

ANIMAL SCIENCE, ISSUES AND RESEARCH

Salamanders

Habitat, Behavior and Evolution



Rashid Gerasimov
Editor

NOVA

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SALAMANDERS

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RASHID GERASIMOV
EDITOR



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PREFACE

In Waterton Lakes National Park, located in southwest Alberta, Canada, under-road crossing structures were installed in 2008 to protect a population of long-toed salamander (*Ambystoma macrodactylum*) at Linnet Lake from mortality during breeding migrations that intersected with the park's entrance road. This compilation first investigates orientation and movement patterns of marked individuals at Linnet Lake in 2013 and 2014, and at a smaller, shallower wetland Stable Pond in 2014 to document in detail how long-toed salamanders used the terrestrial landscape around breeding sites during adult migration and juvenile dispersal.

Following this, the authors use generalized linear models to model salamander occurrence and abundance in the Beaver Hills region of north-central Alberta as a function of land cover type and pocket gopher density.

The authors go on to address whether any ecological barriers exist in the distribution range of the two newt species *N. strauchii* and *N. crocatus*. According to the findings of this study, an identity test was found to be significantly different from the null distribution, whereas a background test was not significantly different from the null distribution.

The concluding chapter summarizes the processes by which light is converted into neural signals by rods and cones and then transmitted to downstream neurons in the retina, with a focus on salamander

photoreceptors. The different types of photoreceptor cells and other light-sensitive cells in salamanders and their role in vision are also discussed.

Chapter 1 - For many pond-breeding amphibians, persistence of a population requires unobstructed movement between terrestrial upland habitats and aquatic breeding sites. Unfortunately, features within the terrestrial environment required for survival for both adults and newly metamorphosed young are often understudied, yet knowledge of terrestrial requirements for movement, foraging, and overwintering are critical in effective conservation planning. In Waterton Lakes National Park, located in southwest Alberta, Canada, under-road crossing structures were installed in 2008 to protect a population of long-toed salamander (*Ambystoma macrodactylum*) at Linnet Lake from mortality during breeding migrations that intersected the park's entrance road. The authors investigated orientation and movement patterns of marked individuals at Linnet Lake in 2013 and 2014, and at a smaller, shallower wetland Stable Pond in 2014 to document in detail how long-toed salamanders used the terrestrial landscape around breeding sites during adult migration (both sites) and juvenile dispersal (Stable Pond). Captures of migrating adult salamanders along drift fences that partially encircled each site revealed that adult movement paths were directional and not evenly distributed along shorelines at either water body. Immigrating and emigrating adults oriented in comparable directions, and the pattern differed between sites. At Linnet Lake, adult movement patterns were the same in both years. At Stable Pond, emigration patterns of adults differed from dispersal patterns of juveniles, but unlike salamanders at Linnet Lake, both age-classes avoided the entrance road. The reasons for the observed patterns are unknown, but salamander movements are likely influenced by the locations of aquatic habitat required for successful egg-laying and larval development, as well as terrestrial habitat offering efficient movement, prey resources, and below-ground overwintering refuges.

Chapter 2 - The western tiger salamander (*Ambystoma mavortium* Baird) is one of the largest species of mole salamanders (Ambystomatidae) in North America and is a top predator in fishless aquatic systems. In addition to aquatic breeding habitat, the species requires adjacent upland

habitat for foraging and for overwintering in subterranean small mammal burrows. In Canada, the western tiger salamander is a federally designated “species of special concern” in the prairie provinces. Degradation of upland habitat is listed as a key reason for the species’ decline, yet patterns of habitat use have never been systematically documented for Canadian boreal or prairie populations. Further, this region lacks detailed distribution data for western tiger salamanders, which are needed to infer trends and make status assessments. Therefore, the authors conducted a survey of western tiger salamander populations in the Beaver Hills region of north-central Alberta by sampling for adults and larvae in 40 potential breeding wetlands across land cover types that ranged from almost pure grassland to continuous forest. Because of their known association with small mammal burrows, the authors also surveyed northern pocket gopher (*Thomomys talpoides* Richardson) density as a proxy for potential overwintering sites. The authors used generalized linear models (GLMs) to model salamander occurrence and abundance as a function of land cover type and pocket gopher density. The authors also assessed occupancy and detectability patterns in order to estimate the number of tiger salamander populations within the 1,572 km² study area. The authors captured western tiger salamanders in 50% of the surveyed wetlands. The proportions of upland grassland or forest adjacent to wetlands were not significant predictors of tiger salamander occurrence or relative abundance of individuals within populations. However, pocket gopher density was a significant predictor of salamander occurrence but not abundance. The authors estimated a minimum of 625 (420-811, 95% CI) tiger salamander populations occupying comparable wetlands in the study area. The authors suggest future actions to locate and conserve populations of this large, cryptic salamander should consider pocket gopher density, in addition to traditionally used coarse habitat proxies such as land cover.

Chapter 3 - *Neurergus strauchii* is an endemic species, narrowly distributed in eastern Anatolia, with an elevation range from 1000 m to 2100 m asl. *Neurergus crocatus* inhabits both southeastern Anatolia and northwestern Iran, and prefers lower altitudes than *N. strauchii*. *N. strauchii* and *N. crocatus* are allopatric salamander species. Although

many phylogenetic studies have been carried out on these species, niche conservatism, which has gained wide attention in recent literature, has not been examined in any studies. The author addresses here whether any ecological barriers exist in the distribution range of the two newt species *N. strauchii* and *N. crocatus*. According to the findings of this study, an identity test was found to be significantly different from the null distribution, whereas a background test was not significantly different from the null distribution. In addition, both linear and blob range-break tests showed that the environmental divergence between *N. strauchii* and *N. crocatus* was not significantly different from the null distribution. These tests can generally be interpreted only in one way: there is no niche conservatism between *N. strauchii* and *N. crocatus*. These allopatric species have similar niche characteristics in the geographic areas they occupy. Therefore, these two species have been separated by non-climatic geographic barriers. Thus, the glacier mountains of Hakkari appear to be an important barrier resulting in the speciation of *N. strauchii* and *N. crocatus*.

