AN ABSTRACT OF THE DISSERTATION OF

Lisa A. Wagner for the degree of Doctor of Philosophy in Environmental Science presented on September 26, 2014.

Title: Life History Variables of *Dicamptodon* Salamanders.

Abstract approved:

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Lynne D. Houck

It is good practice to fully understand components of an ecosystem if we hope to preserve its biodiversity. A problem is that we know very little about some organisms and nothing of others. Studies that investigate an organism's basic biological, ecological and physiological life history variables advance our knowledge of the species of interest and also offer insight about community structure and function. In this thesis I explore courtship behaviors, metamorphic tendencies and hematological parameters in two species of *Dicamptodon* salamanders (*D. copei* and *D. tenebrosus*).

Ecologists examine blood to assess hematological responses to a plethora of factors including hormonal changes, parasites, reproductive status and environmental stressors. While hematologic principles are fairly conserved in vertebrates, blood tissue has evolved to match the organism with its environment and there is considerable diversity in hematological parameters across taxa. Therefore, it is necessary to collect species specific baseline data for comparison. In Chapter 2, I

quantified relative white blood cells and erythrocyte dry volume measurements of both *D. tenebrosus* and *D. copei* in the wild and in captivity. No prior studies have evaluated the hematological parameters of *Dicamptodon* salamanders. My investigation revealed similar leukocyte ratios between species and between wild and captive *D. tenebrosus*, while leukocyte ratios of wild and captive *D. copei* were significantly different.

Dicamptodon species and populations vary in metamorphic tendencies though the reasons for this variation are not known. In Chapter 3 I investigated the difference in metamorphic tendencies of *D. copei* and *D. tenebrosus*. First, I tested the hypotheses that high water inhibits and thermal stress induces metamorphosis. Neither lowered water, nor increased aquatic temperatures induced metamorphosis. Secondly, I compared thermal preferences of both species and found differences in the selection tendencies between species and between sizes of both species. Lastly, I stress responses of both species to 1.66º C, 21.11 º C and 25º C water using a hematological approach. This study revealed significant differences in hematological stress indices between the two species.

In Chapter 4, I explored the courtship behaviors of *D. copei* and *D. tenebrosus* and characterized each behavior and temporal pattern, using a phyloethological approach. I then compared these courtship behaviors between species and to that of the nearest salamander family, *Ambystomatidae*. The courtship patterns and behaviors were similar in both species, but they did not resemble the courtship patterns or behaviors of their sister taxon, *Ambystoma*.

Life History Variables of *Dicamptodon* Salamanders

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Lisa A. Wagner, Author

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Life History Variables of *Dicamptodon* Salamanders.

Chapter 1

General Introduction

If we are to competently preserve biodiversity (which our very lives depend on) we must know as much about as many organisms as possible (E.O. Wilson 2007; Mergeay et al. 2010; Proenka et al. 2013). Studies that investigate the life history variables of individual species provide vital information for understanding their biological, physiological and ecological needs so that we may accurately predict and manage their sustainability over space and time. In this thesis, I explore life history variables of two species of *Dicamptodon* salamanders (*D. copei* and *D. tenebrosus*).

Why Dicamptodon?

Much of *Dicamptodon* biology, physiology and ecology is unknown though, in the Pacific Northwest, they inhabit a large number of headwater streams, rivers and lakes, the hyporeic zones within them and subterranean zones beside them. They are North America's largest terrestrial salamanders, yet terrestrial forms are not easily observed in the wild and very little is known about them. Aquatic *Dicamptodon* are of two forms, paedomorphic and larval, and are often the top predator in streams, accounting for up to 99% of the predator biomass (Davic et al. 2004). Their presence or absence has and does, undoubtedly, affect entire ecosystems. For this dissertation, I investigated aspects of reproduction, metamorphosis and hematological physiology previously unexplored in *Dicamptodon* salamanders.

In Chapter 2, I explore the variable metamorphic tendencies of *D. copei* and *D. tenebrosus* and the possible links to environmental stress. In Chapter 3, I consider the definition of environmental stress in *D. copei* and *D. tenebrosus* by first establishing

baseline hematological parameters in nature and in captivity, and subsequently testing the hematological responses to the three temperatures outside the range of known stream temperatures they inhabit. In Chapter 4, I explore the behavioral and evolutionary aspects of reproduction by documenting and characterizing courtship, mating and oviposition of both *D. tenebrosus* and *D. copei*.

Here, to better understand the context of my experiments, I provide a literature review of *Dicamptodon* biology, systematic and taxonomic history, species distribution and ranges, and the genetic, environmental and hormonal basis of phenotypic variability in salamanders.

***Dicamptodon* taxonomy**

The systematic and taxonomic history of *Dicamptodon* began with confusion and controversy (Nussbaum 1972). Although much of the confusion has been resolved, the familial status of Dicamptodontidae remains unsettled (Pyron et al. 2011, Wake 2012, Frost et al. 2003).

Ambystomatidae once contained two subfamilies, Dicamptodontinae and Rhyacotritoninae, and three genera, *Dicamptodon*, *Rhyacotriton* and *Ambystoma* (Tihen 1958). Regal (1966) eliminated the subfamily Rhyacotritoninae by placing Rhyacotriton within the subfamily Dicamptodontinae. Based on the unique arrangement of spinal nerve pathways, Edwards (1976) determined that *Rhyacotriton* and *Dicamptodon* are morphologically distinct from all other salamanders and thus raised Dicamptodontinae to Dicamptodontidae and placed both *Rhyacotriton* and *Dicamptodon* within the family Dicamptodontidae. Estes (1981) and Duellman and Trueb (1985) agreed with the familial status of Dicamptodontidae. Larson (1991) hypothesized that

Rhyacotriton are not closely related to *Dicamptodon* based on nucleotide sequences and Good and Wake (1992) put *Rhyacotriton* in its own family Rhyacotritonidae. That placement is currently well accepted (Larson and Dimmick, 1993; Weisrock et al., 2005; Wiens et al., 2005; Frost et al., 2006; Roelants et al., 2007).

Until recently, *Dicamptodon* was the considered the only genus within the family *Dicamptodontidae*. There is no disagreement regarding the close taxonomic relationship of *Dicamptodon* and *Ambystoma* (Weisrock et al. 2005, Frost et al. 2006, Wiens et al. 2011). There is, however, apparent contention regarding the placement of *Dicamptodon* within its own family, *Dicamptodontidae*. During a recent renovation of amphibian taxonomic arrangements, Frost et al. (2006), returned *Dicamptodon* to the family *Ambystomatidae* because, (a) they rejected Edward's (1976) hypothesis and (b) they wanted to consolidate two families that each contain only one genus each. Zhang and Wake (2009) disagreed with this placement, citing considerable morphological differences as well as fossil evidence of ancient family divergence (Good and Wake, 1992).

In this dissertation, I consider Dicamptodontidae and Ambystomatidae as distinct families (Vietes et al. 2009, Pyron et al. 2011) because I agree with Zhang and Wake's assessment of their placement based on ancient lineage and morphological differences and because the return of *Dicamptodon* to Ambystomatidae by Frost et al. (2006) was not done on the basis of empirical evidence, but was more likely an effort to simplify the taxonomic arrangement.

Until recently, *Dicamptodon* consisted of a single species, *D. ensatus* (Nussbaum, 1972). Nussbaum (1973) described *D. copei*, thus dividing the genus into two species.

Allozymic studies by both Daugherty et al. (1983) and Good (1989) led to the separation of the genus into four species. Currently, these four distinct species are recognized as: *D. ensatus, D.tenebrosus, D. copei* and *D. aterrimus* (Steele et al. 2005, Good 1989).

Some clarity has been reached, via genetic research, regarding species divergence timing and placement relative to each other (Steele et al. 2005, Daugherty et al. 1983). Steele et al. (2005) determined that the genetic distance between *D. copei* and other *Dicamptodon* spp. supports the hypothesis by Daugherty et al. (1983) that *D. copei* is an ancient lineage that probably diverged prior to the Pleistocene epoch (2,588,000 to 11,700 years ago). Nussbaum (1972) hypothesized that *D. aterrimus* diverged during the late stages of the Cascade orogeny (Upper Miocene-Lower Pliocene). The Pacific Northwest was "broken by volcanism" and the Columbia plateau lifted, producing a rain shadow that isolated the western-most *Dicamptodon* populations. Steele (2006) supported this hypothesis and also determined by genetic analysis that *D. tenebrosus* and *D. ensatus* are sister taxa, and neither are as closely related to *D. copei* as once believed (Figure 1.1).

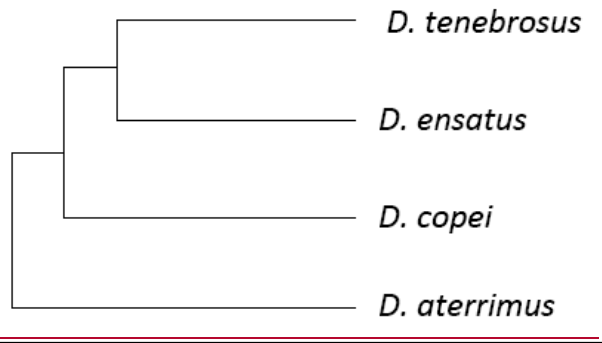

Figure 1.1- Phylogenetic relationships of *Dicamptodon* salamanders (Steele 2006)

Species distributions and ranges

Dicamptodon species are endemic to the Pacific Northwest, each species having a distribution apparently shaped by isolation during Pleistocene glaciations and recent expansions (Steele 2005, Nussbaum 1972).

D. aterrimus inhabit streams in Mineral County, Montana, and Lake Coeur d'Alene River, Idaho, south to the Salmon River, Idaho (Reichel and Flath 1995, Petranka 1998, Daugherty et al. 1983, Nussbaum *et al.* 1983). Disjunctive populations of *D. aterrimus* occur in the Salmon River drainage near Warm Lake, Idaho (Petranka 1998).

D. copei are distributed from the northwestern Olympic Peninsula, Washington to the western side of the Cascade Mountains. Their range extends to the Oregon side of the Columbia River Gorge with a southern boundary of Rhododendron, (Clackamas County) Oregon and an eastern population in Wasco County, Oregon, that is east of the Cascade crest (Jones and Corkran 2002, Steele et al 2006). The southeasternmost record for *D. copei* is 9 km east of the mouth of the White River, Wasco, Co., Oregon (Bury 2014). The *D. copei* are sympatric with *D. tenebrosus* in the southern and eastern most scope of their range (Steele, 2006; Daughtery et al. 1983; Good, 1989; Nussbaum 1970, 1976).

That *D. tenebrosus* and *D. copei* live sympatrically as well allopatrically, provides an opportunity to examine the mechanisms by which they are able to coexist. These mechanisms may be observed when examining life history variables of these species in both sympatric and allopatric conditions. I sampled *D. copei* from multiple localites, from allopatric conditions on the Olympic Peninsula to sympatric populations near the Oregon/Washington boarder where I sampled *D. tenebrosus* as well.

The distribution of *D. tenebrosus*, extends from the south bank of the Frasier River in extreme southwest British Columbia, eastwardly in a narrow band along the Cascade Mountain range. This band is around, but not on, the Olympic Peninsula. Just south of the Olympic Peninsula, the distributional band expands west to the coastal boarder and south to the northern end of Mendocino County. There are two disjunctive populations of *D. tenebrosus* in Oregon: the first occurs just east of the Cascade Crest at Oak Spring, near Maupin, (Wasco Co) Oregon. The second occurs in Shoat Springs, Jackson Co. Oregon (Nussbaum et al. 1983, Leonard et al. 1993). The ranges of *D. ensatus* and *D. tenebrosus* overlap, creating a hybrid zone occurring approximately 10km north of Gualala, Mendocino County, Ca.

The range of *D. ensatus* extends from Sonoma and Napa Counties, Ca. south to Santa Cruz Co., California and Monterey Co., Ca. (Petranka 1998). Their distribution also extents west from near Point Arena Mendocino Co, Ca., east to Lake and Glenn counties, Ca.

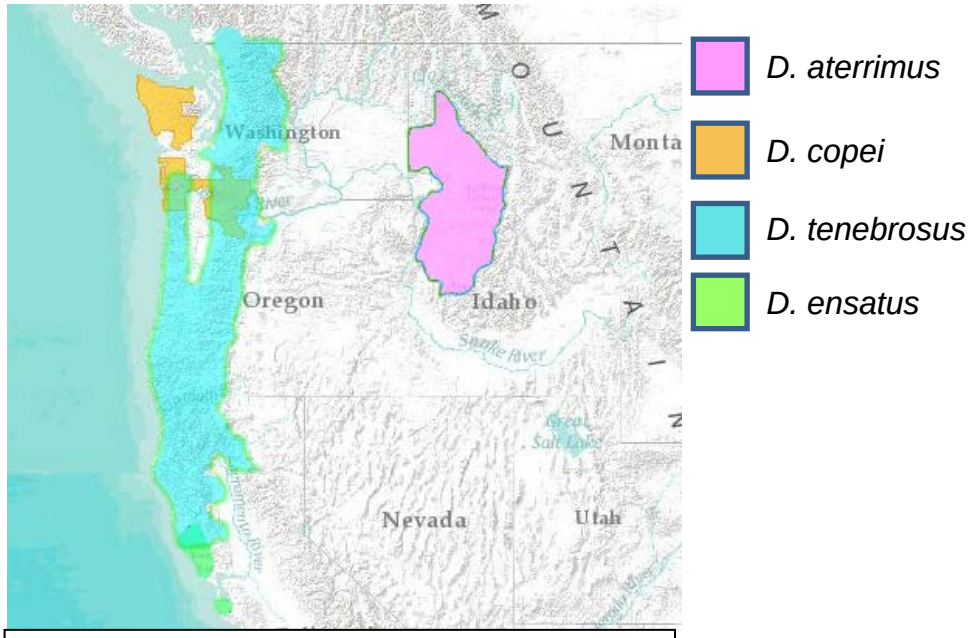

Figure 1.2- *Dicamptodon* distribution

Dicamptodon life history and ecology

Habitat

In general, *Dicamptodon* occur in damp forests and are associated with streams, rivers, mountain lakes, hyporheic zones and riparian zones (Nussbaum et al., 1983; Leonard et al. 1993, Dudaniec et al. 2012). Branchiate individuals usually remain in the water; however, they may be seen on land during rainy nights (pers. obs), presumably hunting. Individuals take cover in the water, beneath rocks or leaf litter and within the interstitial spaces of the hyporheic zone (Feral et al. 2005). Metamorphosed individuals usually inhabit riparian zones where they hide in underground burrows, tree root systems, under rocks, as well as in and along the edge of streams (Dudaniec et al. 2012, Johnston 1998). Metamorphs may also inhabit aquatic environments during particularly dry weather and during mating seasons (pers. observations).

Although there are habitat generalities common to all *Dicamptodon*, the use of species-specific, stream reach, models could be used to accurately predict the presence of *Dicamptodon* because populations often inhabit very diverse and dynamic environments (Dudaniec et al. 2012, Welsh et al. 2002; Tingely et al. 2010). In 2012, Dudaniec et al. reported that the best predictor of *D. tenebrosus* presence within a stream reach was high stream gradient. In addition, higher elevation and greater forest age positively correlate with *D. tenebrosus* presence. Others have reported that branchiate *D. tenebrosus* are most often located in streams where course substrates and large stones are present and fine sediment does not fill their interstices (Welsh 1993, Parker 1991, Nussbaum et al., 1983, Corn and Bury, 1989a; Welsh and Ollivier, 1998).

Unlike the habitat predictions made for *D. tenebrosus,* Sepulveda et al. (2009) reported that the highest densities of *D. aterrimus* were in streams with a high proportion of embedded substrate and fine sediment. The probability that salamanders would inhabit a particular stream were the highest for roadless drainages and the lowest for streams that are spatially isolated or located in an old growth forest (Sepulveda et al. 2009). The authors hypothesized that the positive association with sediment filled, embedded substrate with *D. aterrimus* occurrence may be due to adaptations to high frequencies of landslides and other natural disturbances. Although the Washington State Peninsula has frequent natural disturbances such as landslides, I did not observe this adaptation for *D. copei*.

Unique to *D. ensatus,* Forsman et al. (2007) reported a single case in which a terrestrial adult was found in an arboreal vole nest in a "windswept Douglas-fir", 2.4m above ground. The presence of young voles and the absence of the adult suggest that the "large" salamander was hunting. Nonetheless, the discovery of such climbing abilities suggests that *D. ensatus* may be capable of an arboreal inhabitance.

Fellers et al. (2010) reported aggregations of 23 and 27 metamorphosed *D. ensatus* discovered during two culvert replacements. Metamorphosed *Dicamptodon* had not been observed in large numbers in a single location, previously and the discovery was unexpected due to predatory and territorial behaviors of these salamanders (Fellers et al 2010). Dispersal, displacement and breeding were dismissed as motivating factors for these aggregations (Fellers et al 2010). However, the current study presents evidence that courtship may have been a factor. Regardless of motivation, the aged and leaky culverts apparently provided a high quality habitat for metamorphosed *Dicamptodon*.

Furthermore, terrestrial adults may migrate to quality habitats during dry weather or to seasonally mate.

Prey and predator interactions

Dicamptodon are opportunistic, voracious predators and are considered key ecological members (Davic and Welsh, 2004) because they sometimes comprise up to 99% of the predator biomass and replace fish as the top aquatic predator (Murphy & Hall 1981). *Dicamptodon* can shift their hunting strategies from sit and wait predation to active pursuit and they will hunt continuously (Parker 1994). Darkness probably provides cover from potential predators and *Dicamptodon* have been observed moving about the stream bed and forest floor during the night, presumably hunting.

The primary diet of aquatic *Dicamptodon* includes aquatic insects (Ephemeroptera, Plecoptera, Tricoptera, Diptera, Odonata, Megaloptera, Hemiptera, Coleoptera). However, other prey also are consumed by aquatic *Dicamptodon*: fresh water snails (*Juga*), earthworms (*Lumbricus*), crayfish (*Pacifasticus*) aquatic vertebrates (*Oncorhynchus, Cottus, Dicamptodon*) and non-aquatic insects (Ephemeroptera, Plecoptera, Tricoptera, Diptera, Odonata, Megaloptera, Hemiptera, Coleoptera). *Dicamptodon* also consume terrestrial organisms (Ostracoda, Acarina, Nematomorpha, Turbellaria) (Parker 1994, Bury 1972, 1974, Antonelli et al. 1972).

Terrestrial *Dicamptodon* consume shrews (Sorex), voles (*Microtus longicaudus*) mice (*Mus musculus*), insects (Ephemeroptera, Plecoptera, Tricoptera, Diptera, Odonata, Megaloptera, Hemiptera, Coleoptera), earthworms (*Lumbricus*), snails (Gastropoda), and other amphibians (Parker 1994, Bury 1972, 1974, Antonelli et al. 1972).

Dicamptodon are preyed on by raccoons (*Procyon lotor*), garter snakes (*Thamnophis atratus*), voles (*Microtus*), and fish (*Onchoryncus*) (Welsh and Lind 2010, Parker 1992; Parker, 1991; Lind et al., 1990; Bury 1972).

