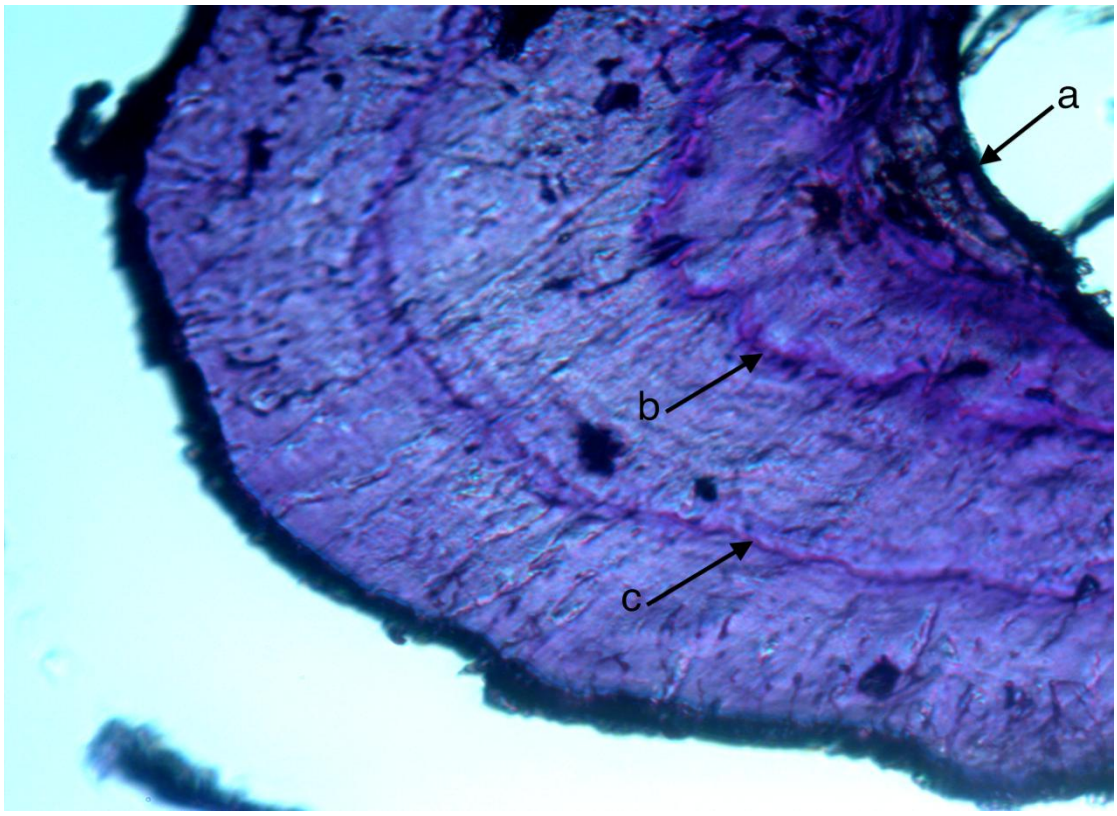

Figure 2. Femur cross-section of a 3rd year Red Eft showing (a) wide and darkly stained endosteum followed by the first periosteal (metamorphic) LAG, (b) darkly stained first true LAG showing invaginations, and (c) the lighter-stained second year LAG.

In this study, age estimates ranged from three years to seven years, with most individuals being three years old (Figure 1). The mean age in this study was 3.5 years. In the study of Forester and Lykens (1991), the age estimates of 32 Red Efts ranged from two years to seven years with the most frequently occurring age being five years old and the mean age being 4.4 years. In the study of Leclair et al. (2005), the age estimates of 226 Red Efts ranged from one year to five years with the most frequently occurring age being four years old. The mean age for 79 Red Efts capable of being sexed from the 226 Red Eft sample was three years. The range of this study's age estimates fits within the

range of age estimates defined by previous studies (i.e., one to seven years). However, the most frequently occurring age in this study is lower than those of previous studies. However, this does not necessarily negate the existence of a relationship between length of time spent in the Red Eft stage and latitude. In examining age means from the three studies, a relationship is suggested between latitude and Red Eft age: Quebec sample = 3.0 years (Leclair et al., 2005), Maryland sample = 4.4 years (Forester and Lykens, 1991), and Tennessee sample = 3.5 years (this study). Again, the thought is the length of the Red Eft stage is likely to be longer at lower latitudes because of periods of drought than at higher latitudes. The difference in the mode and mean of this study compared to that of the Forester and Lykens (1991) study is likely to be the result of biased sampling (non-random selection), sample loss (in this study, $n = 28$) and a small sample size (in both studies).

In the studies of Forester and Lykens (1991) and Leclair et al. (2005), snout-vent length was strongly correlated (i.e., $r = 0.82$ and $r = 0.93$, respectively) with age. However, in this study that was not the case, $r = 0.208$. One possible explanation for this difference relates to how the Red Eft samples were obtained for the skeletochronology studies. In Forester and Lykens (1991) and Leclair et al. (2005) studies, Red Efts were captured alive, were killed, and then immediately preserved. In our study, Red Efts were collected dead as road kill and they had been laying on the road after death for an unknown period of time. Most likely, the result of this was an alteration in body size that varied from individual to individual depending on the number of times it had been run over and how long it was on the road after death. Another variable that may have

contributed to the difference in findings relates to precision of measurement of snout-vent length. In Forester and Lykens (1991) and Leclair et al. (2005) studies, the snout-vent lengths were measured to the nearest 0.5 mm using calipers. In this study, I used a centimeter ruler and measured snout-vent lengths to the nearest mm.

Age and femur length were found to be even less correlated ($r = 0.157$) than was age and snout-vent length ($r = 0.208$). When femurs were removed from specimen bodies, however, they were found to be intact. The correlation between femur length and snout-vent length was also not significant ($r = 0.349$). Of course, the variable alteration of specimen body size and the use of less precise instrumentation to determine femur length and snout-vent length would undermine the validity of these correlation coefficients.

The complex life cycle of the of *N. v. viridescens* is an evolutionary strategy to exploit and survive harsh environments (Noble, 1929) with the Red Eft stage being important for the maintenance of reproductive sinks (Gill, 1978). The metamorphosis of a Red Eft into a sexually mature (aquatic) adult is quite variable with few individuals doing so prior to their seventh year (Forester and Lykens, 1991). This lengthy period of terrestrial foraging is likely to relate to the acquisition of fat stores (Verrell 1982, 1985) and body size (Adams and Rae, 1929) both of which may convey reproductive advantage to the individual animal as an aquatic adult. Selection has favored a complex and variable life cycle for *N. v. viridescens* involving both aquatic and terrestrial environments with many individuals living at least 10 to 15 years (Forester and Lykens, 1991).

Studies of variation in the life cycle of and variation between populations of *N. n. viridescens* are few in number due to the cost and time involved in doing sequential collections and mark-recapture work. Skeletochronology represents a valid and reliable alternative to costly and time-consuming field studies, offering a method to not only assess population structure, but to examine various ecological relationships (Forester and Lykens, 1991).

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