Chapter 4 - Rod and cone photoreceptor cells capture photons of light, transforming light signals into neural signals. Many of the key insights into the structure and function of rod and cone photoreceptors were first obtained by studying retinas of salamanders, especially *Ambystoma tigrinum*. This chapter summarizes the processes by which light is converted into neural signals by rods and cones and then transmitted to downstream neurons in the retina, with a focus on salamander photoreceptors. The authors also discuss the different types of photoreceptor cells and other light-sensitive cells in salamanders and their role in vision.

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Chapter 1

**PATTERNS OF MIGRATION AND DISPERSAL
OF LONG-TOED SALAMANDERS
(*AMBYSTOMA MACRODACTYLUM* BAIRD)
FROM TWO BREEDING SITES
IN THE ROCKY MOUNTAINS
OF ALBERTA, CANADA**

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ABSTRACT

For many pond-breeding amphibians, persistence of a population requires unobstructed movement between terrestrial upland habitats and aquatic breeding sites. Unfortunately, features within the terrestrial environment required for survival for both adults and newly metamorphosed young are often understudied, yet knowledge of terrestrial requirements for movement, foraging, and overwintering are critical in effective conservation planning. In Waterton Lakes National Park, located in southwest Alberta, Canada, under-road crossing structures were installed in 2008 to protect a population of long-toed salamander (*Ambystoma macrodactylum*) at Linnet Lake from mortality during breeding migrations that intersected the park's entrance road. We investigated orientation and movement patterns of marked individuals at Linnet Lake in 2013 and 2014, and at a smaller, shallower wetland Stable Pond in 2014 to document in detail how long-toed salamanders used the terrestrial landscape around breeding sites during adult migration (both sites) and juvenile dispersal (Stable Pond). Captures of migrating adult salamanders along drift fences that partially encircled each site revealed that adult movement paths were directional and not evenly distributed along shorelines at either water body. Immigrating and emigrating adults oriented in comparable directions, and the pattern differed between sites. At Linnet Lake, adult movement patterns were the same in both years. At Stable Pond, emigration patterns of adults differed from dispersal patterns of juveniles, but unlike salamanders at Linnet Lake, both age-classes avoided the entrance road. The reasons for the observed patterns are unknown, but salamander movements are likely influenced by the locations of aquatic habitat required for successful egg-laying and larval development, as well as terrestrial habitat offering efficient movement, prey resources, and below-ground overwintering refuges.

INTRODUCTION

Habitat loss and alteration are among the biggest threats to the persistence of animal and plant populations in North America (Wilcove et al. 1998). Agriculture, water resource development, urbanization, recreational and extractive land uses, as well as infrastructure (including road-building) associated with all these activities represent threats to the habitats of native species. Populations of amphibians, including Caudata,

are no exception (Corn 1994). Much effort has been directed toward amphibian conservation since global declines were recognized in the early 1990s (e.g., Wake 1991).

Planning ahead to mitigate impacts of development on local populations, or for restoration after impacts occur, requires a fundamental understanding of the biology of the affected species (Landres et al. 1999). Depending on the nature of the disturbance, both abiotic and biotic processes may need to be addressed (Hobbs and Norton 1996). Unfortunately, information on fundamental life history characteristics are often lacking for species that currently generate little conservation concern or have no obvious economic value (Tyler et al. 2012). Knowledge gaps can make it difficult to reduce or reverse the impacts of anthropogenic activities and/or implement conservation planning (Cayuela et al. 2009). Amphibians are among those taxa remaining relatively understudied compared to other organisms facing human threats that negatively affect life history processes by destroying or degrading habitat (Cushman 2006; Lawler et al. 2006).

For many temperate, pond-breeding amphibians, especially salamanders, a healthy population requires unobstructed movement between a suitable aquatic breeding site and terrestrial non-breeding habitat (Pittman et al. 2014). Suitable breeding habitats are usually permanent or semi-permanent water bodies lacking fish, but containing aquatic vegetation or other substrates for egg deposition. Terrestrial habitat, utilized for foraging and overwintering, is often conceived as a circular buffer surrounding a breeding site that is estimated to encompass activities for a specified percentage of the breeding population (Semlitsch 1998; Semlitsch and Bodie 2003). A buffer of 500 m has been estimated to include 95% of the breeding individuals for some amphibian species (Scott et al. 2013). However, within a given buffer, not all the available habitat may be used by the individuals of that population. For some amphibian species, movement paths that individuals take between terrestrial home ranges and breeding sites may not represent the shortest, straight-line distances (Shoop 1968; Semlitsch 1981; Pittman et al. 2014; Lee-Yaw 2015). Evidence suggests amphibians in some environments follow

“movement corridors”, presumably because the micro- or macrohabitat within corridors is more conducive to movement based on abiotic or biotic factors. However, the use of defined movement corridors may be a site-specific, not species-specific, trait (Douglas and Monroe 1981; Jenkins et al. 2006).