Reproduction

There is a paucity of published information on *Dicamptodon* reproductive behavior. Though circumstantial, there is evidence that suggests that terrestrial *D. tenebrosus, D. aterrimus* and *D. ensatus* breed during the spring and fall, and that clutch frequency is biennial (Nussbaum and Clothier 1983; Nussbaum, 1969). Nussbaum and Clothier (1983) hypothesized that paedomorphic populations of *Dicamptodon*, primarily, *D. copei* may continue to breed throughout the year (Lohman and Bury, 2005; Nussbaum, 1983) though there is no supporting evidence.

Age at sexual maturity in *D. copei* is unknown. However, 30 sexually mature individuals were discovered, and these ranged from 69 to 104mm snout to vent length (SVL), a size much smaller than other mature *Dicamptodon* (Nussbaum et al. 1983). Size at first reproduction in *D. tenebrosus, D. aterrimus* and *D. ensatus* is thought to occur when the animal is approximately 115 mm snout to vent length (SVL) in both larval and terrestrial forms. Size at first reproduction may vary between and within populations. Because size does not necessarily correlate with age within and between species (Nussbaum, 1970) we cannot infer age from size, though it has been common practice to do so.

Deposition, clutch and egg size

Females typically deposit eggs singly on the underside of objects such as rocks or logs. *Dicamptodon* eggs have been discovered in several aquatic locations including:

under a log in a fast flowing stream (Henry and Twitty 1940), in a stable talus and earth bank adjacent to a stream (Nussbaum 1969), attached to a log in a riffle at the edge of the stream (Jones et al. 1990) and in a rock pile at the base of a waterfall (Nussbaum 1969).

Reported clutch and egg sizes have varied. Nussbaum et al. (1983) reported finding two *D. tenebrosus* (formerly *D. ensatus*) clutches, one contained 146 eggs and the other contained 83 eggs (Nussbaum 1969). Also, Nussbaum (1969) brought a gravid female *D. tenebrosus* to the laboratory and it deposited 185 eggs. Jones et al. (1990) counted 129 eggs from a *D. tenebrosus* clutch. Steele et al. (2003) reported two clutches of *D. copei* one containing 28 eggs while the other had 23 eggs. We do not know the reasons for the differences in clutch sizes. Egg sizes for all species were between 5.5 and 8.5 mm in diameter. Varying developmental stages of the ovipositing female may explain the size variance.

Although Nussbaum indicated that the incubation time is 9 months (Nussbaum 1969, 1987), Jones et al. (1990) estimated that hatching occurs in considerably less time.

To better understand the reproductive biology of this group, I video documented and characterized the courtship and mating behaviors of *D. tenebrosus* and *D. copei* species during staged trials for comparison with their nearest salamander relatives in the family, Ambystomatidae (see Chapter 4).

Metamorphosis and paedomorphosis

Amphibians are so named because they generally spend the embryonic and larval portion of their lives in water and adult stage on land. The transition from one environment to the next involves large-scale morphological and physiological changes that cannot be reversed. As amphibians diversified, adaptive modifications to nearly

every stage of transition evolved so that some amphibians never spend a moment of life in water nor do some ever emerge to live on land. Many amphibians have evolved so that the timing of metamorphosis is timed to or triggered by environmental cues. Salamanders, however, are the only amphibians to exhibit paedomorphosis.

Paedomorphosis describes a life history option to the generalized biphasic developmental pathway of many salamanders. Paedomorphosis is the retention of larval morphology in reproductive adults (Voss, 1995; Semlitsch and Wilbur, 1989; Gould, 1977). Previously, the term "neoteny" was used synonymously. Recent discussion (Denoel et al. 2005; Denver et al., 2002; Denoel and Joly, 2000; Gould 1977) more clearly defines "neoteny" as one of a few ontogenetic (origin and developmental) pathways by which a paedomorphic state is achieved. In particular, body growth and sexual maturation remain unchanged in a neotenic individual, but somatic development is slower than that of a metamorphic individual (Denoel et al. 2005). Changes in developmental timing are known as heterochrony (Denoel and Joly 2000; Ryan and Semlitsch, 1998; Wakahara, 1996; Gould, 1977). Neoteny is one of several heterochronies known to result in paedomorphosis (Denoel and Joly, 2000; Ryan and Semlitsch, 1998; Harris, 1987).

A paedomorphic individual is either an obligate paedomorph, or a facultative paedomorph. Both forms of paedomorphs become mature while retaining larval characteristics such as gills and gill slits, larval hemoglobin, tail fin, and pliability of skeletal structures (Denoel et al. 2001, Semlitsch and Wilbur, 1989; Whiteman, 1994). Obligate paedomorphs are permanently gilled and remain in an aquatic habitat their entire lives (Denoel et al. 2005; Shaffer and Voss, 1996; Whiteman, 1994). Families consisting

entirely of obligate paedomorphs include; Proteidae, Cryptobranchidae, Amphiumidae, and Sirendidae. In nature, these animals never metamorphose and paedomorphosis seems to be genetically fixed. Obligate paedomorphs are also found within Plethodontidae and Ambystomatidae and Hynobiidae (Denoel et al. 2005; Shaffer and Voss, 1996).

Facultative paedomorphs have the ability to become a paedomorph or a metamorph in response to environmental cues (Denoel et al. 2005; Whiteman, 1994; Jackson and Semlitsch, 1993, Semlitsch, 1987). This polymorphism can result in the coexistence of larval and transformed individuals as well as paedomorphic and metamorphic adults in the same populations (Shaffer and Voss, 1996; Whiteman, 1994). Facultative paedomorphosis is found in *Salamandridae, Ambystomatidae, Hynobiidae* and in some *Plethodontidae* (Denoel, 2005; Denoel et al. 2001; Whiteman, 1994; Harris, 1987). Some facultative paedomorphs retain the ability to transform, but will usually only do so after the first breeding season (Denver et al. 2002; Denoel et al. 2001; Ryan and Semlitsch, 1998). Metamorphosis is not reversible.

Facultative paedomorphic populations occur in *D. ensatus* and *D. aterrimus* and *D. tenebrosus. D. copei* have long been considered obligate paedomorphs (Nussbaum 1972). However, an increasing number of transformed *D. copei* have been observed (Spear et al. 2011). These discoveries suggest that at least some populations of *D. copei* are not obligate paedomorphs and that they have retained the ability to transform facultatively.

Genetic basis of paedomorphosis/metamorphosis

Salamanders of the genus *Ambystoma* were used in several crossbreeding experiments to establish a genetic basis for a life history pathway. Thompkins (1978) proposed that homozygous recessive alleles from a single gene are responsible for the obligately paedomorphic state in *Ambystoma mexicanum*. He based this hypothesis on 1:1 mendelian ratios obtained from backcrosses between *A. mexicanum* and *A. tigrinum* (Voss and Shaffer, 1996). Voss (1995) was not able to replicate these findings using the same crossing techniques.

Semlitsch and Gibbons (1985) found that first generation offspring of different populations of *Ambystoma talpoideum* varied in frequency of paedomorphosis in response to pond drying, though they also recognized the potential for nongenetic maternal effects. Other intraspecific crossbreeding experiments (Harris, 1987; Semlitsch 1987a) demonstrated that phenotypic expression of life history pathways varied when populations were raised under different conditions.

Harris (1987) suggested that metamorphosis and paedomorphosis are “threshold traits” whereby each population exhibits a response threshold relative to environmental factors. Constant selection pressure would eventually result in elimination of plasticity. The hypothesis of genetic differentiation in the ability to become metamorphic or paedomorphic was supported by several studies (Voss et al. 2003, Shaffer and Voss, 1996; Harris et al. 1990; Semlitsch et al. 1990; Semlitsch and Wilbur, 1989).

Voss and Shaffer (1997) identified a major quantitative trait locus for the expression of metamorphosis in *Ambystoma mexicanum* using interspecific crossing and genetic linkage analysis. Voss and Shaffer (2000) repeated the experiment using *A. mexicanum*

sampled directly from their natural habitat instead of animals raised in the laboratory. They found no significant association between the gene and metamorphic failure, although a smaller genetic effect was supported.

Even though intra- and interspecific crosses are suggestive of genetic underpinnings for life history phenotypic expression, currently the genetic basis of paedomorphosis is unclear and the genetic architecture unknown.

Hormonal control of amphibian metamorphosis

The majority of research concerning hormonal control of amphibian metamorphosis has been done using anuran models, and assumes validity for both frogs and salamanders. Although anuran species do not exhibit paedomorphosis some populations do exhibit plasticity in metamorphic timing by responding to stress (such as pond drying) in much the same way as do many caudates (Tata, 2007; Denver and Boorse, 2002, Denver et al., 1998; Wilbur, 1987). It appears that anuran endocrine models are valid for both anurans and caudates (Denver and Boorse, 2002). We do not know to what extent the anuran model of metamorphosis applies to caudates.

Amphibian metamorphosis and the timing thereof is controlled by environmental and genetic factors (Denver et al. 2002) but is mediated and carried out by the neuroendocrine axis (hypothalamus and pituitary gland) which controls the activity of the thyroid and interrenal (homologue of mammalian adrenal cortex) glands (Kikuyama et al. 1993; Denver, 1996). German biologist J. F. Gudernatsch (1912) was the first to demonstrate the role of the thyroid in amphibian metamorphosis. He found that feeding mammalian thyroid tissue to tadpoles induced precocious metamorphosis. Further evidence of the relationship between the thyroid and metamorphosis in amphibians came when Allen

(1916) prevented metamorphosis in tadpoles by thyroidectomy (Tata, 2007; Denver et al. 2002; Kanamori et al. 1996). In 1915, an iodine containing protein compound derived from the thyroid and named thyroglobulin was structurally identified by American chemist Edward Kendall (1915) as 3,5,3'5'-tetraiodothyronin, L- thyroxine (T_4). T_4 was thought to be the only active thyroid hormone until 1952 when 3,5,3' –triiodothyronine, T_3 was discovered in the blood and thyroid of lab rats (Tata, 2007; Denver 2002). In 1968, William Etkin proposed a model that explained thyroid hormone activity during metamorphosis in relation to morphological changes that occur (Etkin, 1968). It is now widely accepted that exogenous thyroid hormones (T_3 and T_4) can trigger a cascade of molecular, biological and morphological changes necessary to begin and complete metamorphosis in some amphibians (Boorse and Denver, 2004; Denver et al. 2002; Kanamori and Brown, 1996; Kikuyama et al.1993). Nevertheless, not all salamanders respond similarly to thyroid hormone exposure. Precocious metamorphosis can be induced by exogenous T_3 or T_4 in some paedomorphs, such as *D. tenebrosus, D. ensatus, D. aterrimus, Eurycea tyrenrensis, Typhlomage rathbuni; Gyrinophilus palleucus* and *Ambystoma dumerelii* (Tata, 2007; Denver, 2002; Kanamori, 1996; Shaffer and Voss, 1996; Nussbaum, 1970; Kezer, 1952, Dent and Kirby-Smith, 1963). However, complete metamorphosis in response to exogenous TH exposure varies between and within genera. Obligate paedomorphs (*Amphiuma, Necturus* and *Proteus*) do not undergo metamorphosis but exhibit sloughing of the skin and a slight reduction of the gills (Shaffer and Voss, 1996; Lynn, 1961; Dent, 1968; Harris, 1956). *D. copei* (mature) and *Haideotriton* exhibit partial but incomplete metamorphosis in response to thyroxine treatments (Nussbaum, 1970; Dundee, 1957, 1961). These findings led to exploration of

the mechanistic basis for paedomorphosis. Investigators initially proposed that failure to metamorphose is caused simply by either an insensitivity of tissues to thyroxine or by a lack of thyroxine production (Harris et al. 1990; Harris, 1956). Shaffer and Voss (1996) proposed that different physiological mechanisms have been altered in some salamanders during the convergent evolution of paedomorphosis. Individuals that undergo metamorphosis after treatment with thyroid hormones have a functional metamorphic pathway in the presence of T_4 and therefore the mechanism allowing paedomorphosis lies somewhere "up" the cascade. Those individuals that do not transform even after large doses of TH must have evolved changes further "down" the cascade. Plausibly, any change in the metamorphic cascade could lead to paedomorphosis. Although this model gives us a good starting point, the metamorphic event in *Dicamptodon* is likely to be more complex than this model suggests.

Control of endogenous thyroid hormone (TH) is by thyroid stimulating hormone (TSH) and TH at the pituitary (Denver, 2002). Whereas TSH stimulates the synthesis of TH at the thyroid, excess TH down regulates the production of TSH at the pituitary (Brown and Cai, 2007; Denver et al. 2002; Huang et al. 2001). Hypophysectomized tadpoles fail to metamorphose (Dodd and Dodd, 1976) and injection of TSH in hypophysectomized tadpoles results in a reversal of this condition (Kikuyama et al. 1993).

TSH is controlled further up the hormonal cascade by a neurohormone released from the hypothalamus. Although thyrotropin releasing hormone (TRH) is the primary stimulator of TSH release in mammals, it does not trigger TSH secretion in amphibians (Tata, 2007, 2003; Denver 1996). Instead, TSH is secreted in response to production of

corticotropin-releasing hormone (CRH) (Boorse and Denver, 2004; Denver et al. 2002; Denver, 1997b; Gancedo et al. 1992; Rivier et al., 1984). The primary job of CRH in mammals is the secretion of adrenocorticotropic hormone (ACTH) from the pituitary and as a neurotransmitter at other sites in the nervous system (Seasholtz et al., 2002). In amphibians, however, CRH triggers the release of both ACTH and TSH from the pituitary. Unlike TSH that travels via the blood to the thyroid to stimulate TH production, ACTH is transported to the interregnal gland and stimulates the production of corticoids.

Plasma levels of corticosterone and thyroxine have been shown to increase as a result of stress (Denver, 1997; Newman, 1994; Carr and Norris, 1988; Norman et al., 1987). These hormones rise gradually during metamorphosis and peaking near metamorphic climax (Belden et al. 2005; Glennemeir and Denver, 2002a; Kikuyama et al., 1993). Corticoids are known to accelerate metamorphosis by up regulating TH receptors and promoting conversion of thyroxine (T_4) to the more potent triiodothyronine (T_3) (Darras et al. 2002; Denver, 1997; Kikuyama et al. 1993). The actions of corticoids are bimodal however, inhibiting development during early stages of metamorphosis, and accelerating metamorphosis in advanced stages of development (Hayes, 1997; Denver and Licht, 1989). Excess corticoids may provide negative feedback to both the pituitary and hypothalamus in regulation of CRH and ACTH (Hayes, 1997; Denver, 1993; Gancedo et al., 1992). Blocking the synthesis of corticoids inhibits metamorphosis (Denver, 1997; Kikuyama et al. 1993, Kikuyama et al., 1982). CRH has been implicated as the only known neuroendocrine regulator of stress responses such as metamorphosis (Boorse and Denver, 2002; Denver, 1997).

Several other hormones influence metamorphosis as well. Estradiol and testosterone are known to down regulate circulating levels of TH in amphibians (Denver et al. 2002; Hayes, 1997) and inhibit conversion of T_4 to T_3 (Leatherland, 1985; Maclatchy et al., 1986). Both melatonin and somatostatin are known to hinder metamorphosis by inhibiting TSH secretion (Denver, 1996). Prolactin, once known as the amphibian growth factor (increasing growth prior to metamorphosis and inhibiting growth thereafter) (Denver, 1996) is now thought to work at the tissue level coordinating transformation by regulating TH binding with thyroid hormone receptors (TR) (Tata, 1997; Baker and Tata, 1992). Since different tissues require varying levels of TH throughout the transformation process it has been hypothesized that prolactin acts to regulate TH uptake at the target tissue (Tata, 1997) thus allowing only that TH needed for the specific transformation to bind to receptors.

Environmental factors affecting facultative metamorphosis/paedomorphosis in salamanders

Several studies confirm that pond drying hastens metamorphosis in some salamander species (Semlitsch and Wilbur, 1989; Semlitsch, 1987; Semlitsch et al. 1985). Availability of food, water temperature, density of conspecifics (reviewed by Denoel et al. 2005) and streambed microstructures, such as sediment particle size (Bonett and Chippindale, 2006) also influence metamorphosis in several caudate species. Early researchers of dicamptodontid salamanders (Schuierer, 1958; Kessel and Kessel, 1944) proposed that an aquatic environment that provides year round, well oxygenated water of a somewhat consistent temperature, adequate food and protection from predators is conducive to paedomorph habitation. Kessel and Kessel (1944) also noted a correlation

between rainfall in the preceding spring and a lack of transforming two year old larvae. Stebbins (1951) also suggested that steep stream banks deter metamorphosis.

Models predicting environmental and phenotypic relationships

Evolutionary biologists have long been making predictions regarding selection pressures and life history modes of salamanders. Wilbur and Collins (1973) presented a general model for the timing of metamorphosis in amphibians in which timing is determined by growth rate and size of the animal. If good aquatic conditions such as abundant food, permanent water, low competition, and low level of predation are available, the individual will remain in the water to capitalize on the opportunity for growth. In contrast, metamorphosis would occur as a result of poor aquatic conditions. Based on this model, Whiteman (1994) presented three hypotheses for the maintenance of facultative paedomorphosis in nature, as described below.

The "paedomorphic advantage" hypothesis predicts that a higher ratio of paedomorphs to metamorphs will occur in permanent aquatic environments where the opportunities for growth are good. Fast growing individuals will become paedomorphic while smaller individuals will metamorphose and leave the water to avoid competition from larger conspecifics. The paedomorphic advantage hypothesis predicts that paedomorphs should have higher fitness than metamorphs. An alternative hypothesis is the "best of a bad lot" model, in which salamanders that live in unfavorable conditions and do not reach a critical size for metamorphosis, remain in the water and mature. In this case, maturing in larval form does not provide a fitness advantage but does allow them to reproduce as the "best of a bad lot". Lastly, the "dimorphic paedomorph" model predicts three size classes of larvae occurring in one population. Of the large, medium

and small size classes, the medium is the only class to metamorphose in response to competition, while the large and small size classes become paedomorphic for the reasons described by the "paedomorphic advantage" hypothesis and the "best of a bad lot" hypothesis. There would be no fitness advantage to either phenotype but equal lifetime reproductive success is predicted.

Consistent with the paedomorphic advantage hypothesis are findings from (a) Semlitsch (1987) who found that paedomorphis was more likely in permanent water, (b) Jackson and Semlitsch, (1993) who found that paedomorphis was more likely when no predatory fish are present and (c) Harris (1987) who found that paedomorphis was more likely in low density of conspecifics. Ryan and Semlitsch (1998), however, found no evidence that growth rate influences the probability of becoming a metamorph or paedomorph in *Ambystoma talpoideum*. A further study of growth and expression of alternative life cycles in *A. talpoideum* (Ryan and Semlitsch, 2003) revealed that growth rate during the later portion of the larval period influenced life cycle expression. The "best of a bad lot" is supported by studies in which decreased food levels and low temperatures were conducive to paedomorphic expression (*Ambystoma gracile*, Sprules 1974; *Triturus alpestris*, Denoel et al. 2001). Whiteman acknowledged the lack of empirical support for the "dimorphic paedomorph" hypothesis. Although several studies lend supporting evidence to the validity of the "paedomorphic advantage" and "best of a bad lot" models, there are no studies quantifying, conclusively, lifetime reproductive success indicative of a fitness advantage for either phenotype in any case.

The above models suppose that paedomorphosis is determined only by plastic responses in metamorphic timing to environmental factors. Ryan and Semlitsch (1998)

point out that sexual development responds plastically to selective pressures as does metamorphosis. They argue that age at maturation in facultative paedomorphs is the primary character of selective pressure while morphological change such as metamorphosis is secondary. From a more physiological perspective, Smith-Gill and Berven (1979) suggested that timing of metamorphosis is determined by tissue differentiation rates. In other words, no matter the growth rate, if the tissue is not ready, metamorphosis cannot occur.