Fine-scale use of terrestrial habitat surrounding breeding sites is often difficult to document for individual amphibians due to the technical limitations associated with affixing tracking devices to small-bodied vertebrates either internally or externally. In many cases, habitat-use is inferred from data that are easier to collect for pond-breeding amphibians, such as constructing drift fences to intersect movement and to capture marked or unmarked individuals as they make seasonal overland migrations *en masse* between terrestrial and aquatic habitats. Studies using coarse movement data to investigate habitat-use range from complex designs that isolate specific landscape features (e.g., Gibbs 1998; Regosin et al. 2005) to studies using orientation of migrating adults and dispersing juveniles to infer habitat preferences (e.g., Douglas and Monroe 1981; Homan et al. 2008; Walston and Mullin 2008), to laboratory experiments (e.g., Lee-Yaw et al. 2015). In cases where tracking devices can be used (e.g., Sheppard 1977; Madison 1997; Trenham 2001; Baldwin et al. 2006; Faccio 2003; Hamed et al. 2008; Atkinson-Adams et al. 2018) movement patterns, foraging habitat, terrestrial refugia (including overwintering sites), and sources of mortality can be investigated with more precision. This type of information is invaluable for conservation planning (Trenham and Shaffer 2005).

Ambystomatid salamanders (“mole” salamanders, Family: *Ambystomatidae*, Genus: *Ambystoma*) are physically robust, highly terrestrial and mobile pond-breeding amphibians found only in North America (Stebbins 2003). Species tend to orient non-randomly when entering and exiting breeding sites (Shoop 1968; Simlitch 1981; Douglas and Monroe 1981; Jenkins et al. 2006), use terrestrial habitats selectively during migratory movements (Semlitsch 1981; Douglas and Monroe 1981; Gibbs 1998; Homan et al. 2008) and when foraging and overwintering (Semlitsch 1981; Madison 1997; Trenham 2001; Faccio 2003; Atkinson-

Adams et al. 2018). To date, studies investigating these behaviors have mainly focused on eastern species (but see Trenham (2001) and Trenham and Shaffer's (2005) work on California tiger salamanders (*A. californiense*), and Anderson (1967) and Beneski et al.'s (1986) work on long-toed salamanders (*A. macrodactylum*)).

The long-toed salamander (*Ambystoma macrodactylum* Baird) ranges throughout the Pacific Northwest from California to Alaska, and as far east as the foothills and plains of the Rocky Mountains in Montana (Stebbins 2003). This salamander species consists of five recognized subspecies (Ferguson 1961; Lee-Yaw and Irwin 2012) and occupies a wide range of habitats throughout its range varying from high alpine lakes in the Sierra Nevada mountains in California and the Rocky Mountains from Idaho through Canada, arid sagebrush (*Artemesia* spp.) habitats in the western USA, and temperate rainforests along the west coast of Canada and the USA. Although the species is found in a variety of environments, little is known about its specific micro-habitat requirements, especially at the northern and high elevation limits of its range. Anderson (1967) compared the life-history of two high-alpine (1980 m and 2450 m) populations in the Sierra Nevada, California with two low elevation coastal populations (both at 40 m), Anderson assumed that at high elevations, adult salamanders moved to surrounding forest but was unable to document migration patterns or terrestrial habitat of alpine adults directly. At low elevations, he observed that adult salamanders emerged from ponds directly into connected willow (*Salix* spp.) thickets or remained within bottom substrates in dried breeding-ponds until rain made longer above-ground movements towards wooded areas possible. At a mid-elevation site (850 m) in Idaho, dominated by pine and fir (*Pinus and Abies* spp.), Beneski et al. (1986) found that migrating adult long-toed salamanders oriented randomly and displayed no selection during overland movement among field, wooded, or earthen dam habitats, or between dry and moist soil conditions.

Random orientation during migratory movements in some *A. macrodactylum* populations (Beneski et al. 1986) contrasts with results from orientation studies for other ambystomatids (*A. maculatum*, *A.*

jeffersonianum, and *A. opacum*) in eastern North America (Douglas and Monroe 1981; Rothermel 2004; Jenkins et al. 2006). In these studies, non-random orientation during breeding migrations and juvenile dispersal appear to be the rule, and is hypothesized to reflect the utility of different habitats for movement.

The long-toed salamander is listed as a species at risk, “special concern”, in the province of Alberta, Canada where the species reaches the north-eastern edge of its global range (Graham and Powell 1999). In Waterton Lakes National Park, located in the south-western corner of Alberta, two neighboring long-toed salamander breeding locations, within 1.2km of each other, became of interest in the early 2000’s, largely due to their proximity to the Entrance Road, a known source of mortality during overland movements, and the populations’ strikingly different environmental settings (see “**Study Sites**”). One location, Stable Pond is a shallow semi-permanent wetland, typical of breeding sites throughout the long-toed salamander range (Graham and Powell 1999). The second, Linnet Lake is larger, deeper, and less productive, and thus more similar to alpine breeding sites in this region (Fukumoto and Herrero 1998; Pearson and Goater 2008), but differs in that it is inhabited by fish known to predate salamander eggs and larvae, and has a declining population due to poor recruitment (Pearson and Goater 2009; Pagnucco et al. 2011). Populations at both sites begin breeding migrations as soon as the ice is melted from the margin of the water body and night time temperatures are above freezing, usually early April (Fukumoto and Herrero 1998). Breeding occurs for several weeks, then by the early June most adults return to the terrestrial environment for the remainder of the year while eggs develop, hatch, and larvae metamorphose to disperse into the terrestrial environment mid-July through September (Fukumoto 1995).

Our interest in spatial orientation and movement patterns of long-toed salamanders at Waterton Lakes began when under-road crossing structures (tunnels) were installed in 2008 along the Entrance Road at Linnet Lake to reduce vehicle mortality on salamanders during movements to and from the lake and terrestrial habitat across the road, 13 – 110 m from the lake margin (Pagnucco et al. 2012, Atkinson-Adams 2015). At the time of

tunnel installation, information about movement patterns of salamanders from Linnet Lake was limited to a decade-old study by Fukumoto and Herrero (1998) and on-going observations by park biologists (Parks Canada Agency unpublished data). Three conclusions arose from this information: 1) the majority of the adult population crossed the road during breeding migrations and were consequently subject to road mortality each year, 2) the majority of juveniles crossed the road when dispersing from Linnet Lake following metamorphosis in the fall, and 3) salamanders crossed the road because terrestrial habitat was more favorable or extensive across the road rather than immediately around the lake. Although concentrated movement (non-random orientation) is consistent with observations in other species of ambystomid salamander, these assumptions are not well supported by results from the only detailed orientation study conducted on the long-toed salamander by Beneski et al. (1986) and described above. Movement patterns (random or non-random orientation) during breeding migrations may reflect the location of habitat best-suited for overland movement (e.g., moisture gradients or protection from predators), but may also reflect the spatial arrangement of suitable foraging, and/or overwintering areas on the landscape (Madison 1997); the exact nature of such habitat is largely unknown for the long-toed salamander. After studying the population in 2008–2009, Pagnucco (2010) found evidence that some breeding adults at Linnet Lake do not cross the road, and suggested that future tunnel installations should be preceded by detailed preliminary studies to document movement patterns.

To better understand fundamental patterns of orientation and movement of long-toed salamander in Waterton Lakes, we chose to focus our sampling efforts on Stable Pond. Stable Pond offered an excellent reference location as it: i) did not appear to be a site of vehicle mortality despite being situated even closer to the Entrance Road (< 10 m) than Linnet Lake, ii) was known to have an amply sized breeding population (Pagnucco 2010) allowing documentation of both adult migrations and juvenile dispersal, and iii) was appropriate for intensive surveying due to its small size, shallow depth, and accessibility. We used Linnet Lake, (although of management concern) as a secondary site for investigating

long-toed salamander movement patterns in detail because of its large size (58 times larger than Stable Pond), lack of measurable recruitment to the population (due to fish predation), and low density of adult salamanders (Pagnucco et al. 2011, 2012).

Our overall goal was to explore one aspect of long-toed salamander life history, patterns in terrestrial movement and habitat-use, with the aim of reducing knowledge gaps concerning the species' behavior during adult migration and juvenile dispersal. We aimed to explore links between movement patterns and habitat characteristics, potentially useful for conservation planning in Waterton Lakes National Park, but also in the northern part of this species' range in general. Based on preliminary observations and results from studies on *Ambystoma* species in the eastern USA (e.g., Jenkins et al. 2006), we predicted that: i) adult and newly metamorphosed salamanders would orient non-randomly during immigration, emigration, and dispersal, and ii) patterns of orientation would differ for the two age classes as young-of-year are naïve and would have no previous knowledge of the terrestrial landscape to inform movement decisions as would adults (Madison 1997).

METHODS

Study Sites

We conducted research at two study sites in Waterton Lakes National Park, Alberta, Canada. In 2013, we worked at Linnet Lake only, and in 2014 we worked at Stable Pond and Linnet Lake.

Stable Pond (49° 04' N, 113° 53' W) is a small (0.15 ha), fishless, seasonal pond at an elevation of 1275 m with a maximum depth of 1.6 m; it typically dries by late July/early August (July 22 in 2013, August 7 in 2014) to become a grass-dominated meadow. Stable Pond is surrounded on all sides by poplar (*Populus* spp.) forest and flat terrain. The riparian area quickly breaks into a mix of open grass and low shrubs to the south and poplar forest with small isolated stands of Douglas fir (*Pseudotsuga*

menziesii) to the east. To the west, the pond is immediately bordered (<10 m) by a paved bike path and the Park's Entrance Road, across which lies an open, dry, grassy ridge capped by a stand of Douglas fir. Western toads (*Anaxyrus boreas*) and boreal chorus frogs (*Pseudacris maculata*) also breed in Stable Pond. The breeding population of long-toed salamanders at this site was robust (>600 breeding adults) during our investigations. There has been little observed vehicle mortality of salamander at Stable Pond, but recruitment is reduced in dry years when the pond dries before metamorphosis occurs.

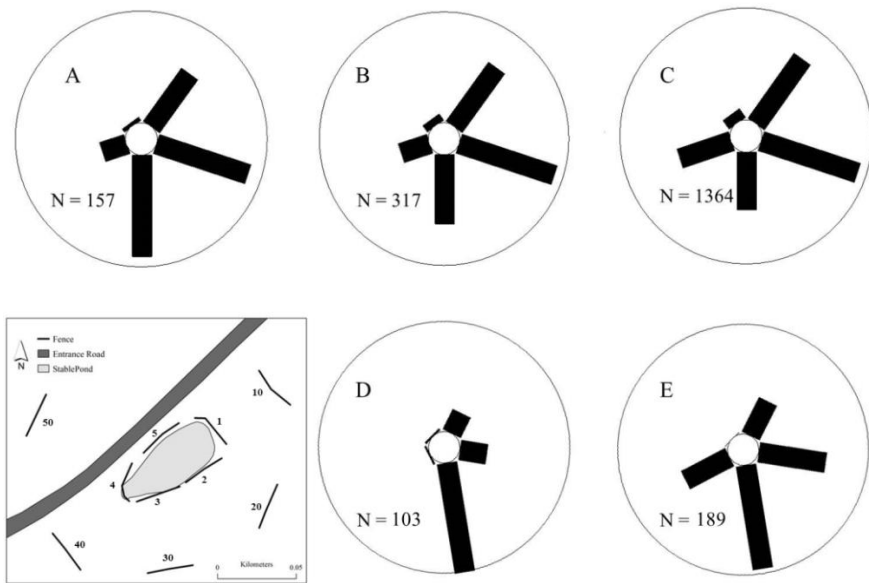


Figure 1. Map of Stable Pond with pitfall-trap fencing (1-5 inner fences, 10-50 outer fences). Five circular histograms of pitfall trap captures show orientation patterns for (A) immigrating and (B) emigrating adults at inner fences, (C) dispersing young-of-year (YOY) at inner fences, and emigrating adults (D) and dispersing YOY (E) at outer fences. Bars represent number of salamanders captured at each fence (Table 1), and correspond spatially with locations on the inset map.