Evolution of paedomorphosis

When either obligate metamorphosis or obligate paedomorphosis has been the ancestral life history pathway of a salamander, selection for facultative paedomorphosis may offer a means for greater fitness when a population is faced with unfavorable environmental conditions (Denoel et al. 2005; Shaffer and Voss, 1996; Whiteman, 1994; Semlitsch et al. 1990; Harris, 1987). Obligate metamorphosis is presumed the typical ancestral life history pathway for most caudates, while facultative paedomorphosis is considered the gateway to either obligate metamorphosis or obligate paedomorphosis (Denoel et al. 2005; Shaffer and Voss, 1996; Whiteman, 1994; Semlitsch et al. 1990). Genera that exhibit each stage in the adaptation of a fixed life history within and between populations, such as Ambystoma or *Dicamptodon*, give us the unique opportunity to witness the environmental and physiological mechanisms by which evolution occurs.

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Chapter 2

Baseline differential leukocyte counts in wild and captive *Dicamptodon copei* and *Dicamptodon tenebrosus*

Abstract

While hematologic principles are fairly conserved in vertebrates, blood tissue has evolved to match the organism with its environment, and thus there is considerable diversity in hematological parameters across taxa. To provide baseline blood data for future intra and inter- species comparisons, I examined the blood cell ratios of *D. copei* from 8 streams across their geographical distribution and blood cell ratios of *D. tenebrosus* from 2 streams in their northern range. I found significant interspecies differences in blood cell ratios from field samples and significant differences between captive and wild samples.

Introduction

In vertebrates, nearly every change in homeostatic balance is reflected in the blood. This condition makes blood analysis a useful means for assessing the intrinsic and extrinsic factors that affect physiological change. Clinicians have long exploited the properties of blood for the evaluation of mammalian health. More recently, scientists have implemented similar studies to assess homeostatic changes in non-mammalian taxa (Davis et al. 2008a, 2008b, 2009; Narayan 2011). Though some characteristics of blood are conserved between taxa, non-mammalian blood varies in form and function (Hightower, 1978), and thus inferences across taxa may be inaccurate. It is therefore necessary to gather species-specific baseline data from which comparisons may be made. For this chapter I examined the blood cell ratios of *D. copei* from 8 streams across their geographical distribution, and *D. tenebrosus* from 2 streams in their northern range.

Amphibian hematology

Amphibians share general blood characteristics with other non-mammalian species and mammals. Common to all vertebrates are four blood components: plasma, red blood cells (erythrocytes), white blood cells (leukocytes) and hemostatic cells (thrombocytes or platelets). However, the shapes, functions and ratios of each component can vary even within amphibian taxa.

Hematopoeisis

In general, blood cells are synthesized (hematopoeisis) in the liver and kidney of juvenile amphibians and in the spleen and liver (and sometimes the bone marrow) of adult amphibians (Campbell 2012; Allender et al. 2008). Plethodontids are the one exception in that hematopoiesis occurs in the bone marrow early in development (Arikan 2014). All blood cells begin as multipotent stem cells called hemocytoblasts. The first phase of differentiation produces either a myeloid progenitor or a lymphoid progenitor. Further differentiation of the myeloid cell produces erythrocytes, megakaryocytes (precursor to thrombocytes) and myoblasts. Terminal myoblast differentiation produces neutrophils, eosinophils, basophils and monocytes. Secondary differentiation of lymphoid cells produces small lymphocytes (T cells and B cells), and large granular lymphocytes (natural killer cells) (Allender et al. 2008). Development of T cells is accomplished in the thymus. Immature forms of blood cells are often found in peripheral blood, making identification somewhat difficult. It is important, therefore, to only count easily identifiable mature blood cells when performing manual differential blood cell counts.

Amphibian erythrocytes

Evolutionarily, erythrocytes (red blood cells) have evolved to optimize gas exchange in their host (Glomski et al. 1997). Erythrocytes transport oxygen and waste to and from tissues. The amount of oxygen the erythrocytes carry is dependent on the cell's hemoglobin carrying capacity and the hemaglobin's binding affinity (Feder et al. 1992). The amount of hemoglobin a cell carries and the efficiency of gas exchange is determined by the size, shape and number of erythrocytes (Glomski et al. 1997, Hillman et al. 2009).

Urodeles, have the largest erythrocytes among vertebrates. Similar to other all non-mammalian vertebrates, most amphibians have nucleated erythrocytes that are oval, ellipsoidal and biconvex near the nuclear bulge. The large size of urodele erythrocytes is correlated with genome size (Olmo et al. 1975; Olmo et al. 1978; Olmo et al. 1989) and can be attributed to the large amount of DNA contained in the nucleus (Glomski et al. 2006). For example, *Amphiuma means,* has nearly 25 times the amount of DNA of humans, and their erythrocytes are 150 times the size of human erythrocytes (Mirsky and Ris 1951). Furthermore, diploid, triploid and tetraploid offspring of diploid salamander crosses (Ambystoma sp.) have erythrocytes with increasingly larger dimensions (Glomski et al. 1997). Erythrocyte size is inversely proportional to basal energy expenditure (Smith 1925), and the smallest erythrocytes have evolved in the highest energy-consuming species.

Not all amphibian erythrocytes are nucleated. Most urodeles have a small number of anucleate erythrocytes in circulation. Furthermore, the majority of erythrocytes are anucleate in lungless salamanders. In fact, up to 90% of slender salamander (*Batrachoceps*) erythrocytes are anucleate. Anucleate erythrocytes are believed to have

evolved as a compensatory mechanism for oxygen acquisition without the use of lungs (Turner 1988).

Erythrocyte counts are inversely proportional to their size (Arikan et al. 2014, Turner 1988). This measure is commonly expressed as numbers of erythrocytes per unit volume of blood in cubic millimeters (rbc/mm^3). Logic follows that among amphibians, amphiumids have the least number of erythrocytes (30,000 rbc/mm^3). In contrast, the female Indian Skipper frog (*Rana tigerina*), has the smallest erythrocytes of all amphibians (16.4 x 10.0) and has the greatest number of erythrocytes (2,060,000 rbc/mm^3) (Glomski et al. 1997). Inter- and intraspecific erythrocyte counts vary by sex, ontogeny altitude and season (Glomski et al. 1997). Elevated reticulocyte (immature red blood cell) and polychromatic cell counts are indicative of a compensatory response to anemia. For example, warm water carries less dissolved oxygen than cold water. When less oxygen is available in the environment then more erythrocytes are produced to provide more oxygen to the tissues. Conversely, the appearance of hypochromatic cells and low erythrocyte counts may indicate non-regenerative anemia as a result of chronic inflammatory diseases and iron deficiency, decreased hormonal production, malnutrition, tissue toxicity and neoplasia (tumor formation) (Allender et al. 2008, Campbell et al. 2004).

The erythrocyte is shaped by the cytoskeleton. The components of the cytoskeleton provide an internal framework that is both resistant to bending yet flexible enough to return the cell to equilibrium shape when deformation occurs (Glomski et al. 1997). The cytoskeleton is present in denucleated amphibian cells of (a) species with primarily nucleated erythrocytes, and (b) of species in which the majority of erythrocytes

are nucleated (Glomski et al. 1997). Erythrocyte shape has evolved for optimal transportive flow and optimized oxygen transport and capillary diffusion (Feder et al 1992, Wang et al. 1992). Misshapen erythrocytes may indicate physiological distress. However, analysis of erythrocyte shape may be difficult to assess unless changes throughout ontogeny are documented as larger sized animals may have more round erythrocytes than smaller ones (Davis 2008).

Thrombocytes

Amphibian thrombocytes are the functional equivalent of mammalian platelets and are similar in structure to those of reptiles and birds. The thrombocytes are nucleated and ellipsoid or fusiform. Thrombocytes are similar to small lymphocytes though they become clumped in blood smears, making them easy to identify (Claver et al. 2009). The nuclear chromatin is densely packed and, when stained are deep blue, often appearing near black. The thrombocyte cytoplasm is colorless and contains sparse small red granules (Campbell et al. 2004). The platelet carries out primary homeostatic functions involving adhesion, aggregation and secretion (Jagadeeswaran et al. 1999). Platelets are not found in abundance unless there is homeostatic imbalance.

Agranular Leukocytes : lymphocyte, monocyte

Lymphocyte and monocyte morphology is fairly conserved across vertebrate taxa (Davis 2008). The small lymphocyte is often confused with the immature thrombocyte; however, the lymphocyte is more round with a nucleus that is also very round and large, almost filling the entire cell, such that there is very little cytoplasm. Large lymphocytes have more spherical nuclei that are commonly not centered as in the small lymphocytes.

Lymphocytes are the most common leukocyte in most amphibian blood smears (Davis et al. 2008). These cells produce hematopoietic growth factors and function in immune responses (Arikan et al. 2014). B lymphocytes respond to bacterial infections by phagocytizing bacteria and by releasing microbicides that lyse or mark the bacteria for other macrophage consumption (Barreda et al. 2006). T lymphocytes respond to viruses in much the same way and are known to be critical to amphibian recovery during ranovirus infection (Kolias et al. 1984). Therefore, lymphocytes may be abundant in blood samples during bacterial and viral infection.

On average less than 5% of leukocytes in amphibian blood smears are monocytes. Monocytes are small or large and usually have a proportionally smaller nucleus than that of lymphocytes. Monocyte nuclei may change shape throughout development and is often described as kidney shaped or horseshoe-shaped when mature (Turner 1988, Allender 2008, Arikan et al. 2014). However, round nuclei also are found in mature monocytes of *Notopthalamus* (Turner 1988).

Monocytes are most abundant in amphibians during metamorphic climax and are known to phagocytize bacteria and cellular debris (Davis et al. 2008). Monocyte derived macrophages (osteoclasts) play a role in limb regeneration, by eroding the cut site to release bone building cells (osteoblasts) to aid in limb repair (Mescher 1996). Monocytes are ambulatory and chase prey by deforming a section of the cell membrane and extending this section while pulling the cytoplasm along. The cellular extension is called a pseudopode and the presence of deformed monocytes may indicate bacterial infection. Once the monocyte has captured its prey, the cytoplasm often appears frothy as vacuoles become abundant and the prey is digested.

Granular Leukocytes: neutrophils, eosinophils, basophils

The granules associated with granular leukocytes appear on the cell's surface as the cells differentiate. Some evidence that basophils and eosinophils differentiate from neutrophils (Hightower 1978), but mature cells have granules on their surface that impart unique capabilities to each cell type. Granulocytes are named by the color of stain taken up by the cell's granules. Granular color and size, and nuclear morphology make these cells easily identifiable.

The second most common leukocyte in amphibian blood smears are neutrophils (Davis 2008). The neutrophilic granules are small, round, pink and abundant. The nucleus is lobed and the number of lobes varies during cellular development. Mature neutrophils often have characteristic bands of chromatin connecting the nuclear lobes so that they look like a string of pearls. The term heterophil is often used synonymously with neutrophil. Neutrophils and heterophils function similarly, but the morphology differs slightly as heterophil granules are cylindrical. There is little consistency among authors regarding the application of the term "heterophil" as some authors consider heterophils unique to birds and reptiles (reviewed in Davis et al. 2008).

Phagocytosis is the primary function of the neutrophil. In humans, however, neutrophils also may transport material to lymph nodes, trap the material, or mark it with antigens for destruction by other cells. Neutrophilia describes the proliferation of neutrophils in response to infections, inflammation or stress (Allender et al. 2008, Davis et al. 2008) and the levels of circulating stress-related hormones can accurately be assessed relative to neutrophil/lymphocyte ratios (Davis et al. 2008).

Eosinophil counts vary among amphibians but in general range between 1-15% in amphibian blood smears. Ambystomatid eosinophil counts apparently are much higher (20-50%) (Davis et al. 2009). Eosinophil counts increase during metamorphosis. When properly stained, eosinophils are remarkable and easily identifiable. The granules are large and bright red. These granules often crowd the cytoplasm and make it nearly impossible to see the nucleus (which is lobed).

Parasitic infections trigger eosinophilia (eosinophil proliferation). Until recently, the defense against parasitic invasion was the only function associated with eosinophils. We now know that eosinophils initiate and control the immune and inflammatory responses during metamorphosis (Adamko et al. 2005; Rothenberg and Hogan 2006). Davis (2009) hypothesized that the granules of the eosinophils release the chemicals necessary to carry out this function. Increased numbers of eosinophils may indicate parasitic infection, inflammatory response, or metamorphic processes; decreased counts may indicate thermal intolerance.

Basophils are usually rare in amphibian blood samples (Davis 2009) but are identifiable by the dark-blue to purple granules on the cell surface. The basophil is comparatively small compared to other granulocytes, and the larger granules often obscure the non-lobed nucleus and the cytoplasm. Remarkably little is known regarding this blood cell. However, Cannon et al. (1988) studied the basophil granules of *Bufo marinus.* His study revealed various amino acids also present in human basophils, including arganine, tyrosine, and lysine. Histamines were also present in the granules, suggesting a role in inflammatory responses. Interestingly, several types of basophils have been identified in the amphibian pituitary, leading some authors to suggest that

basophils function during metamorphosis by releasing thyroid stimulating hormone from its granules (Cardell, et al 1964, Reichen et al. 1965).

Methods

Capture and husbandry

I collected *D. copei* and during August, September and October of 2009, 2010 and 2012 from eight streams throughout their range in Washington State (Mason Co., Grays Harbor Co., Skamania Co., and Lewis Co.) and *D. tenebrosus* from five streams (Skamania Co., Washington) in their northern range (Figure 2.1).

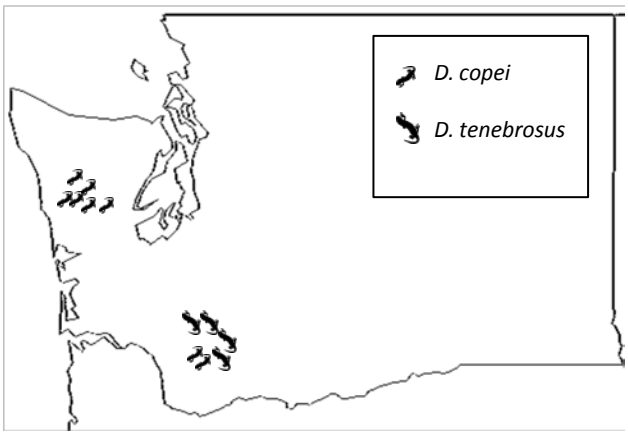

Figure 2.1. Approximate sampling locations of *D. copei* and *D. tenebrosus* 2009-2012

I located salamanders during daylight hours using goggles and transparent thermoplastic-bottomed buckets. While looking through a bucket or goggles, I lifted rocks until we located a salamander and captured it using an aquarium net. I used coolers to transport the salamanders to Oregon State University in coolers and housed them in a climate-controlled room with natural photoperiod and the temperature maintained between 11°C and 14°C. Each salamander was individually housed in a 38 liter aquarium (50.8 cm x

27.9 cm x 33.0 cm), with rocks as cover objects and a charcoal filter to aerate and clean the water. I cleaned the aquaria and filters every two weeks and fed the salamanders twice per week, each time feeding them until they stopped accepting food. Wax worms (Galleria sp.) provided the primary diet throughout captivity. However, their diet also included: earthworms (Toutellus spp.), crickets (Grillus spp.), aquatic insects (Hesperoperla spp., Perlinodes spp., Isoperla spp., Ephemerella spp, Calineuria spp., Doroneuria spp.) and goldfish (Carassius spp.).

Anesthesia and measurements

I measured the total length of each animal and assigned each to one of three size classes, small (≤ 100mm total lenth (tl)), medium (101- 200 mm tl) and large (>200mm tl). I then anesthetized each animal using tricaine methanesulfonate (MS-222). To decrease chances of accidental overdose, initial doses were at least100 mg/L less than veterinary standards for "newts and tadpoles" (Mitchell 2009; Kohn et al. 1997). I combined stream water and pre-measured MS-222 doses in reclosable plastic bags and I buffered the solution with sodium bicarbonate to achieve a neutral pH. The temperature and pH of the stream were documented, and the anesthetic solution was kept at stream temperature by submerging the sealed bags containing the anesthesia solution in the stream or in an ice-filled cooler, prior to and during induction. I placed salamanders singly, according to size, in the anesthetic solutions. Small salamanders were immersed in 50 mg/L solution, medium salamanders were immersed in 100 mg/L MS-222 and stream water, solution and large salamanders immersed in 200 mg/L solution. I prepared new anesthetic solution for each treatment and collected used solutions in a container for future disposal in the laboratory.

I monitored induction time, gular movements and withdrawal reflex. When I observed no gular movements and no response to toe-pinching, the animal was removed from the solution, weighed and measured from the tip of the snout to the distal edge of the vent and then quickly submerged in an open plastic container filled with 2-3 liters of fresh stream water. Each animal was placed dorsal surface up such that the animal was completely submerged. The recovery water was changed every 10 minutes throughout the recovery. I monitored recovery and considered a salamander fully recovered when it began moving about the container and was able to right itself continuously.

When an animal was sedated in less than five minutes or not sedated within ten minutes, I removed the animal from the solution and placed it in fresh water as described above. Subsequent anesthetic treatments for animals of similar length (±5mm) were 50 mg/L higher or lower in concentration.

Blood samples

I obtained blood samples 1-2 hours after capture from 62 *D. copei* and 35 *D. tenebrosus* and after at least 6 months in captivity of 62 *D. copei* and 18 *D. tenebrosus* in the laboratory. Six microscope slides were laid flat (3 for receiving blood and 3 for spreading blood) on the left side of the blood drawing station. The maximum blood quantity (MBQ) that could safely be taken from each animal was calculated based on United States Geological Survey guidelines (2001): (10% of body mass /2) = MBQ in milliliters). I planned to collect enough blood from each animal to produce 3 blood smears (3 drops ~4mm in diameter); the quantity of blood obtained was safely below the maximum blood limit in all cases. In some cases, however, I was unable to obtain blood.

Once anesthetized, the animals were placed on water-soaked paper towels, so that the ventral surface of each salamander was accessible for venipuncture. Heparinized insulin syringes (31 gauge x 5/16, 0.5-1cc) were used to obtain blood from the veins, and heparinized capillary tubes were used to collect any blood that leaked from the puncture site, after the needle was removed. All blood samples were taken from the ventral tail vein, which runs ventrally along the caudal vertebrae. I selected the venipuncture site by visually plotting a distance of one full cloaca length distal to the posterior end of the cloaca. Following the USGS standard operating procedure for blood collection from amphibians (Feb, 2001), I aligned the needle with the midline of the tail. The needle was inserted at a near 45 degree angle (with the head of the needle toward the cloaca). The needle was inserted into the tail until it contacted the vertebral column. Suction was created within the syringe to obtain 0.1cc of blood; the needle was then withdrawn from the tail until it was slightly above the vertebral column. If no blood flowed into the syringe, the needle was rotated 180 degrees. If blood still did not flow into the syringe, the needle was withdrawn (without exiting the skin) and moved longitudinally along the vertebral column. Straight, deliberate, downward movements were taken with the needle to pierce the caudal vein, and care was taken not to tear the vein by crossing it transversely. If still no blood was collected, a second venipuncture site was chosen closer to the end of the tail and the previous steps were repeated. If three unsuccessful attempts to collect blood were made, the salamander was placed in chemical-free water to recover. Collected blood was quickly transferred to microscope slides (25x75mm) for future analysis.

Blood smear preparation

Following the blood smear preparation protocol provided by the Cornell University website (ahdc.vet.cornell.edu), I placed one drop (~4mm in diameter) of blood approximately one third of the distance from the edge of a microscope slide. The blood was spread by: (a) placing another microscope slide at a 30-45 degree angle in the middle of the slide containing the blood, (b) backing up the slide to make contact with the drop of blood, (c) allowing the blood to spread by capillary action along the contact point of two slides, and (d) dragging the blood forward until the end of the slide was reached. Blood that was spread on a slide was allowed to air dry at room temperature. Each blood slide was fixed within 4 hr of collection by dipping the slide in 100% methanol (MeOH) for 30 seconds. All slides were labeled and placed in a dry slide box. Within 2 weeks the slides were placed in slide racks and stained. Prior to staining slides for analysis, test slides were selected from low quality blood smears to determine the quality of various stains. The criterion for stain selection was the ease with which the blood cells were identified (Heatly et. al, 2009). Slides for analysis were stained in modified Field's stain (Azure II replaced Azure I). Adequate immersion times varied with the age of the stain and the number of the times the stain had been used (1-1.5 minutes in Field's stain I and 2-3.5 minutes in Field's stain II). All stains were filtered and tested before each use. When stain precipitants appeared on a test slide or when blood cells could not quickly be identified due to staining issues, I replaced the old stain with new stain. Stained slides were air dried and glass cover slips were adhered to them with Cytoseal mounting medium.

Transport and husbandry

15 of 49 *D. tenebrosus* were returned to the capture site directly following blood sampling procedures. All others were transported to Oregon State University in coolers and housed them in a climate controlled room with natural photoperiod and the temperature maintained between 11°C and 14°C. Each salamander was individually housed in a 37.9 liter aquarium (50.8 cm x 27.9 cm x 33.0 cm), with rocks as cover objects and a charcoal filter to aerate and clean the water. One to two times per week I used a suction hose to remove large organic debris and old water and then replaced the old water with dechlorinated water. I fed the salamanders twice per week, each time feeding them until they stopped accepting food. Their diet included earthworms (Toutellus sp.), crickets (Grillus sp.), aquatic insects (Hesperoperla spp., Perlinodes spp., Isoperla spp., Ephemerella spp, Calineuria spp., Doroneuria spp.) and goldfish (Carassius spp.); however, wax worms (Galleria spp.) provided the primary diet throughout captivity.

One worker visually counted blood cells from all blood films and recorded them using a blood cell counter that I created in Visual Basic programming language within Excel. A starting point was randomly selected by obtaining 2 numbers within the Vernier coordinate range, using a random number generator; the numbers were assigned to the X and Y coordinates of the starting point. A grid overlay and the contents of a slide were projected on one computer monitor with an AmScope 10MP microscope eyepiece camera mounted on a Zeiss Axiostar plus light microscope at 40X, and the blood cell counter was projected on a second monitor. The slide was moved in a zig-zig pattern, left to right and top to bottom until 100 leukocytes had been counted. The slide was moved continuously

with one hand while the other hand entered the quantity and the type of the cells into the blood cell counter. A sound was generated by the blood cell counter to alert the worker that the target number of cells had been reached. The data was then transferred automatically to a spreadsheet by clicking an update button on the blood cell counter. The counter was then cleared, and the steps were repeated with another slide. Two counts were taken for each animal. When two or more slides were available, one count per slide was done; when only one slide was available, two counts were taken from the same slide, each count having a different starting point.

Leukocyte differential counts

One worker visually counted blood cells from all blood films and recorded them using a blood cell counter that I created, in Visual Basic programming language within Excel. Using a random number generator a starting point was randomly selected by obtaining 2 numbers within the Vernier coordinate range. The numbers were assigned to the X and Y coordinates of the starting point. A grid overlay and the contents of a slide were projected on one computer monitor with an AmScope 10MP microscope eyepiece camera mounted on a Zeiss Axiostar plus light microscope at 40X. The blood cell counter was projected on a second monitor. The slide was moved in a zig-zig pattern, left to right and top to bottom until 100 leukocytes had been counted. The slide was moved continuously with one hand while the other hand entered the quantity and the type of the cells into the blood cell counter. A sound was generated by the blood cell counter to alert the worker when the target number of cells had been reached. The data were then transferred automatically to a spreadsheet by clicking an update button on the blood cell counter. The counter was then cleared, and the steps were repeated with another slide.

Two counts were taken for each animal and average counts were obtained from both samples.

Erythrocyte measurements

The ToupView measurement tool was calibrated using a Motic calibration slide. Step-by-step instructions were provided by the software as I proceeded with the calibration. The protocol of Felip et al. (2009) was followed to select the number of erythrocytes to measure for each animal in the study. For each blood related experiment, in this study, 35 erythrocytes were measured per animal. Four measurements were made using the ToupView measuring tool: a) cell long axis diameter b) cell short axis diameter, c) nucleus long axis diameter, and d) nucleus short axis diameter. For comparisons, I calculated the dry cell area (A_c) and dry nucleus area (A_n) using the formula for the area of an ellipsoid area ($A = \pi(LD/2)(SD/2)$. Where LD= long diameter and SD=short diameter. All variables were entered in a pre-programmed Excel spreadsheet to calculate cell and nucleus diameters. Diameters are in µm, areas are in µm2, and volumes are in µm3

Data analysis

Erythrocyte dimensions – All statistical procedures were performed using STATGRAPHICS centurion statistical software. To approximate normal distributions of erythrocyte dimensions, I log transformed the data prior to analysis. I obtained 95% confidence intervals for each variable from field and captive samples ($LD_{c,n}$, $SD_{c,n}$, $A_{c,n}$,$V_{c,n}$) using a multiple variable analysis approach. I backlogged the results and reported the diameters in µm, areas in µm2, and volumes in µm3. I performed a Mann-Whitney (Wilcoxon) W-test to make interspecies comparisons of the median nucleus areas and

median cell areas of wild populations.

White blood cell (WBC) proportions - To approximate normal distributions of WBC proportions, relative numbers of each white blood cell type count were arcsine square transformed in Microsoft Excel. I calculated the mean, lower and upper confidence limits of the transformed data and then back-transformed and squared these results.

I fit a general linear model (ANOVA) relating both size (SVL) and stream to the percentage of leukocyte types to examine their effects on the relative numbers of each white blood cell type. Then, I used a multiple sample comparison approach (Multiple Range Test) to determine which means were significantly different (Fisher's least significant difference (LSD) procedure) between wild and captive populations and between species.

Results

95% confidence intervals for mean field erythrocyte dimensions are reported in Table 2.1. Interspecies comparisons revealed no significant differences (W= 1.45857E6, P = 0.178247) in median cell areas of wild *D. copei* (2.86) and *D. tenebrosus* (2.86), and a significant difference between median nucleus areas of wild *D. copei* (2.21µ) and *D. tenebrosus* (2.22µ) (W = 1.49E6, P = 0.008).

Wild	*Mean*	*Lower limit*	*Upper limit*	Captive	*Mean*	*Lower limit4*	*Upper limit5*
LDc cop	41.33	40.98	41.69	LDc cop	41.89	41.51	42.27
SDc cop	20.04	19.81	20.28	SDc cop	21.82	21.59	22.05
Ac cop	650.84	641.19	660.64	Ac cop	718.12	706.8	729.62
LDn cop	18.65	18.52	18.78	LDn cop	18.082	17.91	18.25
SDn cop	9.81	9.72	9.91	SDn cop	10.53	10.38	10.68
Anc cop	143.83	142.04	145.65	Anc cop	149.6	147	152.25
LDc ten	40.77	40.41	41.12	LDc ten	41.1	40.61	41.6
SDc ten	22.18	21.93	22.43	SDc ten	22.59	22.36	22.83
Ac ten	710.39	699.72	721.2	Ac ten	729.52	717.57	741.66
LDn ten	18.76	18.61	18.92	LDn ten	18.05	17.89	18.21
SDn ten	11.12	10.99	11.26	SDn ten	10.83	10.69	10.99
An ten	164.04	161.56	166.55	An ten	153.69	151.1	156.32

Table 2.1. Mean wild and captive erythrocyte dimensions in *D. copei and D. tenebrosus*. LD_{cn}= long diameter (µ), SD_{cn}= short diameter (µ), A_{cn}=Area (μ^2).

The general morphology of each leukocyte type was similar to typical urodele leukocytes (Fig. 2.2) (Thrall 2004). Relative abundances of each leukocyte type for wild and captive *D. copei* and *D. tenebrosus* are shown in Table 3. In both species, and in wild and captive measurements, the mean lymphocytes were the most commonly encountered leukocyte, ranging from 37.93% (± .06%) in wild *D. tenebrosus* to 45.63% (± .02%) in captive *D. copei*. Eosinophils were the second most commonly encountered leukocyte in all populations, ranging from a 29.31% (± .04%) in wild *D. copei* to 35.83% (± 1.98%) in captive *D. tenebrosus*. Neutrophils composed the third most commonly encountered leukocyte in all populations, ranging from 14.71% (± .02%) in captive *D. copei* to 20.97%(± .02%) in wild *D. tenebrosus*. Basophils were the fourth most abundant leukocyte in all populations, ranging from 5.07% (±.03%) to 1.61% (± .01%) except in captive *D. tenebrosus* in which monocyte (2.22% ± .54%) and basophil (2.08%

± .4%) abundance were similar. Monocytes were the least abundant leukocyte in all but captive *D. tenebrosus*, ranging from 0.69% (± .4%) in wild *D. tenebrosus* to 0.81% (± .01) in *D. copei*.

There was no significant difference between leukocyte ratios of different sized animals in either wild *D. tenebrosus* ($F_{5, 28}$ =0.29, P = .91) or wild *D. copei* ($F_{5, 56}$ = 0.60, P = 0.7). There was no significant difference between leukocyte ratios from different streams in either wild *D. tenebrosus* ($F_{5, 28}$ =1.84, P = .13) or wild *D. copei* ($F_{5, 56}$ =0.89, P = 0.49).

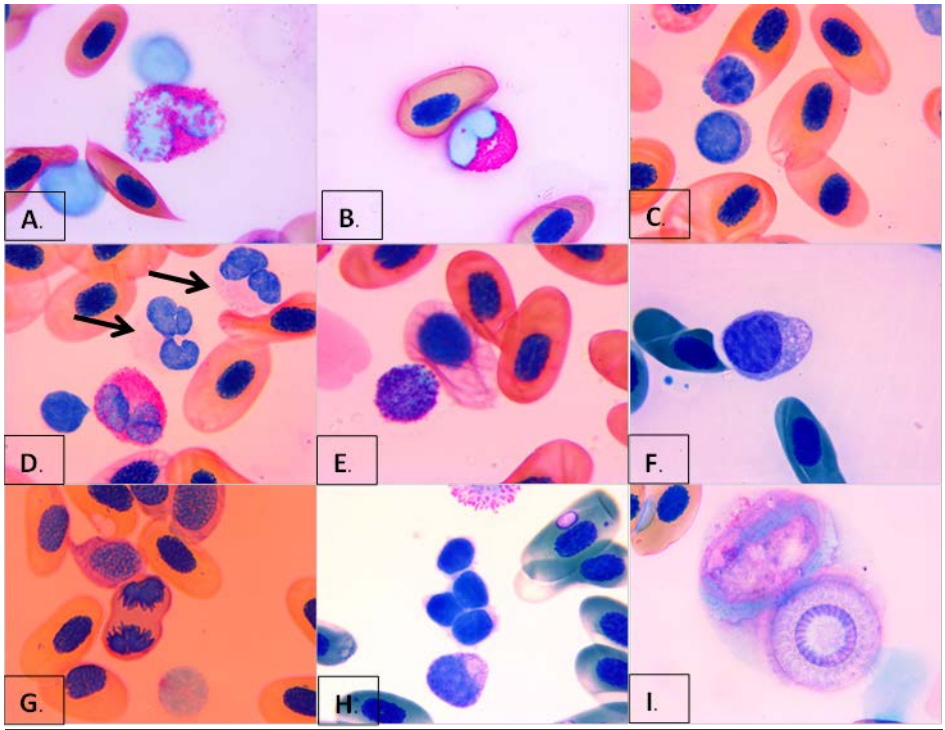

Figure 2.2- *Dicamptodon* leukocytes, thrombocyte and erythrocyte: A-large eosinophil with large bright red granules, B- small eosinophil with tight granules, C- small lymphocyte, D- two polynucleated neutrophils with lightly stained pink granules, E- Basophil showing single large nucleus and dark tightly packed granuoles, G- Monocyte showing frothy vacuoles, G- Erythrocyte in mitotic division, H- Thrombocytes adhering to one another, L- parasite found in several blood samples (Trichodina).

The multiple sample comparisons (Multiple Range Test) revealed no significant ($p>.05$) difference in mean leukocyte abundance between wild *D. copei* and wild *D. tenebrosus* and no significant ($p>.05$) difference in mean leukocyte abundance in captive *D. copei* and captive *D. tenebrosus*. There was no significant ($p>.05$) difference between any leukocyte ratios except basophil ratios in captive and wild *D. tenebrosus*. There were significant ($p\leq .05$) differences in estimated differences in all leukocyte ratios except monocytes between wild and captive *D. copei*.

***D. copei* Wild**	**Mean**	**SE**	**Min**	**Max**
Neutrophils	22.73%	0.01%	21.09%	24.43%
Lymphocytes	40.45%	0.03%	37.27%	43.68%
Eosinophils	29.31%	0.04%	25.81%	32.93%
Basophils	3.93%	0.02%	3.03%	4.94%
Monocytes	0.81%	0.01%	0.55%	1.12%
***D. tenebrosus* Wild**	**Mean**	**SE**	**Min**	**Max**
Neutrophils	20.97%	0.02%	18.57%	23.47%
Lymphocytes	37.93%	0.06%	33.34%	42.63%
Eosinophils	32.64%	0.07%	27.79%	37.69%
Basophils	5.07%	0.03%	3.64%	6.71%
Monocytes	0.69%	0.02%	0.33%	1.17%
***D. copei* Captivity**	**Mean**	**SE**	**Min**	**Max**
Neutrophils	14.71%	0.02%	12.81%	16.73%
Lymphocytes	45.63%	0.02%	42.82%	48.46%
Eosinophils	35.32%	0.03%	31.87%	38.84%
Basophils	1.61%	0.01%	1.26%	2.00%
Monocytes	0.79%	0.00%	0.56%	1.05%
***D. tenebrosus* Captivity**	**Mean**	**SE**	**Min**	**Max**
Neutrophils	16.94%	1.54%	13.70%	20.19%
Lymphocytes	42.92%	1.79%	39.13%	46.70%
Eosinophils	35.83%	1.98%	31.66%	40.01%
Basophils	2.08%	0.40%	1.23%	2.93%
Monocytes	2.22%	0.54%	1.09%	3.36%

Table 2.2 – Summary of leukocyte differentials for wild and captive *D. copei* and *D. tenebrosus*

Discussion

This study is the first to document leukocyte differentials and erythrocyte measurements in both captive and wild populations of *Dicamptodon* salamanders. These parameters provide baseline data for future studies of captive and wild populations of *Dicamptodon* and for comparison with other urodele families.

There is an extensive range and variation in intra- and inter specific amphibian erythrocyte morphology and size (Arikan et al. 2009). The size and shape of erythrocytes are indicative of both the level of ploidy and the metabolic potential of an animal (Arikan et al. 2011). In this case the erythrocyte cell areas are similar between species which indicates similar rates of O_2 and CO_2 diffusion; however *D. tenebrosus* nuclei were significantly larger than *D. copei* nuclei. There are several possibilities that may be considered. The difference in nucleus size suggests a difference in the amount of genetic material contained within it. Brinkman et al. (2002) reported supernumerary chromosomes in *D. tenebrosus* but this condition is unknown in *D. copei*. Alternatively, the difference in nucleus sizes may indicate a difference in sex chromosomes as both ZZ/ZW and XX/XY systems exist within and between amphibian species and the sizes of sex chromosome types differ (Nakamura 2009). Lastly, the large size of the *D. tenebrosus* nuclei may be indicative of greater metabolic needs related to body size, large prey consumption or a warmer aquatic habitat.

Wild aquatic *D. copei* and aquatic *D. tenebrosus* share similar leukocyte profiles although they live in varied geographical locations and in varied thermal conditions. These parameters are also similar to those of aquatic ambystomatid salamanders (Davis 2009) which are their nearest relative. This similarity suggests that these profiles are

fairly conserved between and within these families. However, there is no documentation of leukocyte profiles in aquatic *D. ensatus*, aquatic *D. aterrimus* nor in any terrestrial *Dicamptodon*. While this study can serve as a general reference for future hematological studies of aquatic *D. copei* and aquatic *D. tenebrosus*, a thorough analysis requires baseline data for all *Dicamptodon* species and forms.

The comparison between wild and captive *D. copei* revealed a significant difference in mean leukocyte ratios. These differences likely reflect a captive stress response; however, there was not a significant difference in leukocyte ratios between wild and captive *D. tenebrosus* which suggests that there is a difference in physiological responses to an environmental condition in their captive habitat. Davis et al. (2008) proposed two possible explanations for the difference between leukocyte profiles of wild and captive *Ambystoma talpoideum*; first, that there was an acute stress response to capture and removal from their natural habitat, and second, that suboptimal laboratory conditions resulted in hematological stress responses. In this study all captive blood samples were obtained between 8 months and 4 years of captivity and no acute responses were assessed. Therefore, the observed blood parameters are probably due to suboptimal captive conditions. As laboratory conditions did not mimic natural conditions sufficiently, several conditions may be invoked as possible causes for physiological responses by captive *D. copei*. First, because wild aquatic *Dicamptodon* live hidden within the interstices of rock piles and in hyporheic zones in their natural habitat, a lack of sufficient cover may have affected blood parameters. Second, as flowing water dilutes and relocates feces in their natural habitat, changing pH levels caused by fecal matter may have affected captive blood parameters. However, there was a difference in

leukocyte profiles in *D. copei* and *D. tenebrosus* yet both were held in identical conditions, it is likely that differences in wild habitats of each species caused differences in captive physiological responses. Noticeably, the aquatic temperature in the laboratory was comparatively warmer than wild conditions for *D. copei* than for *D. tenebrosus* and thus a difference in thermal tolerance range is likely.

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Chapter 3

Stress and metamorphosis in *D. copei* and *D. tenebrosus*

Abstract

Both *Dicamptodon tenebrosus* and *Dicamptodon copei* metamorphose in nature, albeit to varying degrees. Though early studies suggested that *D. copei* is obligately paedomorphic, recent evidence shows that some populations have retained the ability to transform. The environmental factors that trigger metamorphosis/paedomorphosis in *Dicamptodon* are unclear, though several environmental factors including habitat desiccation (Kessel and Kessel 1944) and sensitivity to thyroxine (Nussbaum 1970) were previously invoked.

To evaluate relationships between environmental factors and metamorphic tendencies in *Dicamptodon*, I tested the effect of increased water temperature and decreased water level for the morphological phenotype of *D. copei* and *D. tenebrosus*. I examined thermal selection and assessed hematological responses of both species to three temperature regimes (1.7º C, 21.1 º C and 25º C). Neither increased temperature nor decreased water level induced metamorphosis of any animal. Interspecies comparisons revealed significant differences ($p<0.05$) in temperature selection tendencies between *D. tenebrosus* and *D. copei*, and significant differences ($p<0.05$) in hematological responses to temperature change within and between species.