Linnet Lake (49° 04' N, 113° 54' W) is a small (3.9 ha), foot-shaped, shallow (5 m maximum depth) lake at an elevation of ~1260 m in a bowl-like catchment. The vegetation around the lake is dominated by stands of Douglas fir and poplar, with an understory of small trees and shrubs, e.g.,

chokecherry (*Prunus virginiana*), saskatoon (*Amelanchier alnifolia*), and snowberry (*Symphoricarpos albus*), and open grasslands.



Figure 2. Map of Linnet Lake with 16 lake-side drift fences; fences numbered in italics (even numbers) were deployed in 2013 only. Two circular histograms present locations of hand-captures during visual searches along drift fences for 2013 (N = 129, 16 fences) and 2014 (N = 54, eight fences). Histogram bars represent the number of salamanders captured at each fence (Table 1), and correspond spatially with lake-side fences on the map.

The lake is surrounded by moderately steep-sloping (up to 15%) hillside except on the north end, which is a flat, low-lying area containing a paved parking lot (~30 x 70 m) adjacent to Middle Waterton Lake. The Entrance Road parallels the west side of the lake at a straight-line distance of 13 – 110 m and a road leading to the Prince of Wales Hotel passes 50 m south. Entrance Road is punctuated by four salamander tunnels (ACO Technologies, Shefford, UK) with 50 cm x 33 cm openings spaced ~80 – 110 m apart (see Figure 2) and described in detail by Pagnucco et al. (2012). Linnet Lake is inhabited by three fish species: two species of sucker (Catostomidae) and one species of chub (Cyprinidae), as well as western toad and western tiger salamander (*Ambystoma mavortium*). Investigations since 2008 have failed to document any young-of-year, likely due to recruitment being suppressed on eggs and larvae by fish (Pagnucco et al. 2011), thus we collected orientation data from Linnet Lake for adult migrations only.

Salamander Capture: Stable Pond

To capture salamanders at Stable Pond, we installed 10 1-m high drift fences (operated April 17 – August 22). Drift fencing was composed of 1-m high silt fencing buried to a depth of 5 – 10 cm and stapled to wooden stakes for support. Five 30-m fences, 5 – 8 m apart were built around the pond within 3 m of the high water line beginning in mid-April (referred to as “inner fences” at this site). By the beginning of May, we installed five more 30-m drift fences 50 m away from the high water line (“outer fences”) and approximately in line with a corresponding inner fence, except for one fence on the other side of the Entrance Road that was offset 20° SW due to an exposed rock outcropping (Figure 1). Outer fences were only used to capture emigrating salamanders because of their late deployment compared to inner fences. We used 50 pitfall traps buried along the fences to capture salamanders terrestrially. Pitfall traps were made from #10 food service cans containing a stick that served as a ramp for mammals incidentally trapped and a small piece of sponge (4 x 7 cm),

wetted as needed with pond water, to provide moisture and cover for trapped amphibians. In addition to fences and pitfall traps, we placed 10 Gee minnow-traps (42 x 19 cm, 6.4 mm mesh, 2.5 cm openings) in the pond evenly spaced around the perimeter and at varying distances from shore depending on pond depth to trap breeding adults. An air space was provided in minnow traps to prevent animals from drowning. When the water table was too high for pitfall traps to be functional, they were replaced temporarily with minnow traps placed against and parallel to the drift fence. In mid-June we doubled-over the fencing material (fence reduced to a height of 0.5 m) at the 50-m distance to reduce wind damage.

We focused capture effort on migrating adult salamanders from April 17 to June 14 by placing eight pitfall traps along inner fences, four on each side evenly spaced (~7.5m apart), and placing two pitfall traps along outer fences, one at each end on the side facing the pond. We used minnow traps in the pond from April 12 to June 14. As the pond dried, we removed some traps to keep the set evenly spaced. From July 16 to August 22, we focused capture effort on dispersing young-of-year (YOY) salamanders using pitfall traps installed only on the pond-side of all drift fences.

When traps were in place, we checked them daily, usually within 1 h of sunrise. All captured salamanders were placed in a small plastic container with moist paper towel for transport to and from the lab where we took measurements and marked individuals (see below). We immediately released any other animals present in traps on the opposite side of the fence or directly into the pond at the trap location.

Salamander Capture: Linnet Lake

To capture salamanders to investigate orientation at Linnet Lake, we installed a series of drift fences around the lake (April 29 – August 30, 2013; April 23 – July 11, 2014) to create a temporary barrier to salamander movement during breeding migrations using the same fencing materials and methods as at Stable Pond, but without pitfall traps. In 2013 we installed 16 30-m drift fences around Linnet Lake spaced 15 m apart and

ranging between 10 m and 25 m from the lake edge. In 2014 we installed eight of the original 16 fences around Linnet Lake. We did not install drift fencing at the north end of Linnet Lake due to the presence of the paved parking lot (Figure 2).

Fences were walked nightly beginning the first night a salamander was encountered during preliminary surveys (April 25 in 2013, April 17 in 2014) and we collected all salamanders encountered. We continued night searches until five consecutive nights passed with no salamander encounters, which happened in late June both years. We then re-initiated night searches on the next rainy night and continued nightly until no salamanders were encountered (July 8 in 2013, July 2 in 2014). We used the same materials and methods to hold and transport captured salamanders as at Stable Pond.