Background

Many amphibians are able to respond plastically to environmental cues. For example, spadefoot tadpoles metamorphose more quickly when temperatures rise or their aquatic environment begins to dry. Facultative paedomorphic/metamorphic salamanders

sometimes respond morphologically to conditions in their habitat that may compromise their reproductive fitness by altering life history pathways (Denoel et al. 2005; Whiteman, 1994; Jackson and Semlitsch, 1993, Semlitsch, 1987). A variety of environmental factors affect the frequency of paedomorphic and metamorphic salamanders. Sprules (1974b) found a higher incidence of paedomorphosis in *Ambystoma gracile* raised in 12°C than those raised in 19°C water. Harris (1987a) reported higher incidence of metamorphosis in *Notopthalmus viridescens dorsalis* raised in high conspecific densities, and higher incidences of paedomorphs in low density populations. Similarly, Semlitsch (1987a) found that *Ambystoma talpoideum* raised in high larval density were more likely to metamorphose, and those raised in low density were more likely to express paedomorphosis. Low food availability (Ryan and Semlitsch 2003) and habitat desiccation (Semlitsch 1987a) also resulted in higher incidences of metamorphic individuals. Because facultative paedomorphosis evolved under diverse ecological circumstances in a number of salamander families (Wells 2007), it is plausible that the ranges of tolerance and physiological responses to environmental conditions also vary. However, there are few comparative studies addressing these differences. The *D. tenebrosus* and *D. copei* live allopatrically and sympatrically and have facultative and obligate populations. This situation offers unique opportunities to investigate and compare the selective pressures producing these different phenotypes.

Early investigations of metamorphosis in *Dicamptodon* focused primarily on the size of *D. ensatus* at metamorphosis (Storer 1925; Kessel and Kessel 1944). From this work, Kessel and Kessel (1944) hypothesized that certain environments provide a physiological block to metamorphosis. They held four *D. ensatus* (perceived as second

year larvae by their size) in individual aquaria and observed the duration of transformation, the behaviors during the transition and the changes in external morphology occurring throughout their transition. Salamanders were placed singly in an aquarium. "Salamander A" and "Salamander B" were placed in one inch of water. The water was at one end of the aquarium and sand and rocks were piled up above the water line at the opposite end. Complete metamorphosis was accomplished in 11 and 18 days respectively. "Salamander C" was not provided sand or rocks, and the entire floor of the aquarium was covered with two and one half inches of water. Though metamorphosis was initiated in "Salamander C", 6 months passed without complete transformation. After 6 months, most of the water was removed and the salamander completed metamorphosis within a week. "Salamander D" was placed in an aquarium with 5 inches of water only. In this case, metamorphosis did not begin for over a month and only partial transformation was accomplished after 6 months. The water was then removed and "Salamander D" completed metamorphosis in a few days. Kessel and Kessel (1944) concluded that larval salamanders unable to exit their aquatic habitat would become "axolotl-like". Furthermore, they posited that paedomorphic salamanders would likely inhabit streams where there is enough rainfall to keep the creeks high. While statistical confirmation of their hypothesis was lacking, this was the first time researchers linked the occurrence of paedomorphosis/metamorphosis with environmental factors in *Dicamptodon*.

Consistent with Kessel and Kessel's (1944) hypothesis, Nussbaum and Clothier (1973) studied population structure in *D. ensatus* and they reported that the frequency of paedomorphs was extremely low in small permanent streams, occasional in medium

streams and high within large permanent streams and lakes. Conversely, metamorphosis was common in streams that occasionally dried up, and also in medium streams. In large permanent streams, however, growth was slower and metamorphosis occurred later or not at all. In this study, Nussbaum and Clothier (1973) invoked both stream size and growth rate as possible causes of paedomorphosis/metamorphosis.

In addition to environmental factors, physiological control of metamorphosis has been studied in *Dicamptodon*. Gudernatsch (1912) discovered that by feeding *Rana temporaria* tadpoles thyroid tissue extracted from calves and horses, the tadpoles quickly transformed to frogs. This discovery led to the assessment of metamorphosis in salamanders, including *Dicamptodon,* via exogenous thyroid hormones (Brown and Cai 2007). Nussbaum (1970) assessed the sensitivity to thyroid powder in *D. copei* and *D. ensatus* from one location in Washington (Marrata Creek, Cowlitz Co.). He immersed similar sized *D. ensatus* and *D. copei* in a solution of water and a "small amount" of bovine thyroid powder. He observed that *D. copei* transformed much slower than did *D. ensatus*. Nussbaum (1970) noted that, during the experiments, all *D. copei* were sexually mature while *D. ensatus* were immature. However, immature *D. copei* that were tested separately responded similarly to mature *D. copei*. Additionally, sexually mature and older *D. ensatus* transformed more slowly than did immature *D. ensatus*, but more quickly than *D. copei*.

Nussbaum (1972) subsequently investigated geographic variation in sensitivity to thyroxine in *D. copei* from six locations (Maratta Creek, Cowlitz Co., Washington; Oneota Gorge, Multonomah Co., Oregon; Nine Foot Creek, Skamania Co., Washington; Wahkeena Falls, Multinomah Co., Oregon; Saddle Mountain, Clatsop Co., Oregon and

Merriman Creek, Grays Harbor Co. Washington). The specimens used in this study were of mature size, based on size at maturity at Marratta Creek (pers. com. 4/14/08). Nussbaum (1972) recognized that size and age may not correlate between populations but estimated age by size to be similar in this study. Treated *D. copei* from three locations (Oneota Gorge, Saddle Mountain and Wahkenna Falls) responded similarly to all specimens from Marratta creek. Transformation of specimens from Nine Foot creek, however, was even less sensitive to treatment than all others. Contrarily, *D. copei* from Merriman Creek were more sensitive to thyroxine treatment than all other populations, yet transformed more slowly than *D. ensatus*.

Based on Nussbaum's (1970, 1972) results he proposed that *D. copei* do not transform in nature. Since then, many authors (Jones et al. 1989; Sever 1992; Steele et al. 2005, Steele 2006) have referred to *D. copei* as obligate paedomorphs. However, an increasing number of documented reports (Spear et al. 2005, Nussbaum et al. 1983; Jones and Corn, 1989; Loafman and Jones, 1996) and undocumented reports (Beatty pers. com. 2010; Leonard pers. com 2009; Bury pers. com 2014) of transformed *D. copei* have been surfacing. In one case, more than 50 post metamorphic *D. copei* were discovered in the Willapa Hills of southwestern Washington (Spear et al. 2005).

In the laboratory, Coriell (2003) examined the rate of metamorphosis in *D. tenebrosus* larvae from medium and large streams. He reported that animals from medium streams metamorphosed 36% more often than did those from large streams. In addition he assessed the relationship between feeding and metamorphosis and found that the low feeding group metamorphosed significantly more often than the high-feeding group.

Section 1 – Morphological responses to water level and water temperature

I tested Kessel and Kessel's (1944) hypothesis that water level affects metamorphic tendencies in *Dicamptodon*, by replicating Kessel and Kessel's (1944) original experiment with *D. copei* (n=15) and *D. tenebrosus* (n=6). I subsequently tested the hypothesis that water temperature affects metamorphic changes in *D. copei* (n=15) and *D. tenebrosus* (n=15). Below, I describe the methods of two experiments and then the results and conclusion for these experiments.

Methods

I captured *Dicamptodon copei* (n=24) and *Dicamptodon tenebrosus* (n=7) during late August and throughout September and October of 2009 by hand and using dipnets (methods described in Chapter 2 of this thesis). The animals were housed in a temperature controlled laboratory (avg. temp= 12°C±1) with a natural photoperiod. Each salamander was placed in a 10 gallon aquarium (elevated 2.54cm at one end) containing water roughly 10cm deep at its deepest point. Aquarium gravel was piled at the elevated end of the aquaria, which provided egress from the water for each animal. Charcoal filters cleaned and aerated the aquaria water. I fed the animals three times a week with a variety of foods, including aquatic insects (Plecoptera, Ephemeroptera, Trichoptera, and Odonata) and earth worms (Oligochaeta). I placed food portions in front of the animal's snout until it stopped accepting food. Any food left uneaten after 30 minutes was removed. The feeding regime was consistent throughout captivity and during experimental treatments.

Assay 1: Does water level affect the timing of metamorphosis?

In early November 2009, one *D. copei* from Cabin Creek (Mason Co., Washington) unexpectedly showed signs of metamorphosis. I observed that the gills were shorter than previously observed and the animal was increasingly keeping its head above water. The next day I randomly assigned one set of 5 *D. copei* and 2 *D. tenebrosus* to each of three water level treatments (2.54cm, 6.35cm or 12.7cm) and immediately lowered the water levels accordingly. Following Kessel and Kessel's (1944) protocol, I observed the animals daily over 6 months for signs of transformation. The criteria for metamorphic initiation were: (1) decrease in gill size, (2) eyes protruding above the surface of the snout, and (3) decreased tail fin height.

After 6 months I began to lower the water in each tank. On May 15, 2010, I decreased the water level of all experimental treatments 0.5cm per week until the water was no lower than the animal's mid-lateral abdomen. Observations were made nearly every day over a 4 month period. Water levels were returned to the initial depth on September 15, 2010.

Assay 2: Does increased water temperature induce metamorphosis?

During September and October of 2010, I captured an additional 13 *D. copei* and 14 *D. tenebrosus*. As in the previous year, one newly captured *D. copei* began transformation in early November. All animals were monitored almost daily for signs of metamorphosis. In late February I randomly assigned as set of 5 *D. copei* and 5 *D. tenebrosus* to each of three temperature treatments (15± 1°C, 18± 1°C, 20± 1°C). I prepared the experimental aquaria 3 days prior to the beginning of observations (no animals were in the aquaria during these 3 days). A standard aquarium heater was placed

in each aquarium, turned on and set to ambient air temperature (12°C). The height of the water in all experimental aquaria was 10cm at its deepest point and egress was provided as described above. Also, I provided refugia in the form of rocks of various sizes piled in the pool end of the aquaria and provided additional air that bubbled in from a tube connected to the building air supply. I used the same mercury glass thermometer to take measurements in all aquaria throughout the study. Prior to observations, I measured the water temperature and placed the animals in their assigned aquaria on March 2, 2011. I increased the water temperature by 1°C daily until the target temperature was reached (3, 6 and 8 days). Additional containers of water were warmed to the three experimental temperatures each morning prior to water exchanges. Twice per week, I fed the animals and exchanged aquaria water. Near daily observations were made over an 8 month period.

Results

No signs of metamorphosis were observed in any animals during any water removal or temperature modification treatment. During the first month of the temperature increase trials, one *D. copei* from the 20± 1°C treatment group became systemically bloated and was removed from the study. I placed the animal in cool water (12°C) and within a week it recovered fully. In the fourth month of that same study, a second *D. copei* from the 20± 1°C treatment group also became bloated, was subsequently removed from the study and placed in cool water (12°C). This animal did not recover.

Conclusion

I was unable to replicate Kessel and Kessel's (1944) results. Failure of decreased water level to induce metamorphosis in either species during this study suggests that (a) either the metamorphic tendencies of *D. copei* and *D. tenebrosus* differ from metamorphic tendencies of *D. ensatus*, or (b) that response to environmental stressors responsible for triggering metamorphosis differ between species or (c) that the metamorphic changes experienced during Kessel and Kessel's (1944) study were coincidental. One notable difference in salamander morphology during these studies was that all salamanders in Kessel and Kessel's (1944) study began to transform during the holding phase of the study. This may indicate that *D. ensatus* had reached a physiological or developmental threshold prior to or during captivity, or that the population of *D. ensatus* was composed of obligate metamorphs and would transform in any conditions.

That a single *D. copei* outside of the study began transformation a few weeks after capture suggests that physiological factors for transformation were met for some individuals within this population. In my studies *D. copei* of similar size, from the same stream, participating in the study did not show signs of transformation. This phenomenon may indicate the presence of both obligate and facultative paedomorphs within the population. To confirm the existence of metamorphic individuals in this population, future sampling should include a thorough search along the stream edges and within interstices of piled rocks and tunnels surrounding this stream.

Failure to induce metamorphosis in either species in response to 15°C, 18± 1°C, 20± 1°C aquatic environments suggests that either temperature does not affect

metamorphic tendencies, or that these temperatures are not stressful enough to induce metamorphosis in *D. copei* and *D. tenebrosus.* That another *D. copei* from a different stream began transformation within weeks of capture was very surprising, given the scarcity of previous reports of transformation in the species and the insensitivity to thyroxine reported by Nussbaum (1970). Further investigations into the metamorphic tendencies and environmental factors influencing *D. tenebrosus* and *D. copei* is vital to resolving questions still surrounding this life history variable.

Section 2: Thermal selection of aquatic *D. copei* and *D. tenebrosus*

Introduction

I assessed the behavioral responses of *D. copei* and *D. tenebrosus* to varied thermal environments to test the hypotheses that: (1) thermal selection will differ between *D. copei* and *D. tenebrosus* and (2) *D. copei* is likely to select colder temperatures more often than *D. tenebrosus*.

Thermoregulation is paramount to an organism's survival and reproductive fitness because physiological systems have evolved to function within a range of temperatures (Wells 2007). The range of temperatures in which an organism is able to carry out survival and reproductive activities is known as the range of thermal tolerance (Wells 2007). Within the range of thermal tolerance, each physiological system operates most effectively at an optimal temperature. Thus, as the internal temperature of an organism diverges from its optimal temperatures the functional efficiency of physiological systems decrease (Hillman et al. 2009). These decreases in physiological function can, in turn, trigger both behavioral and physiological stress responses (Feder et al. 1992; Wells 2007).

All animals have a suite of behavioral and physiological responses used to maintain physiological functionality. Mammals have evolved internal mechanisms that help maintain thermal homeostasis. Ectotherms like amphibians, however, must rely primarily on their environment to thermoregulate. Behavioral thermoregulation can allow amphibians to select environments where physiological needs may be met. Aquatic amphibians that are constrained by the temperature in their local habitat must move, when possible, between microhabitats to avoid physiological stress (Wells 2007).

To understand and predict stress responses in amphibians, we must first identify the environmental conditions that produce these types of responses. Because an animal is likely to choose a thermal environment that is least stressful, thermal selection experiments can be used to infer what temperatures are within an organism's thermal tolerance range. In this experiment, I examined the thermal preferences of two species of *Dicamptodon* salamanders to establish temperature ranges in which stress responses are not likely.

Most thermal aspects of *Dicamptodon* life history are unknown. Thus far, three previous studies examined thermal aspects of *Dicamptodon* spp. life history (Brattstrom 1963; Huff et al. 2005; Bury 2008). Brattstrom (1963) reviewed the thermal requirements of 99 species of amphibians from various parts of North and Central America. Body, air, soil and water temperatures were taken and Brattstrom (1963) reported that *Dicamptodon* spp. larvae were collected in streams with temperatures of 12.0- 16.2°C. Interestingly, the body temperatures of *Dicamptodon* spp. (taken internally) were identical to stream temperatures. Furthermore, extended contact with a salamander resulted in increased temperatures, presumably caused by heat transferred

from a workers hand. Brattstrom (1963) deduced that most salamanders have no preferred temperature and will accept the available temperatures, but that aquatic salamanders (like the *Dicamptodon* sampled) select specific temperature regions or levels within ponds.

Unfortunately, there is no indication of the sample locations for the larval *Dicamptodon* sampled by Brattstrom (1963) and thus it is impossible to determine which species were sampled. At least two sampling locations were in central Oregon (Lane Co., and Tillamook Co.) and one in California (Humboldt Co.). These sampling locations are within known *D. tenebrosus* distribution (and no other *Dicamptodon* species). These individuals were all terrestrial. *Dicamptodon ensatus* were among the sampled salamanders, however, Brattstrom (1963) did not describe all of the sampling locations and *D. ensatus* is currently split into four distinct species.

Huff et al. (2005) predicted the realized thermal niches of 14 aquatic vertebrate species by gathering temperature data and species abundance data across five geographic ranges. The realized thermal niche was described as the range of temperatures that an animal used in natural aquatic conditions including conditions known to thwart preferential temperature selection. *Dicamptodon* spp. were sampled from 100 streams, and daily stream temperatures were averaged over a 7 day period and the temperature averages where *Dicamptodon* were found ranged from 7.9°C to 20.2°C and each region varied within this range. Huff et al. (2005) provides evidence of upper temperature tolerances for *Dicamptodon* spp. over a wide geographical scale and from a large sample size. However, the study took place during the warmest part of the year (July-Sept.) and the results may not reflect the actual minimum temperatures within the *Dicamptodon*

thermal niche. Another issue with the study was that the authors documented sampling *D. ensatus.* However, the sampled streams were outside of the range of *Dicamptodon ensatus* and within the range of *D. tenebrosus* and *D. copei,* so it is unclear to which species these data apply.

Bury (2008) tested the upper thermal tolerance of *D. tenebrosus* using standard protocols for determining Critical Thermal Maxima (CT_{max}). The CT_{max} and Critical Thermal Minima (CT_{min}) are measurements commonly used to predict species ranges of temperature tolerance. These measurements reflect the maximum and minimum temperatures that an animal can withstand before it loses righting ability and would expire with continued exposure. The ranges of thermal tolerance, however, are sometimes "plastic" in the sense that amphibians can adjust their thermal tolerances to match sustained ambient temperature changes. This phenomenon is known as thermal acclimation (Duellman and Trueb 1994; Hillman et al. 2009; Wells 2007). Bury (2008) tested 12 *D. tenebrosus* from a single stream in Oregon (Douglas Co.). The animals were acclimated in the laboratory at 14°C for 3 weeks prior to the beginning of the study. Bury (2008) reported that the CT_{max} ranged from 28.7-29.3°C. Though the animals were acclimated prior to the study, I am unaware of the thermal history of this population of *Dicamptodon* and do not know how these results compare to the CT_{max} of their wild counterparts because high thermal acclimation tends to result in high CT_{max} and low thermal acclimation tends to result in low CT_{min} (Hillman 2009).

The magnitudes of thermal acclimation ranges are adaptive and vary widely between taxa (Wells 2007; Hillman et al. 2009). Duellman and Trueb (1994) hypothesized that amphibians from small gene pools (thus having lower heterozygosity)

have less ability to respond plastically to environmental temperature changes and have smaller ranges of thermal acclimation. In contrast, wide-ranging species with higher levels of heterozygosity may have broader ranges of thermal acclimation. Steele et al. (2009) determined by genetic analysis that of *D. copei* and *D. tenebrosus*, *D. copei* had a small distribution, a low level of dispersal, and a high level of genetic structuring, as compared to *D. tenebrosus* that had a much larger distribution, a high level of dispersal and a low level of genetic structuring.

Thermal tolerance ranges also are adaptive and greatly influenced by historical environmental temperatures (Feder et al. 1992; Hillman et al. 2009). Though I am unaware of historical stream data for the streams in which *Dicamptodon* have existed for these millions of years, recent accounts are known. Nussbaum et al. (1983) reported the range of stream temperatures that *D. copei* are known to inhabit as 8-14ºC. However, the *D. copei* sampled for this study were discovered in colder stream temperatures (3-10ºC) than reported by Nussbaum. Also the *D. tenebrosus* captured for this study were discovered in streams ranging in temperature from 13-18ºC. However, the Washington Department of Ecology (2014) reported seasonal stream temperature maxima for Lewis River where I sampled *D. tenebrosus* to be 24-29ºC for the years of 2001-2009, temperatures that near CT_{max} (2008) and exceed the temperature that I observed during capture in 2010 and 2012.