Salamander Processing: Measurements and Marking

Upon arrival at the Parks Canada laboratory 100 m from the Linnet Lake parking lot and 1.1 km from Stable Pond, Adult and immature salamanders were anaesthetized by immersion in 1 g/L trimethane sulfonate (TMS) solution until unresponsive to prodding (typically 6 – 10 min). We then recorded weight, total length (TL, measured as the distance from tip of snout to distal end of tail), and snout-vent length (SVL, measured as the distance from tip of snout to posterior edge of vent), and determined sex and age-class (i.e., young-of-year, juvenile, or adult). We considered salamanders with swollen vents (able to be sexed) to be mature adults and smaller salamanders without swollen vents (unable to be sexed) to be juveniles. We classified juvenile salamanders captured at Stable Pond in July and later, that had gill-remnants behind the jaw, as YOY; we observed no YOY at Linnet Lake. To uniquely mark an individual, we inserted a 12 x 2.12 mm sterile half-duplex PIT tag (Texas Instruments purchased through Oregon RFID, Portland, Oregon, USA) into the body cavity via a 3 mm incision, made using a fresh #11 scalpel blade, just anterior to the right hind leg and slightly toward the midline. We double-

marked these individuals by either clipping toe three of the right hind leg at the second phalangeal joint, or injecting red (2013) or orange (2014) visual implant elastomer (VIE) (Northwest Marine Technologies, Shaw Island, Washington, USA) sub-dermally at the ventral base of the tail just posterior to the vent. We closed the PIT tag incision with Vetbond™ Tissue Adhesive (3M, St. Paul, Minnesota, USA), applied to the dried incision while holding the opposing edges of the incision together with forceps. For brief periods each year we had no PIT tags, therefore we created individual marks with VIE by making a series of three or four dots and dashes (e.g., $- \cdot -$, $- - -$, $\cdot \cdot \cdot$) at the base of the tail. After marking, which usually took 1 – 3 min, we placed individuals in a slanted container and immersed them in non-chlorinated water with their heads above water until they recovered from anesthesia (typically 10 – 15 min). Once they recovered (awake and responsive to prodding), we placed individuals in their original containers for release as near as possible to the point of capture. Individuals remained in captivity for 45 min to 3 hr.

Orientation Data Analysis

We used chi-squared goodness-of-fit tests, similar to Douglas and Monroe (1981) and Jenkins et al. (2006), to test for a non-random orientation of movement for immigrating and emigrating adults (both sites) and dispersing YOY (Stable Pond only), and to test for non-random capture among aquatic traps (Stable Pond only). We used chi-squared contingency tests to examine differences in capture patterns between adult migration phases (immigration vs emigration, both sites), between life stages (adult emigration vs YOY dispersal, Stable Pond only), with distance from the water body (inner fences vs outer fences, Stable Pond only), and between years (Linnet Lake only). We grouped captures by fence location for orientation analysis at both sites.

For the Stable Pond population, we also tested terrestrial movement path-fidelity for recaptured individuals (YOY and adults separately) by calculating the difference in angular degrees between the trap of first

capture (set to 0°) with the trap(s) of subsequent capture. We used trap rather than fence location because it gave finer angular resolution. If an individual was captured more than two times, we added the angular differences between subsequent capture events to get a total change in bearing as a measure of angular departure from the original movement path.

RESULTS

Salamander Orientation

We captured a total of 184 migrating adult long-toed salamanders at Linnet Lake (129 in 2013 and 55 in 2014), and a total of 619 migrating adult long-toed salamanders and 1460 young-of-year at Stable Pond (2014) (Table 1).

At Stable Pond, adult salamanders trapped April 17 – June 14 exhibited non-random orientation among the five fences both during immigration ($\chi^2 = 67.36$, $df = 4$, $p < 0.001$, inner fences only) and emigration (inner fences: $\chi^2 = 109.51$, $df = 4$, $p < 0.001$; outer fences: $\chi^2 = 158.50$, $df = 4$, $p < 0.001$). We captured more immigrating and emigrating adult salamanders along the three eastern inner fences (fences 1 – 3) than expected (90% of immigrants and 86% of emigrants), and more emigrating adults than expected (68%) at the southern outer fence (fence 30) (Table 1; Figure 1). These fences were all within stands of poplar, with the exception of fence 30, which extended ~5 m beyond the poplar stand into the adjacent mixed shrub-grassland.

The orientation pattern of adults at Stable Pond did not differ between the two modes of migration at inner fences ($\chi^2 = 5.85$, $df = 4$, $p = 0.211$; Table 1). The concentration of emigrating salamanders shifted slightly to the north compared to immigrants, but movements were still focused to the east, opposite the road into stands of poplar bordering the pond (Figure 1).

Table 1. Long-toed salamander captures used for orientation analysis for Linnet Lake (2013 – 2014), and Stable Pond (2014)

	2013		2014		
Fence	Immigrants	Emigrants	Immigrants	Emigrants	Young-of-Year
Linnet Lake					
1	2	2	0	3	—
2	3	4	—	—	—
3	3	3	9	4	—
4	3	1	—	—	—
5	4	2	2	1	—
6	2	6	—	—	—
7	7	42	12	10	—
8	8	9	—	—	—
9	3	4	5	1	—
10	1	2	—	—	—
11	2	1	2	2	—
12	2	0	—	—	—
13	2	3	1	1	—
14	1	3	—	—	—
15	1	1	0	2	—
16	1	1	—	—	—
Stable Pond					
1	—	—	35	82	356
2	—	—	52	114	456
3	—	—	54	78	258
4	—	—	14	34	246
5	—	—	2	9	48
10	—	—	—	14	28
20	—	—	—	17	49
30	—	—	—	70	76
40	—	—	—	1	36
50	—	—	—	1	0