Examining habitat selection in sympatric congeners provides knowledge that helps explain the variability in life history patterns of closely related species. I chose to examine the thermal selection of *D. copei* and *D. tenebrosus* because they live sympatrically and vary in developmental aspects of life history. By comparing thermal

selection in *D. copei* and *D. tenebrosus,* I hoped to gain insight to the selective pressures that produce these phenotypes.

D. copei captured during this study inhabited cooler streams than did the *D. tenebrosus* captured during this study. Thus, I predicted that the thermal preferences of *D. copei* and *D. tenebrosus* would differ and that *D. copei* would select colder temperatures more often than *D. tenebrosus*.

Materials and Methods

Study animals

I collected *Dicamptodon copei* and *Dicamptodon tenebrosus* during late August-October of 2009, 2010 and 2012 by hand and dipnet (methods described in Chapter 2). The animals were housed (methods described in Chapter 2) in a temperature controlled laboratory with natural photoperiod. The animals acclimated at 12ºC ±1 for at least one month prior to this experiment. The animals were maintained individually in aerated, 10 gallon aquaria and fed 2-3 times weekly. Ninety *D. copei* and 22 *D. tenebrosus* participated in this experiment.

Aquarium construction

I constructed a temperature choice aquarium from ¼" thermoplastic (1.22m x 1.83m x 30.48cm) and created three sunken pools within the arena. Three holes, parallel and equidistant from one another were cut in the floor of the aquarium, and metal pans (25.09cm L x 12.7cm W x 6.98cm H") were placed in the holes so that the flared top of each pan contacted the floor of the arena. I sealed the joint between the pans and aquarium floor with 100% silicon. To decrease convection and mechanical mixing of the water while still allowing the animals to move between the pools, I installed small,

rectangular, ceramic walls between the pools. In addition transparent, blue, square bowls, (with holes cut for easy access to the pools for salamanders) were placed over the pools to provide cover and also allow video monitoring. Ceramic tiles lined the walls of the aquarium to provide insulation and to give the animal spatial perspective. The arena was placed atop six 10 gallon aquaria. Three aquaria provided structural stability while three aquaria provided temperature regulated baths in which the metal pools were suspended. I used Aqueon ProHeater, aquarium heaters and an Aqua Euro USA Max Chill Aquarium Chiller maintained the temperatures of the baths.

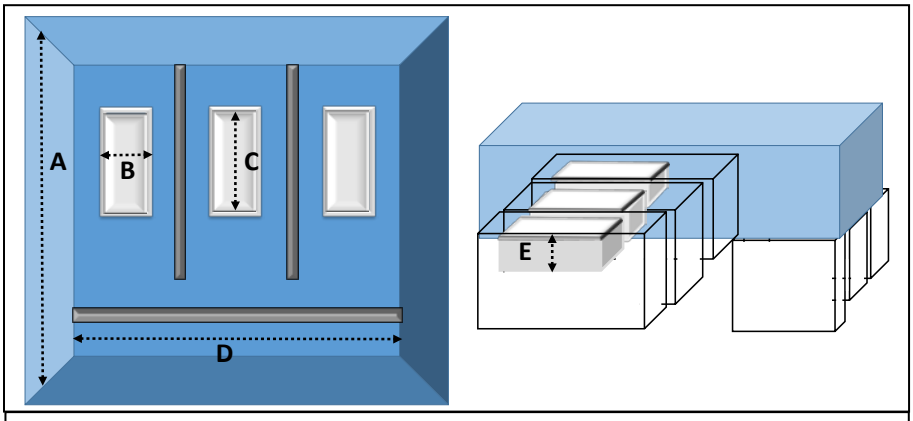

Figure 3.1- Dimensions of the temperature selection arena top view and side view. A= 1.83m B= 12.7cm C= 25.09cm D= 1.22m E= 6,98cm

Aquarium conditions

The water depth in each pool was 15.87cm and the water level surrounding the temperature choice pools were 8.89cm deep. Each evening, the water was changed and the arena was washed with a damp cloth to help control for possible confounding scent affects and to allow time for water to reach the desired temperatures in each pool. The temperatures of the pools were selected so that warmest and the coolest temperatures were slightly warmer and slightly colder than the stream temperatures from which the animals were collected. Pool temperatures were 6ºC± 1º C, 13ºC± 1º C, and 20ºC± 1º C.

For simplicity, these temperatures will be represented as 6°C, 13°C and 20°C. The pool temperatures were arranged from coldest to warmest with the midrange temperature between them. Digital aquarium thermal sensors (Digital aquarium thermometer ST-3 with ±1 °C accuracy) were attached by suction cups to the bottom of each pool and the digital display was placed in view of the monitoring camera.

Experimental procedure and data collection

Experimental trials were completed between the hours of 0800 and 1800 during September and October of 2013. One salamander participated in each experimental trial and I selected each animal using a random number generator. Prior to the beginning of the experimental trial, an individual was placed in the arena farthest from the temperature selection pools. A habituation period of an hour allowed the animal to explore the arena and shuttle between the pools. Each trial was monitored using a Carl Zeiss Tessar, HD 1080p, wide angle webcam (Logitech), suspended above the arena. Used in conjunction with the ISpy surveillance software (open source http://www.ispyconnect.com/) and Hewlett Packard Compaq personal computer, the camera took a still photo each minute for 90 minutes and stored it on an external hard drive for later viewing.

To convert the pictures to data I first created a photo album and slide show of each trial using in Microsoft Power Point software. Then, using a Visual Basic macro (created in Microsoft excel) I registered the position of the animal with a keystroke while simultaneously viewing the slide show.

Data analysis

To determine whether the 3 pool temperatures were equally selected, I performed a Chi-square test of goodness-of-fit. I used a multinomial logistic regression in "R" statistical software to predict the probabilities of different possible outcomes.

Results

Chi square test

A Chi-square test of goodness-of-fit was performed to determine whether 3 pool temperatures were equally selected. Preference for the pool temperatures was not equally distributed in either *D. copei* X^2 (2, N = 90) = 822.643, p < .0001 or *D. tenebrosus*, X^2 (2, N = 22) = 356.134, p < .0001.

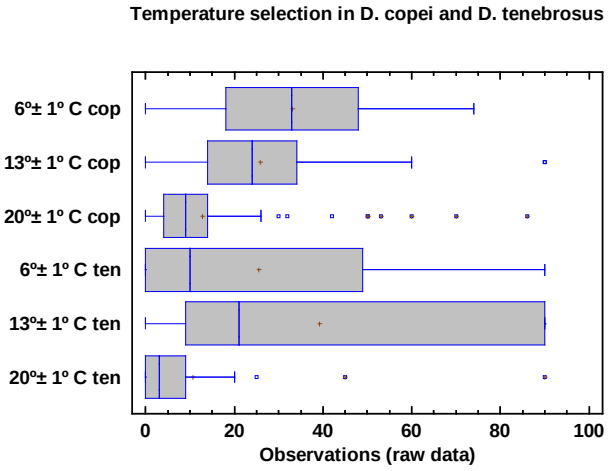

Figure 3.2 - Temperature selection observations: one observation per minute over 90 minutes total.

Multinomial logistic regression

There was a significant difference between the temperature selections of averaged sized *D. tenebrosus* and *D. copei*. The *D. tenebrosus* were more likely to be found in 13ºC (p<0.01) and 20ºC (p<0.01) pools than were *D. copei*. Average sized *D. tenebrosus* were most likely to be found in the 13ºC (p<0.01) pool over both 6ºC and 20ºC pools,

and 1.7 times more likely to be found in the 20ºC pool than the 6ºC ($p < 0.001$) pool. An average size *D. copei* from a stream with the average temperature is equally likely to be found at any of the 3 temperatures, and was 69 times more likely to be moving between pools ($p<0.001$) than to be found in any one pool.

Larger *Dicamptodon* (after controlling for species and the stream temperature they were found out) of both species were equally likely to be found in the 6ºC pool and the 13ºC pool. These larger animals were 1.02 times more likely to be found in the 6ºC pool than the 20ºC pool and are 1.02 times more likely to be found in 6ºC than moving between pools.

Dicamptodon from higher stream temperatures are equally likely to be seen in 6ºC, 13ºC, or 20ºC pools, but they are 1.09 times less likely to be observe moving between pools than to be in 6ºC.

Model coefficients	Estimate ±SE	*p* Value
13ºC± 1º C		
Intercept	0.47 ± 0.49	0.128
Species (tenebrosus)	1.82 ± 0.17	* 0.000
SVL	1 ± 0.002	0.064
Stream	1.02 ± 0.01	0.066
20ºC± 1º C		
Intercept	1.08±0.63	0.904
Species(tenebrosus)	1.657127 ± 0.22	* 0.021
SVL	0.99 ± 0.003	* 0.000
Stream	1± 0.014	0.698
Shuttle		
Intercept	69.21 ± 0.59	* 0.000
Species (tenebrosus)	5.49 ± 0.21	* 0.000
SVL	1± 0.002	* 0.000
Stream	1 ± 0.01	* 0.000

Table 3.1- Coefficients from the regression model of impact of Species, SVL and Stream on temperature selection. * indicates significant *p* values. A positive value indicates a greater likelihood of pool selection.

Conclusion

Behavioral responses to thermal options differed significantly between species. Average sized *D. tenebrosus* were more likely to select warmer temperature pools than average sized *D. copei*, though they were acclimated at the same

temperature. However, SVL influenced thermal selection, and large animals of both species behaved similarly. Large *Dicamptodon* selected the coldest temperatures most often, and *D. tenebrosus* that were average size, selected the "medium" pool most often. In contrast average sized *D. copei* were most often found moving between the pools. I propose several explanations for these results that may be investigated in future studies. First, varied thermal responses by different sized animals may reflect natural feeding behaviors. *Dicamptodon* are gape-limited predators that feed on nearly anything that they can fit in their mouth. Large animals are able to eat larger prey that inhabit the colder, deeper portions of the stream while smaller animals are limited to smaller, size appropriate prey that are most easily caught in the warmer, shallows of the streams. By remaining in the warmer, shallower water, smaller animals may actively hunt small prey while avoiding becoming prey to a larger *Dicamptodon*. Alternatively, large *Dicamptodon* salamanders may conserve metabolic energy by resting in cold water during the day and emerging to hunt in all possible locations of the stream in the evening. I have observed large aquatic *Dicamptodon* in shallow water and even on land in the evening and in deeper water during the day, thus it is possible that *Dicamptodon* shift hunting strategies and thermal environments with the time of day and location of large prey.

Although the largest animals of both species showed preference for the coldest water, overall the species varied significantly in thermal selection. The *D. tenebrosus* were more likely to choose the warmer pools (13ºC and 20ºC), indicating that these temperatures are likely within their thermal tolerance range. Though the *D. copei* were significantly less likely to be found in the warmer pools than were *D. tenebrosus* this may

be explained by the fact that they were 69 times more likely to be observed moving about the aquarium than to be in any pool. It is possible, however, that from the populations sampled, *D. copei* have evolved a lower thermal tolerance range than the sampled populations of *D. tenebrosus.*

That *D. copei* was 69 times more likely to be found moving about the aquarium than to be in any pool suggests that either an optimal thermal choice was not available in this study or that average sized *D. copei* are more active than large *D. copei* and *D. tenebrosus* of all sizes.

While this study provides evidence of differential thermal tolerance ranges between species, it is likely that thermal preferences differ with each set of thermal conditions across *Dicamptodon* geographical ranges. Furthermore, a model that includes the effects of age, sex, reproductive status and the overall health of each animal may uncover underlying relationships between these factors and thermal selective pressures.

Section 3: Hematological stress responses of *D. copei* and *D. tenebrosus* to 1.66 ±1°C, 21.11±1°C and 25±1°C environments

Abstract

Environmental stress is known to bring about alterations in fitness-related life history traits, such as the metamorphic and paedomorphic tendencies in salamanders. The relationship between stress and metamorphosis/paedomorphosis in salamanders is unclear because we do not know the threshold at which environmental factors become stressful. In this study, I relate neutrophil to lymphocyte ratios (N/L) to assess stress responses of *D. tenebrosus* and *D. copei* to three temperatures (1.66 ±1°C, 21.11±1°C,

25±1°C). Changes in N/L differed significantly within species, between treatments and between species.

Introduction

Amphibians are ideally suited for testing the effects of stress because they are sensitive to the environment and often able to alter life history traits to escape detrimental conditions (Denver 1997; Van Buskirk 2000; Relyea 2002). In amphibians, stress is mediated by the release of the stress hormone corticosterone (Denver 1997; Boorse et al. 2003). A common method of stress assessment in amphibians is to measure corticosterone in the blood. As stress increases, the levels of corticosterone also increase (Denver 1997; Boorse et al. 2003). A problem with this method of stress assessment, however, is that levels of corticosterone rise quickly in response to stress. Simply capturing or handling an animal may contribute to an inaccurate assessment (Davis and Maerz 2008). It is possible, however, to assess stress by examining leukocyte profiles. Leukocyte profiles change relative to stress. But, unlike with corticosterone, there is a delayed response. This response lag allows for baseline measurements to be taken. Specifically, neutrophils increase and lymphocytes decrease as stress increases. The ratio of neutrophils to lymphocytes (N/L) before and after a stressful event can be used to infer the intensity of the stress response (Davis and Maerz 2008). Davis and Maerz (2008) reviewed previous studies and reported similar hematological stress responses in various amphibian species for limb amputation, osmotic stress, photoperiod stress and temperature extreme stress. In this study, I compare N/L ratios of *D. tenebrosus* and *D. copei* to test the hypotheses that (a) N/L ratios will increase relative to increased

temperatures in both *D. tenebrosus* and *D. copei* and (b) that stress responses will differ between species in each treatment.

Methods

I selected treatment temperatures (1.66 ±1°C, 21.11±1°C, 25±1°C) based on (a) stream temperature ranges documented by Nussbaum et al. (1983), (b) Bury's (2008) assessment of critical thermal maxima, (c) my field observations and (d) the temperatures selected by the salamanders during the temperature selection trials (see section two of this chapter) For simplicity, these temperatures will be represented as 1.7°C, 21°C and 25°C. Ninety *D. copei* and 25 *D. tenebrosus* participated in the 21°C, treatments and 35 *D. copei* and 21 *D. tenebrosus* participated in the 25°C trials during November and December of 2013, with a two week recovery period between treatment applications.

To obtain blood in a timely manner following treatment, I divided the *D. copei* into 3 groups by random selection, and placed one group in the same temperature treatment per day, over a three day period. I placed all *D. tenebrosus* in the same treatment on the 4th day. During each trial, the salamanders were placed individually in plastic boxes (15.24 cm x 30.48 cm x 10.16cm) with 3 inches of water. I then placed each box in an environmental chamber. To avoid shocking the animals, I placed the salamanders in the environmental chamber at the ambient temperature of the laboratory (~13°C) and allowed the temperature in the chamber to increase or decrease gradually. The air temperature in the chamber reached the desired temperature within an hour and the salamanders remained in the chamber for a total of 24 hours. Following treatment, I anesthetized each salamander and obtained blood (as described in Chapter 2 of this thesis) from all animals within ~2 hours of treatment. The salamander groups

participated in each temperature trial, in the same order for each trial. The 35 *D. copei* that participated in the 25°C experimental trials were randomly selected from all captive *D. copei*. Blood differentials and erythrocyte measurements were performed in the same manner as described in Chapter 2 of this thesis.

Data analysis

The variables of interest in this study were: Log transformed N/L, Vc, and Vn for each of wild and captive populations and 3 temperature treatments (1.7 °C, 21°C, 25°C). All statistical analyses were performed using STATGRAPHICS centurion statistical software. To approximate normal distributions, all data were log transformed prior to analyses. To visually assess differences and look for outliers I generated box and whisker plots of the log-transformed data. I used a Multiple Variable analysis to obtain the 95.0% confidence intervals for the means and standard deviations for each of the variables. I used a multiple sample comparison (Multiple Range Test) approach to determine which means (within and between species and treatments) were significantly different. To determine the possible effects of size and species on the N/L ratios of each temperature treatment, I performed an analysis of variance (ANOVA).

Results

Among *D. tenebrosus* there were no significant differences between N/L ratios of the wild and the 21°C treatment nor the captive and 21°C treatments. There were significant ($p \leq .05$) differences between the baseline and the 1.7°C treatment and the baseline and the 25°C treatment. There were also significant ($p \leq .05$) differences between all treatments (1.7 °C, 21°C, 25°C).

Among *D. copei* there were significant differences ($p < 0.05$) between the wild and captive N/L ratios, wild and each treatment, captive and each treatment and between all treatments (1.7 °C, 21°C, 25°C).

Interspecies comparisons revealed significant differences ($p < 0.05$) in N/L between *D. copei* and *D. tenebrosus* in wild, captive, 1.7°C and 21°C treatments. There was no significant difference between *D. copei* and *D. tenebrosus* for the 25 °C treatment.

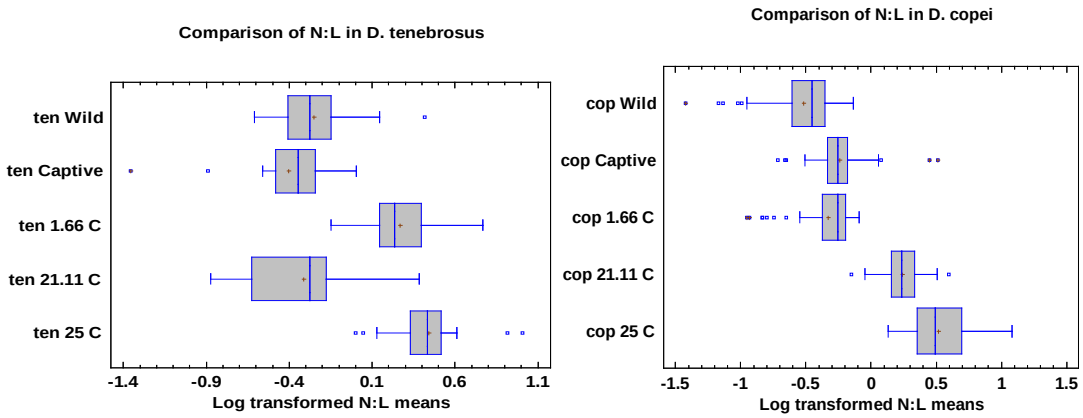

Figure 3.3- Standardized neutrophil/lymphocyte mean ratios in *D. tenebrosus* and *D. copei* for wild and captive baselines and three temperature treatments (1.7°C, 21°C, and 25°C).