At Linnet Lake, fences were placed 10 – 25 m from the edge of the lake. Only eight of the 16 fences from 2013 were also used in 2014. At Stable Pond, fences 1 – 5 were within 3 m of the shoreline at high water and fences 10 – 50 were 50 m from the shoreline at high water. Adult immigrants and emigrants were captured April 25 – July 8, 2013 and April 17 – July 2, 2014 at Linnet Lake, and April 17 – June 14, 2014 at Stable Pond. Dispersing young-of year were captured July 16 – August 22, 2014 at Stable Pond

During emigration, patterns differed between inner and outer fences ($\chi^2 = 66.05$, $df = 4$, $p < 0.001$) with salamander captures heavily concentrated toward the south at outer fences. We also found, by comparing captures

among 10 minnow traps from April 12 – June 14, adults in the water were not distributed evenly along the perimeter of the pond ($\chi^2 = 280.71$, $df = 9$, $p < 0.001$). More adults were captured along the southeastern pond edge than expected.

YOY trapped July 16 – August 22 at Stable Pond exhibited non-uniform patterns of dispersal at both inner and outer fences (inner fences: $\chi^2 = 337.09$, $df = 4$, $p < 0.001$; outer fences: $\chi^2 = 82.35$, $df = 4$, $p < 0.001$), and patterns differed between the two fence types ($\chi^2 = 53.18$, $df = 4$, $p < 0.001$; Table 1, Figure 1). At inner fences YOY captures were concentrated in poplar stands to the northeast (almost 60% on fence 1 and 2), with most of the remainder of captures (37%) distributed between the two fences (3 and 4) in poplar stands in the south and southwest, and far less than expected (4%) captured at fence 5 along the road. At outer fences, YOY captures were concentrated to the south (85%) at fences 20, 30, and 40 located in poplar forest and grassland. Patterns of YOY dispersal differed from adult emigration at both inner and outer fences, with differences being more pronounced at outer fences (inner fences: $\chi^2 = 13.26$, $df = 4$, $p = 0.010$; outer fences: $\chi^2 = 31.98$, $df = 4$, $p < 0.001$). Compared to adults, YOY captures at inner fences were shifted northward, and less focused to the south at outer fences (Figure 1).

At Linnet Lake, patterns of immigration and emigration did not differ (2013: $\chi^2 = 23.21$, $df = 15$, $p = 0.080$; 2014: $\chi^2 = 9.37$, $df = 7$, $p = 0.228$) with captures being similar at each fence for immigrants and emigrants (Table 1, Figure 2), thus we pooled captures between the two modes of migration at each fence to test for uniformity of movement patterns for each year. Although all fences were located within continuous forest canopy, we found that the orientation of movement differed from random each year (2013: $\chi^2 = 246.44$, $df = 15$, $p < 0.001$; 2014: $\chi^2 = 53.85$, $df = 7$, $p < 0.001$), and that orientation patterns did not change between 2013 and 2014 ($\chi^2 = 11.60$, $df = 7$, $p = 0.114$). In all cases, captures were concentrated (52%) at fences 6 – 8 at the southwest corner of the lake above the shoreline where driftwood tends to accumulate (Figure 2).

Path Fidelity for Individual Movements

For adults at Stable Pond, values of angular departure followed lognormal distributions (180° maximum) and cumulative angular departure ranged from $0^\circ - 164^\circ$ ($n = 45$) with a median of 29° . For individuals first caught immigrating, then emigrating, at inner fences only, angular departure ranged from $0^\circ - 94^\circ$ ($n = 19$) with a median of 38° . When we pooled immigrant and emigrant captures of adults at inner fences and compared them with capture locations at outer fences, angular departure ranged from $0^\circ - 164^\circ$ ($n = 18$) with a median of 29° . Adult emigrants captured at inner fences then subsequently captured at outer fences ranged in angular departure from $0^\circ - 76^\circ$ ($n = 11$) with a median of 10° . Recaptured YOY at Stable Pond departed from original dispersal directions from $0^\circ - 177^\circ$ ($n = 89$). The angular departure values also followed a lognormal distribution with a median of 30° . For initial captures at inner fences versus recaptures at outer fences, angular departure again ranged from $0^\circ - 177^\circ$ ($n = 61$) and followed a lognormal distribution with a median of 21° .

DISCUSSION

Orientation: Adult Migrations and Young-of-Year Dispersal

As expected, long-toed salamander movements were oriented non-randomly at both study sites in Waterton Lakes National Park. Additionally, the adult orientation pattern was consistent between years at Linnet Lake. These findings agree with informal observations for the species (Anderson 1967), and patterns seen in other eastern *Ambystoma* species. For example, Jenkins et al. (2006) found that adult and YOY marbled salamanders (*A. opacum*) oriented non-randomly at nine breeding ponds in Massachusetts, but adults oriented differently from YOY at 52% of sites; adult marbled salamanders maintained a similar pattern of orientation from year to year at each pond. At Stable Pond, capture patterns

at fences 50 m away from the pond edge indicated that orientation shifted for both emigrating adults and dispersing YOY as they moved away from the breeding site. Based on the distribution of captures at inner fences, when treated as a group, adults immigrated then emigrated in the same direction along the pond shoreline, but during emigration they shifted their direction from east to south as they progressed further away (50 m) from the pond at outer fences. YOY oriented differently than adults at both distances from the pond, but both shifted their movements southward at outer fences. Although these findings are comparable to studies of other *Ambystoma* species, they differed from the findings of Beneski et al. (1986) for long-toed salamanders in Idaho that found breeding adults orienting randomly, with no consistent orientation as a population, during both immigration and emigration.