Environment	Mean N/L	Stnd. error	Lower limit	Upper limit
cop Wild (4-12°C)	-0.52	0.03	-0.59	-0.45
cop Captive (12 ± 1 °C)	0.66	0.06	0.53	0.78
cop 1.66 ± 1 °C	0.51	0.02	0.47	0.55
cop 21.11 ± 1 °C	1.84	0.09	1.65	2.03
cop 25± 1 °C	3.84	0.47	2.86	4.81
ten Wild (15 -17 °C)	0.66	0.08	0.50	0.81
ten Captive (12 ± 1 °C)	0.47	0.06	0.35	0.59
ten 1.66 ± 1°C	2.04	0.19	1.64	2.44
ten 21.11 ± 1°C	0.64	0.13	0.37	0.91
ten 25 ± 1 °C	3.25	0.48	2.25	4.24

Table 3.2- log transformed, 95.0% confidence intervals showing changes in N:L in *D. copei* and *D. tenebrosus* from wild, captive 1.66 ± 1 °C, 21.11± 1 °C and 25± 1 °C environments. Red text highlights extreme changes in N:L indicating stress.

Conclusion

The *D. copei* and *D. tenebrosus* clearly differed in thermal stress responses. While *D. copei* were significantly more stressed ($p \leq 0.05$) in response to the mid (21°C) and warmest treatments (25°C), *D. tenebrosus* were significantly more stressed ($p \leq 0.05$) in the coldest (1.7°C) and warmest (25°C) environments. These responses suggest a difference in thermal tolerance ranges of these salamanders. While CT_{min} and CT_{max} are the lethal limits of thermal tolerance, there is a range of temperatures just above and below these thermal limits in which salamanders may survive, but would not choose to endure if there were a less stressful option. These temperatures are called the zones of resistance (Hillman 2009). Though acute exposure to temperatures within the zones of resistance may not be lethal, prolonged exposure would certainly affect the efficiency of most physiological processes. That both species were similarly stressed at 25°C indicates that this temperature lies within the upper zone of resistance. Remarkably, *D. tenebrosus* was nearly as stressed by the coldest water as the warmest water. This is likely a population level phenomenon because many populations of *D. tenebrosus* inhabit very cold streams. Nevertheless, if cold temperatures are stressful to *D. tenebrosus* there may be behavioral or physiological effects resulting from this thermal stress. Though the effects of warm temperatures on amphibians are fairly well studied, nothing is known of the effects of cold water on *Dicamptodon*. The *D. copei* were not stressed by exposure to 1.7°C, but were stressed by 21°C, which suggests that 1.7°C is within *D. copei's* range of thermal tolerance, that the lower range of thermal tolerance is below 1.7°C and the optimal range of temperatures lies somewhere between these temperatures. The *D. tenebrosus* were not stressed by 21°C, and this indicates that this highest temperature lies

within the optimal range of temperatures in which the *D. tenebrosus* from these populations can survive, grow and reproduce.

Neutrophil/lymphocyte ratios change relative to circulating stress hormones. This phenomenon is fairly consistent and conserved across taxa, thus making it possible to compare stress responses between species to answer a variety of research questions. In this case, I used a hematological approach to (a) define thermal stress in *D. copei* and *D. tenebrosus,* and (b) to assess differences in stress responses of these species. Clearly, *D. copei* and *D. tenebrosus,* from the populations sampled, differ in thermal stress responses and their physiological systems operate most efficiently within different temperature ranges. It is reasonable to suggest therefore, that the varied response to thyroxine treatments in *D. copei* and *D. tenebrosus* (Nussbaum 1970) may have been a result of suboptimal conditions for thyroxine sensitivity in *D. copei.* I also propose that the spontaneous transformation of *D. copei* subsequent to capture was a threshold response (to either captive or thermal stress) that confirms *D. copei* has retained the ability to metamorphose given optimal environmental conditions.

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Chapter 4

Courtship behavior of paedomorphic Pacific Giant Salamanders, *Dicamptodon*

Abstract

Characterization of dicamptodontid courtship provides an excellent opportunity to compare their courtship behaviors with that of their sister taxa, Ambystomatidae, thus strengthening our understanding of the deep evolutionary history of salamander courtship. Courtship behaviors have not been previously described from *Dicamptodon.* Indeed, most aspects of *Dicamptodon* reproductive biology are poorly understood. I characterized the respective courtship behaviors of two species, the Pacific Giant Salamander (*Dicamptodon tenebrosus*) and the Copes Giant Salamander (*Dicamptodon copei*) by observing staged male-female encounters in the laboratory. Ten male-female encounters (five *D. copei* and five *D. tenebrosus*) were videotaped for analysis. Five courtship stages were consistently present among all mating pairs: recognition/persuasion, primary stimulation, spermatophore deposition, post deposition maneuvering, and secondary stimulation. The occurrence of sperm transfer was observed a total of three times: once in *D. tenebrosus* and twice in *D. copei*. Although the general type of behaviors observed were similar in both species at each stage, pairs varied in the duration and number of times a particular behavior was displayed. Notably, male *D. tenebrosus* deposited more spermatophores per courtship trial, and the female of the species was often more aggressive toward the male than was in the case of *D. copei*. I conclude that, in a phyloethological context, *D. copei* and *D. tenebrosus* courtship are similar to one another and both differ greatly from ambystomatid courtship behaviors.

Introduction

Urodeles demonstrate diversity in courtship behavior and in modes of sperm transfer. Three families of salamanders exhibit external fertilization, while seven families employ internal fertilization, but without a copulatory organ, via indirect sperm transfer (Wells 2007). For insemination to occur the male must deposit a gelatinous, sperm-capped mass (spermatophore) to the substrate, and a female must lodge the sperm cap into her cloaca. While this method of insemination is common to all internally fertilizing salamanders, a plethora of behavioral, morphological and chemical innovations have evolved in the service of sperm transfer (Halliday 1998; Houck & Arnold 2003; Houck 2014). Some innovations are conserved within and among families while others are unique to families, genera, species, or even to populations (Arnold 1977; Houck & Arnold 2003). Consequently, we can compare courtship behaviors of closely related taxa to inform our understanding of behavioral traits that may have evolved millions of years ago.

Dicamptodontidae and Ambystomatidae are sister families that diverged about 115.8 million years ago (Vietes et al., 2009). Although early field observations indicated that dicamptodontid salamanders deposit numerous spermatophores during a single courtship, no other dicamptodontid courtship behaviors were known prior to this study (Arnold 1977). Fortunately, courtship behaviors for nearly all species of *Ambystomatidae* have been observed (Salthe 1967; Arnold 1977; Houck and Arnold 2003), thus offering an opportunity ripe for phyloethological discovery and comparison.

Neither Dicamptodontidae nor Ambystomatidae possess a mental gland or nasolabial glands that might be used for pheromone delivery during courtship (as do

plethodontid salamanders). However, Sever (1992) compared the cloacal anatomy of dicamptodontid and ambystomatid salamanders and reported that males share similar cloacal morphology. Like most urodele males (Wells 2007), *Dicamptodon* and Ambystoma possess kingsbury glands, anterior and posterior ventral glands, dorsal and lateral pelvic glands and vent glands (Sever 1992). Females of both genera each possess spermatheca and ventral glands; however, dicamptodontid females lack a dorsal gland that is present in many ambystomatid females (Sever 1992). Sever did not report on the function of cloacal glands in these two families, however, all but the vent glands in male salamanders are assumed to function in spermatophore production (Wells 2007). Sever (2003) proposed that the vent glands of the plethodontid, *Eurycea cingulatum*, secrete courtship pheromones. He also speculated that the ventral glands of the female salamandrid, *Euproctus asper* must also secrete mating pheromones. Ambystomatid courtship behaviors, such as the "cloaca tail nudging walk" and "pheromone wafting", suggest that the male vent glands also secrete pheromones that serve in orientating the female toward the spermatophore (Arnold 1977).

Houck and Arnold (2003) postulated that the ancestral courtship behaviors of ambystomatids occurred in water, and progressed in the following sequence: (1) the male approaches the female and rapidly nudges her, (2) the male taps the female on the head with his tail and (3)the female indicates receptivity by following the male while nudging his cloaca, (4) the male deposits spermatophore after spermatophore in the female's path during this walk and, (5) the female may or may not insert a sperm mass into her cloaca. Evolutionary modifications on this general sequence involve the loss of the cloacal-tail nudging walk in five species, introduction of dorsal amplexus in three species, and

shoving transport of the female in three species (Houck et al. 2003, Arnold 1977). Two ambystomatid species, *A. opacum* and *A. cingulatum* have evolved terrestrial courtship (Hill 2013; Krenz et al. 1994). All extant ambystomatids deposit multiple spermatophores during a single courtship (Houck & Arnold 2003).

Based on these observations, I predicted that dicamptodontid and ambystomatid courtship behaviors would be similar because Dicamptodontidae and Ambystomatidae are morphologically similar sister families that are uniquely capable of depositing numerous spermatophore during a single courtship.

Materials and Methods

Capture and husbandry

The *D. copei* and *D. tenebrosus* for this study were captured and housed as described in Chapter 2 of this thesis (methods described in section 1).

Mating pair selection

Because *Dicamptodon* are nearly monomorphic, I based sex identification on reproductive morphology (Greven 2003) and behavioral cues. The female oviducts extend from the anterior end of the body cavity to the opening at the cloaca. As the eggs mature they increase in size and fill the body cavity, causing lateral distention. Distention decreases at the inguinal and axillary regions, and this feature gives the salamander a rounded appearance. The males lack this rounded appearance, and may or may not have a swollen cloaca. Clothier (1966) initially described cloacal papillae in older males. However, further investigation revealed characteristic papillae for males of all sizes. I thus examined each animal's cloacal region and scored it as female or male.

To establish potential mating pairs, I randomly selected two salamanders and placed them together in a small aquarium. I then observed subsequent behaviors of the paired animals for ~10-20 minutes. The one exception to random pairing was that I re-paired animals if one of the selected animals was only half the size of the other (because the smaller one could potentially be eaten by the larger). If the pair fought, or if there was no courtship interest at all between the pair after three to four hours, each was placed back in its respective tank. If at least one of the two animals continually attempted to place its snout on the other without biting, these two animals were selected as a potential mating pair.

Behavioral Trials

I staged courtship trials between the dates of October 2011 and March 2013. A potential mating pair was placed together in a 61cm x 33cm x 43.2cm (75.7 liter) aquarium equipped for video surveillance. I created a courtship chamber, within the aquarium, by piling rocks and leaving space in the center of the rock pile. The size of the chamber was determined by the size of the salamanders, and was made large enough to allow pairs to move freely and to accommodate a clear path for videotaping. The pair was free to enter and exit the chamber at will.

Over a period of three years, I recorded thirteen courting pairs of *D. tenebrosus* and ten courting pairs of *D. copei*. However, filming conditions posed a number of challenges that made it difficult to quantify some of the courtships. Furthermore, I did not provide cover for the animals during some of the early trials and this stress may have altered the female's behaviors. Of the twenty-three staged courtships, I included five *D. copei* and five *D. tenebrosus* in our data analysis based on the direct observation of

spermatophore deposition regardless of whether or not I was able to observe sperm transfer. I established the beginning of a courtship trial to be when the male began to pursue the female, and the trial ended after 48 sequential hours of courtship inactivity.

Videography

I recorded continuously using at least four low-light security cameras (Logitek, WLPC-810i) that were placed in varied locations around the tank. The cameras were connected to a Hewlett Packard personal computer, equipped with Wilife command center security software, provided with the security camera. To capture video throughout the night, low wattage red lights remained on when the main lights (controlled by a photocell) automatically shut off. Each morning, I examined the video clips for evidence of courtship activity. Each trial video was then transferred to an external hard drive for scoring. I continued to videotape until there was at least seven days of courtship inactivity, in case a pair would re-mate.

Data collection

From the video footage, I documented and characterized individual behaviors and courtship patterns to determine the components and stages of courtship. I observed and scored courtships in which included at least one full sequence of courtship behaviors, from the initial male-female interactions to spermatophore deposition, and secondary stimulation. Included in the video footage were the few courtships that led to insemination. I scored the time spent in each of the following stages: Recognition/Persuasion, Primary Stimulation, Sperm Deposition, Post Deposition Maneuvering, and Secondary Stimulation. Sperm transfer occurrence was also noted. I

calculated the mean durations, standard deviations and duration range for each of the courtship stages in Microsoft Excel.

Results

In total, I included five sequences for *D. copei* and five sequences for *D. tenebrosus* in our results. I observed sperm transfer three times, once in *D. tenebrosus* and twice in *D. copei*. Closer scrutiny of the many hours of video footage, however, may reveal additional occurrences. I recorded the results of the courtship observations in an ethogram format (as in Arnold, 1972). In this format, the male and female behaviors are listed separately for each species. The *D. copei* and *D. tenebrosus* courtship behaviors are nearly the same, with a few notable differences in some courtships. First, *D. tenebrosus* deposited 9-13 spermatophores per courtship trial, while *D. copei* deposited six to nine spermatophores per courtship trial (Table 1). Secondly, *D. tenebrosus* spent less time courting between spermatophore depositions (Table 1). Thirdly, female *D. tenebrosus* contacted the male with their snout more often than did female *D. copei*. This female assertiveness was observed most often during the recognition phase. On one occasion, a female *D. tenebrosus* initiated contact and briefly pursued the male. This female was considerably larger than the male and size may have influenced the behavior. Subsequent to the female's aggressions, the male reciprocated contact and began (or resumed) courting her. Because the courtship behavior of the two species was so similar (occurring in the same order), I present a single ethogram that includes general behaviors of both species.

Ethogram of Male Behaviors

Head contact. The male contacts any region of the female with his head.

Snout to Tail Contact. The male places his snout directly in contact with the female's tail. His snout may remain stationary or he may move it along the lateral surface of the tail.

Snout to Cloaca Contact. The male slides his snout under the female until his nares are in contact with her cloaca.

Snout Sliding. The male slides his snout along the female's tail and/or body.

Pushing. The male uses his snout to force either part or all of the female to move.

Shoveling. The male lowers his snout (mouth closed) and pushes it beneath the female. He then lifts his snout such that his nares are higher than his gills.

Rubbing. The male strokes the female with his snout.

*Open mouth contact***.** The male contacts the female with his mouth but without biting.

Biting. The male closes his mouth on some part of the female.

Body contact. The male's body comes in contact with the female.

Dorsal Sliding (DS) The male maintains contact with the ventral surface of the female, with his dorsum while either he or she is in motion. There are two types of dorsal sliding.

DS1. After accessing the female's ventral surface, the male slides transversely under the female while attempting to maintain contact with her ventral surface.

*DS2***.** After accessing the female's ventral surface by shoveling, the male extends

Corralling. The male flexes his body laterally around the female.

C 1. The male curls his tail laterally around one end of the female and curls his head around the other. The male then moves laterally such that the female is displaced.

C 2. The male gains access to the female's ventral surface with his snout. He moves transversely beneath her body. He curls this head and thorax back toward the female while his tail is still under her. He places his head beneath her and then lifts the female using both his head and his tail.

Spermatophore deposition. The male aligns himself parallel to the female, either head-to- tail or head- to- head. He lowers his body by rocking fore and aft several times and eventually coming to rest with his cloaca pressed against the substrate. He does not move and gill pumping stops. He remains in the same spot for one to four minutes (Table 1) while he deposits one spermatophore. Gill pumping resumes directly after he has deposited the spermatophore. He lifts off of the spermatophore by raising his hind quarters and tail, and proceeds forward. He leaves the spermatophore adhering to the substrate. He deposits one spermatophore between the stimulation and maneuvering stages, and the spermatophores are deposited in nearly the same spot each time. On a few occasions, I observed the male release a spermatophore during stimulation or while transporting the female. Sometimes the spermatophore adhered to the substrate, but in other instances the spermatophore remains unattached to the substrate.

Sperm transfer. The male pushes and shovels until he has positioned the female so that the spermatophore inserts in her cloaca.

Ethogram of Female Behaviors.

Traveling. The female walks or swims about the tank, often in a direction away from the male.

Head contact. The female contacts any part of the male with her head.

Snout contact. The female's snout is in direct contact with any part of the male's epidermis.

Snapping. The female aggressively turns her head toward the male, opens her jaws and lunges, open mouthed, toward the male while closing her jaws.

Vent exposure. The female extends her legs and raises her body. Three variations of this behavior are described below.

V1. The female raises her body by extending all four legs vertically, while also lifting her tail.

V2. The female raises her hind quarters by extending her hind legs, while also lifting her tail.

V3. The female raises her thorax and head by extending her front legs.

Sperm mass pick-up. After the male aligns the female's cloaca with his spermatophore, the female is either backed onto or she lowers her cloaca to accommodate the sperm cap within her cloaca. When the sperm cap has been transferred, the female moves away or is pushed from the spermatophore base.

Courtship stages and temporal organization

During a particular stage, some behaviors occur more often, but the expression of one particular behavior did not seem to exclude the expression of another behavior during any given stage (with the exception of spermatophore deposition). Common behavioral

trends were noted, but these behaviors may, or may not, lead to sperm transfer. The following stages were observed in all courtship trials, and the activities during each stage varied in terms of participation and duration. No obvious signal(s) preceded the end of courtship.

Recognition/Persuasion

Generally, courtship began when the male contacted the female with his snout, first with hesitation but then with increasing vigor. At some point, the male escalated his courtship efforts by (a) pushing the female around the aquarium with his snout, or (b) snout sliding. The female sometimes defied the male's advances by escaping, or by snapping and biting him. On a few occasions the female turned toward the male and made snout to tail contact (STC) or snout to cloaca contact (SCC). The female traveled about in the aquarium and the male followed. When the female successfully escaped the male, he searched for her and regained STC or SCC. The Recognition/Persuasion phase was the shortest phase, lasting an average of 11 minutes (1 - 23 min).

Primary Stimulation (PS)

After a time, the female no longer defied the male's advances and she allowed him to stimulate her. Primary stimulation was the longest courtship phase, lasting an average of over five hours (Table 1). During PS, notable changes occurred in the structure and appearance of the male and female cloacae; the cloacae of both males and females became engorged, causing the cloacal lips to spread and appear red.

During PS, the male broadened the regions of contact and increased the amount of SCC. Thereafter, the male increased the number of stimulation strategies and varied their order of occurrence. Behaviors included Shoveling, DS1, DS2, and open mouth contact.

During late stages of primary stimulation, the female sometimes exhibited vent exposure postures (VEP). These postures resulted in greater access by the male to her ventral surface.

Spermatophore deposition (SD)

The time between recognition/persuasion and the first SD was highly variable between trials, but similar between species (Table 1). Typically, the male oriented laterally to the female, either head-to-head or head-to-tail. The male rocked forward and back several times before settling with his cloaca pressed on the substrate. Gular pumping ceased and he remained completely still. The male then lifted his abdomen and tail while walking forward, leaving the spermatophore undisturbed. The sticky base of the spermatophore adhered to the substrate and the sperm was contained in an apical cap.

The female generally remained still during SD, but on occasion she moved a short distance (~4-5 cm) from the male, yet she remained in the courtship chamber. Spermatophores were not continuously deposited, instead a male would move to PDM.

Post deposition maneuvering (PDM)

After the male deposited the first spermatophore, he immediately turned toward the female and began PDM. He pushed and lifted her toward the spermatophore. If the female attempted to move away, the male corralled her toward the spermatophore. The male used open-mouth contact and closed mouthed snout contact as he positioned the female. The female either a) became rigid and she allowed the male to maneuver her, or b) she moved away from the male. On a few occasions the female snapped at the male. This behavior momentarily caused retreat by the male, however, PDM quickly resumed.

Throughout PDM, the male located the spermatophore with his own snout and when the spermatophore became dislodged or damaged he abandoned PDM and once again began stimulation behaviors.

Secondary stimulation

The behaviors observed during secondary stimulation can be characterized as more focused and less variable than those observed during primary stimulation. The male constantly pushed on the female's ventrolateral edge, (usually near the cloaca) presumably to gain access the ventral surface of the female. If his attempt to slide beneath her was difficult, he quickly rotated his body from side to side, or he bit the female's leg until the resistance was overcome. After gaining access to the female's vent the male engaged in Dorsal sliding (DS). DS was often the primary stimulation tactic during the secondary stimulation phase and the male implemented DS most often near the female's cloacal region. The male sometimes displayed any or all other stimulation behaviors during secondary stimulation, albeit to a lesser degree. Secondary stimulation continued until the male deposited another spermatophore.

Sperm transfer

At no time did a female move toward a spermatophore without male physical manipulation. If the male was able to maneuver the female over the spermatophore and if the female was receptive then the cloaca-to-sperm-cap contact resulted in sperm transfer.

Discussion

Male salamanders have limited sexual resources and the likelihood of reproductive success may depend on what form the resources take and how they are allocated (Halliday 1976). Not all salamanders are equipped with the same resources,

which suggest that both sexual innovations and the allocations thereof have emerged from varying selective pressures, or that taxa have evolved differential responses to similar pressures. When compared to one another, however, *D. copei* and *D. tenebrosus* have distinctly different courtship behaviors from any other ambystomatid salamanders. Our observations suggest that, despite morphological similarities and close genetic relatedness, *Ambystoma* and *Dicamptodon* have evolved different courtship innovations for similar tasks, and different resource allocation patterns as a result of varied selective pressures.

Competitive behaviors and breeding season

Ambystomatids typically breed in polyandrous congregations due to short breeding periods (Houck et al. 2003). This pattern sets the stage each breeding season for intense male-male competitive behaviors. Although I did not stage male-male encounters during the mating trials, I made two observations suggesting that *D. copei* and *D. tenebrosus* may minimize competitive interactions by allocating reproductive resources differently than ambystomatid salamanders. First, I observed that in *D. copei*, the cloacae of both sexes were nearly monomorphic, unless the animals were in the presence of a potential mate. During male-female encounters, the cloacae of both sexes became engorged and, swelling decreased only subsequent to the encounter. Secondly, I observed, that all 50 *D. copei* females bore yoked eggs. Ova sizes were relative to animal size (snout to vent length range 83mm-119mm). The *D. tenebrosus* females also bore ova though the eggs of each clutch varied widely in size and were presumably in stages of resorption. The pattern of cloacal swelling was different for *D. tenebrosus* than for *D. copei* during the time in captivity. Cloacal swelling was increasingly apparent in male

and female *D. tenebrosus* as the animals increased in age and size and the pre-courtship swelling was independent of the presence of a potential mate. During courtship, cloacal swelling increased and subsequently decreased as in *D. copei*. Though statistical confirmation is needed, this discovery suggests that *D. tenebrosus* may court after a certain age is reached.

For opportunistic or asynchronous breeding to occur, the salamanders must be physiologically capable of mating throughout the year. I observed both *D. copei* and *D. tenebrosus* courting each month of the year in the laboratory. If *Dicamptodon* court seasonally, they may engage in competitive behaviors much like ambystomatids. However, Nussbaum (1971) suggested that courtship takes place in well-hidden chambers deep in the interstices of rock piles. It is plausible that *Dicamptodon* males avoid competition during courtship by establishing territory and maintaining a courtship chamber throughout the year. During mate selection, I observed both male-male sex biased aggressions and apparent chamber guarding.

Courtship duration

Staged *Dicamptodon* courtships were lengthy (Table 1) when compared to other urodele courtships. Prior to this study, the plethodontid, *Aneides ferreus* was the only salamander species known to court longer than seven hours (Sapp and Kiemnec-Tyburczy 2011). Though I terminated the courtship trial when I observed no courtship behaviors for 48 hours, the salamanders often resumed courting when left together for an extended period. I did not consider this as part of the trial; however courtship behaviors often resumed and would continue for up to a week or more. For the male, the courtship bouts were vigorous and nearly continuous. I tested the hypothesis that the animals

paused between courtship bouts because their metabolic and organic resources were depleted because when I fed a pair *ad libum* immediately after a courtship trial, courtship typically resumed. I fed them every two days thereafter and returned them to separate housing aquaria when I observed no courtship activities for a week. In this fashion, the pair often courted for over a week at a time with several courtship bouts.

Spermatophore allocations and male-female interaction

Ambystomatids vary in allocation of spermatophore resources and male-female interactions as the proportion of one increase the other decreases. For example, *A. texanum* are explosive breeders that compete for reproductive success by depositing numerous spermatophores consecutively, and the male may or may not have contact with a female. On the other end of the spectrum, *A. tigrinum,* also an explosive breeder, employs the cloaca tail nudging (CTN) walk whereby the female places her snout on the male's cloaca and follows him forward as he leads her over his newly deposited spermatophore. Ambystomatids that engage in the CTN walk spend more time interacting with the female per spermatophore deposited (Houck & Arnold 2003).

Dicamptodon males seemed to allocate resources intermediate to ambystomatid males because they deposited numerous spermatophores per courtship bout (Figure 1), and also dedicated a considerable amount of time toward male-female interactions. *Dicamptodon* males deposited numerous spermatophores during a courtship trial, but deposited one spermatophore per courtship sequence (beginning with primary stimulation and ending just prior to secondary stimulation). Remarkably the male continually returned to the spermatophore during maneuvering presumably to monitor its condition and check on its location and presence. The male did not deposit an additional

spermatophore until the prior spermatophore was either dislodged from the substrate or destroyed. Each spermatophore was typically deposited within 0.5 to 2 inches from the female and in the nearly the exact location of the previously deposited spermatophore.

The female remained mostly passive while the male deposited a spermatophore and maneuvered her to the spermatophore. Consequently, this behavior eliminates the need for coaxing behaviors by the male toward the female for sperm retrieval. In this way, the male is also better able to monitor the condition and presence of his spermatophore. No other urodele has been observed guiding the female to the spermatophore in a similar manner.

Tactile and chemical communication

Our observations suggest tactile behaviors that may comprise chemical communication in *Dicamptodon*. For example, upon first approach the male often placed his snout on the female's tail. Subsequent behaviors apparently were based on information received during this interaction. Epithelial cells located on the tail are likely to provide chemical cues necessary for identification of both species and sex. Furthermore, as courtship progresses, the male increasingly contacted the female's cloaca with his head and dorsum. The apparent results were (a) cloacal swelling and, (b) a change in the female's behavior in response to the male's advances. This proposal is highly speculative and further investigation is needed to assess its accuracy. A plausible explanation for these behaviors is that cloacal stimulation of the female triggers the release of hormones that induce her receptivity and prepare the cloacal opening for sperm retrieval. As the female does not have vent glands, perhaps the interest of the male is triggered by dermal pheromones and it is maintained by pheromones released by the

ventral gland as Sever (2003) suggested for *Euproctus asper*. I do not know if increased hormonal release occurs with tactile stimulation, or if increased hormonal release triggers swelling in *Dicamptodon*. However, Wells (2007) reported that cloacal glands in all other male salamanders studied enlarged with increasing hormone levels and the cloacae of all animals observed in this study swelled during the courtship trials.

Ancestral Courtship

Our observations revealed similar courtship behaviors of *D. copei* and *D. tenebrosus.* That these species diverged over 2.5 million years ago (Steele et al. 2005) in variable environments and yet they share very simple, nearly identical, courtship behaviors suggests that these behaviors have gone unchanged and must have arose in or before their common ancestor. To gain insight to the pressures that may have influenced the courtship of ancient *Dicamptodon*, I examined the environment of the first known articulated fossil specimen, *D. antiquus.* I propose that *Dicamptodon* courtship was shaped by predation.

Dicamptodon antiquus was discovered in the Paleocene lakebed sediments of Alberta, Canada. The Paleocene strata contain the most diverse freshwater assemblages known from North America (Brinkman 2013). Included in the fossil record are: *D. antiquus* (Naylor & Fox 1993), the crocodile *Borealosuchus griffithi*, the first known pike, *Esox tiemani*, numerous undescribed osteoglossomorph and percopsid fishes, a snapping turtle, *Protochelydra zangerli* (Wu et al. 2001), the trionychid turtle *Aspideretes superstes* (Russell 1930), at least three non-trionychid turtle taxa, an indeterminate aenid, and a macrobaenid, *Judithemys backmani*, (Brinkman 2013, Naylor & Fox 1993, Wilson 1980).

If dicamptodontid courtship behaviors arose in this environment, these salamanders certainly would have benefited by courting discretely. This seems to be the case as *Dicamptodon* court beneath rocks, they lack elaborate courtship dances or tail waving and they have not been observed in large mating congregations that might draw attention from potential predators.

Paedomorphic *Dicamptodon*, *Cryptobranchus*, *Amphiuma* and *Necturus* are the only salamander genera that contain living species known from skeletal fossils as old as the Paleocene (Naylor et al. 1993, Estes 1981). It is therefore likely that dicamptodontid courtship behaviors evolved prior to the diversification of ambystomatid courtship behaviors and it is also likely that courtship behaviors known to ambystomatids were not lost by dicamptodontid salamanders. A more complete analysis of *Dicamptodon* courtship awaits field observations of all four *Dicamptodon* species.

D. copei

	R/P	PS	ST	M	S1	SS	S2+	TCT	SP
Ave	0:11:27	5:35:28	0:02:32	0:29:38	6:34:01	1:15:13	1:48:01	17:00:29	7
Min	0:01:09	4:07:49	0:01:36	0:11:20	4:48:59	0:09:50	0:41:01	14:47:35	6
Max	0:26:07	7:08:00	0:03:22	1:33:21	7:38:44	2:32:54	4:09:18	20:15:29	8
Stdev	0:10:07	1:06:08	0:00:29	0:13:45	1:04:09	0:25:00	0:34:26	2:47:47	1
Med	0:11:25	6:02:00	0:02:36	0:27:26	6:42:22	1:10:42	1:45:07	15:09:49	7

D. tenebrosus

Ave	0:24:47	5:14:57	0:02:35	0:20:34	6:04:18	0:31:56	0:54:56	15:24:41	11
Min	0:04:00	3:12:34	0:01:06	0:03:25	3:48:23	0:09:22	0:18:15	13:33:02	9
Max	1:00:20	6:58:34	0:04:00	1:06:23	7:51:15	2:17:11	2:49:21	16:10:17	13
Stdev	0:22:25	1:32:15	0:00:54	0:08:46	1:45:39	0:19:29	0:21:55	1:04:06	2
Med	0:16:17	5:21:43	0:02:27	0:21:18	6:00:53	0:30:49	0:54:17	15:41:11	11

Table 4.1- Duration of courtship stages for five *D. copei* and five *D. tenebrosus* courting pairs.
R/P= Recognition/Persuasion, PS=Primary Stimulation, M=Post spermatophore maneuvering, S1= total time of initial sequence, SS= Secondary Stimulation, S2+=total time of sequences involving Secondary Stimulation, TCT= total courtship time, SP= number of spermatophore deposited during trial

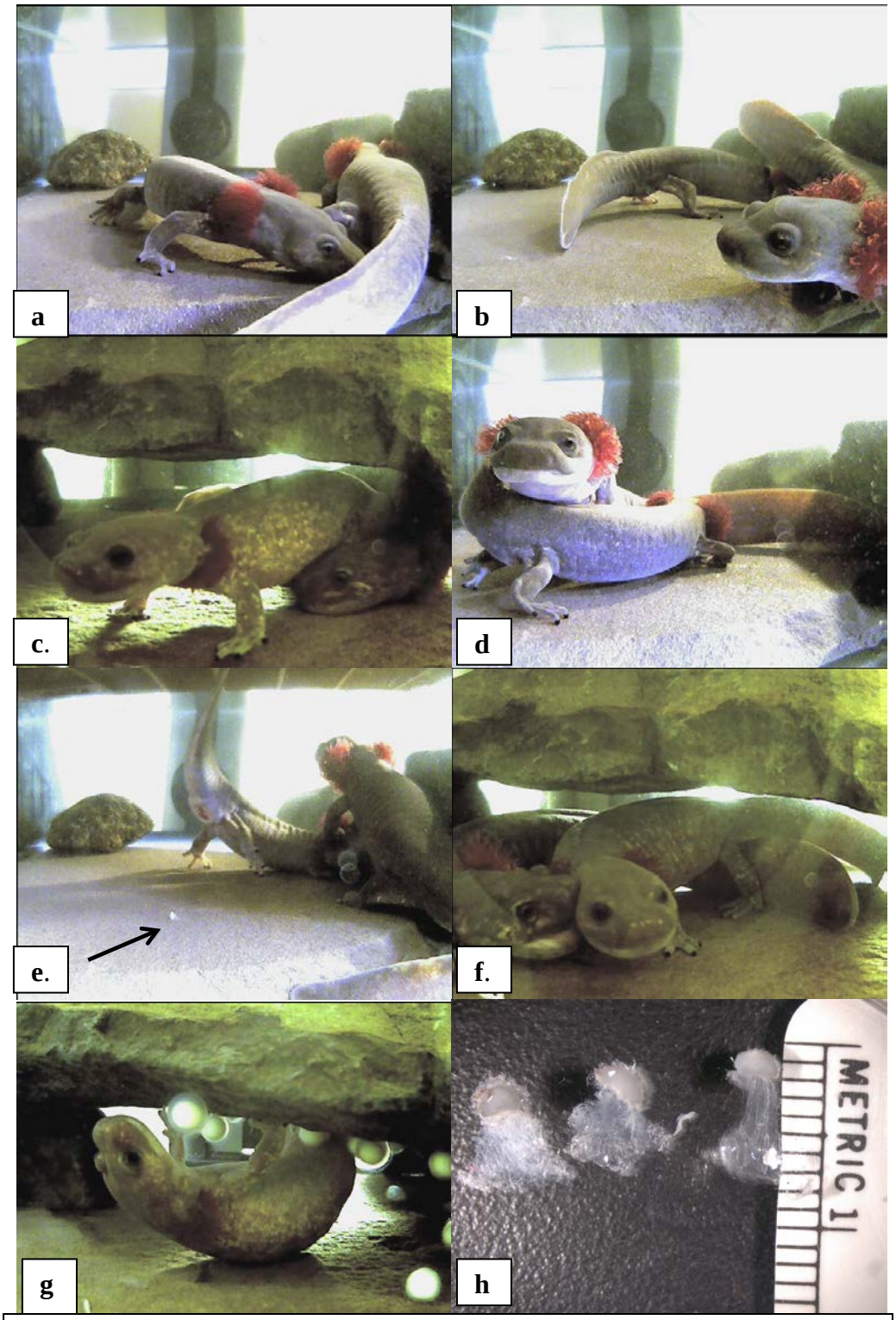

Figure 4.1- *Dicamptodon* courtship behaviors: a) Recognition (*D. tenebrosus*) b) primary stimulation (*D. tenebrosus*) c) Lifting (*D. copei*) d) Corralling (D. tenebrosus) e) Post spermatophore maneuvering (*D. tenebrosus*) f) Secondary stimulation (*D. copei*) and g) Oviposition (*D. copei*) h) Spermatophore morphology (*D. tenebrosus*).

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Chapter 5
Conclusion

In this thesis, I investigated hematological, physiological and behavioral aspects of *Dicamptodon* life history in *D. copei* and *D. tenebrosus*. In particular, I (a) obtained baseline leukocyte differential counts and erythrocyte dimensions in both wild and captive populations (b) documented and compared the morphological, behavioral and hematological responses of both species to environmental temperatures and (c) I characterized and compared the courtship behaviors of *D. copei* and *D. tenebrosus*. My results indicated that (a) *Dicamptodon* leukocyte ratios differed between wild and captive populations of *D. copei* but were similar between wild and captive populations of *D. tenebrosus* (b) behavioral and hematological responses to thermal environmental conditions differed between species and that (c) courtship behaviors of *D. copei* and *D. tenebrosus* were similar to one another, but not similar to the courtship behaviors of other salamander families.

Dicamptodon salamanders are abundant in many aquatic systems in the Pacific Northwest yet very little is known about these salamanders. Because of their abundance and predatory behavior, it is likely that their presence or absence greatly affects the ecosystems in which they live. The purpose of this study was to broaden our knowledge of these salamanders so that we do not mismanage nor disregard their ecological needs. In Chapter 2 I obtained baseline differential counts for both *D. copei* and *D. tenebrosus* that are useful for assessing stress levels and the overall health of these species. I found that these species share similar leukocyte profiles in the wild. However, the leukocyte profile of *D. copei* differed significantly between wild and captive samples. This

difference indicates the need for both field leukocyte baseline measurements and captive baseline leukocyte measurements when hematological parameters are used as physiological indicators. The erythrocyte measurements revealed similar sized erythrocyte measurements between these *Dicamptodon* species, however, the nuclear area of *D. tenebrosus* erythrocytes were larger than the nuclear area in *D. copei* erythrocytes. The reason for varied nuclear sizes has not been established. However, *D. tenebrosus* are known to have a large number of supernumerary chromosomes and it is likely that varied nuclear size is a result of a difference the amount of genetic material within the nucleus.

In Chapter 3 I took a three step approach to investigating the metamorphic tendencies of *D. copei* and *D. tenebrosus*. First, I tested their morphological response to both decreased water level and increased temperature and found that the salamanders showed no signs of metamorphosis even after months of treatment. However, two *D. copei* transformed spontaneously after two weeks of captivity. Though previous studies indicated that *D. copei* are obligate paedomorphs, their spontaneous transformation is evidence that they are facultative paedomorphs and that given optimal conditions, they can and will transform. The set of environmental and physiological conditions necessary for metamorphosis remains unknown.

Secondly, I examined thermal preferences of both species and found differences in thermal selection behaviors between species. While *D. tenebrosus* was most likely to be found in the mid- temperate pool, *D. copei* was most likely to be observed moving about the experimental pool than to be in any one pool. These results indicate a significant difference in thermal preferences between these species. However,

physiological factors, such as reproductive status, size, age and health may influence thermal selection in nature.

Lastly, I assessed thermal stress using relative leukocyte abundances to indicate stress levels relative to three temperatures. I found that stress levels differed significantly between *D. copei* and *D. tenebrosus* in both the coldest and the mid-temperature treatments, but were similar in the warmest treatment. *D. copei* was increasingly stressed as the temperature treatments were warmer. Interestingly, *D. tenebrosus* was similarly stressed in the warmest and coldest temperature which indicates a narrow thermal tolerance range for this population. As *D. tenebrosus* inhabit varied environmental conditions, these results may have differed given a more geographically diverse sample.

In Chapter 4 I documented and described the courtship behaviors of both *Dicamptodon* species. These behaviors were not previously known and this documentation gives insight to adaptive reproductive behaviors of this salamander family. I found that the courtship behaviors of *D. copei* and *D. tenebrosus* are similar between these species, but that they differ from the courtship behaviors of other salamander families. Remarkably, the female of both species is mostly passive throughout the courtship thus allowing the male to stimulate and maneuver her for insemination.

The information that I have collected and presented in this dissertation was not previously known. Each study in this dissertation opens new lines of inquiry and may even spark controversy. This is important if we hope to understand and thus preserve these unique animals and the ecosystems in which they live. We should take into account, however, that these studies were accomplished in artificial conditions that did

not mimic the natural conditions of *Dicamptodon* habitats. To most accurately assess life history variables of these animals, future studies should be performed in the field where natural environmental conditions allow for more accurate assessments.

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