Individual Patterns of Directionality

At Stable Pond, directionality was maintained at the individual level for immigrant and emigrant adults at shore-side inner fences. Other studies have documented individual adult spotted (*A. maculatum*), marbled, mole (*A. talpoideum*), and Jefferson (*A. jeffersonianum*) salamanders entering and exiting breeding sites from the same locations along drift fences (Shoop 1968; Shoop and Doty 1972; Semlitsch 1981; Douglas and Monroe 1981). We found that 19 adults encountered at inner fences during immigration changed direction a median of 38° upon exiting the pond. On the ground, this is a small directional change. Traps were on average 19° apart, and following the logic of Shoop (1965), if an immigrating salamander encountered a fence 1° to the right of a trap then turned right along the fence before falling into the next trap (18° away), and upon emigration changed its bearing by 2° from its initial immigration route and turned right again upon encountering the fence, it would have appeared to deviate from its immigration bearing by 38° even though in reality it only deviated by 2°. Therefore, as Shoop (1965) did for spotted salamanders, we consider an individual captured entering and exiting the pond within a

three-trap section of fence (38° angular width on average) to be entering and exiting from virtually the same location.

Individual adults ($n = 11$) and YOY ($n = 61$) that were captured leaving the pond at inner fences, then captured again at outer fences, changed direction by a median of 10° and 21° , respectively. These results suggest that as salamanders move further from the pond, adults have a stronger directional focus in their movements than YOY, as was reported by Jenkins et al. (2006) for marbled salamander. It should be noted, however, that captures for adults and YOY were much lower at outer versus inner fences (Figure 1), and sample sizes for individuals captured at both sets of fences as they left Stable Pond was small, especially for adults.

Orientation Mechanisms

The mechanisms driving non-random orientation and differing capture patterns at inner versus outer fences at Stable Pond are not known, but may be related to salamanders' perception of habitat characteristics and landscape features along movement paths (Rittenhouse and Semlitsch 2006; Lee-Yaw et al. 2015), or by the distribution of clumped resources on the landscape (i.e., below ground refugia) (Trenham 2001; Regosin et al. 2003; Atkinson-Adams et al. 2018). Cues and perception may differ between adults and YOY (Homan et al. 2008). Some evidence suggests salamanders will travel along "movement corridors" or "conduits" (Shop 1965, 1968; Gibbs 1998), and that habitat edges can act as either barriers or conduits to salamanders and other amphibians, depending on species. For example, in Connecticut, USA Gibbs (1998) found that pickerel frogs (*Rana palustris*) preferred to move along stream beds, marbled salamanders moved along stream beds and forest-residential edges, and eastern newts (*Notophthalmus viridescens*) moved through forest interior habitats and avoided forest edges. Forest-road edges acted as strong barriers to these and three other migrating amphibian species.

At Linnet Lake, all fences were located under a forest canopy of coniferous or deciduous trees and shrubs with varying density of

understory plants. At Stable Pond, all inner fences were located in continuous-canopy deciduous forest, but three of the five outer fences (in the south and west) were separated from inner fences by ~ 17 – 67 m of open grassland; the northwest fence (50) required animals to cross a 12-m wide paved road before encountering it. Only one adult (no YOY) was captured at fence 50. But rather than moving through the continuous forest that connected Stable Pond to outer fences 10 and 20, the majority of individuals in both life stages traversed up to 33 m of open habitat and were captured at fence 30. Ambystomid salamanders will readily cross open habitat during migratory movements (Anderson 1967 (long-toed); Shoop 1968 (spotted salamander); Ryan and Calhoun 2014 (blue-spotted salamander (*A. laterale*)) even though open habitat typically poses the largest risk of desiccation for amphibians, especially young-of-year (Semlitsch 1981; Rothermel and Semlitsch 2002). In a laboratory experiment with the long-toed salamander, Lee Yaw et al. (2015) found that salamanders chose substrates, such as moss and grass, which balanced movement efficiency with desiccation risk. Migratory movements typically occur at night, reducing desiccation and possibly predation risk, and concurrent movement of many individuals (mass movement) is often linked to precipitation, which reduces the threat of desiccation in habitats characterized by low humidity. We regularly observed pulses of movement of long-toed salamanders at Linnet Lake during and after rain (Atkinson-Adams 2015).

Amphibians may treat roads similarly to open-grass habitats. Gibbs (1998) and deMaynadier and Hunter (2000) found that roads act as strong barriers to movement. It is possible that the combination of a paved road and open habitat impeded most passage from Stable Pond to fence 50. At Linnet Lake 1.2 km away, however, a large portion of the adult population has historically migrated over a paved road bisecting the terrestrial habitat on one side of the lake, and based on captures along roadside fencing and at tunnel entrances, most road-crossing at this site occurs along the segment of road with the highest continuous forest and shrub cover on both sides (Pagnucco et al. 2012; Atkinson-Adams 2015). Interestingly, these heavily-used crossing sites are not aligned with the near-lake drift fences

with the highest capture rates, possibly indicating that adults emigrating from Linnet Lake also change direction as they move further into the terrestrial environment. At Stable Pond, the lowest inner-fence captures for both age classes occurred at the fence bordering the road (2.3% of adult captures; 3.5% YOY captures) and we only encountered one vehicle-killed adult during the breeding migrations and four juveniles (one confirmed YOY) in August. Two adults were captured across the road at fence 50 during 2014, and we received one report by Park staff of an adult salamander seen crossing the road near fence 50 (Figure 2) in May, 2014. It appears some adults successfully cross the road at Stable Pond and YOY will attempt to, but the road and neighboring grassland may be perceived as a barrier or unfavorable destination by the majority of adult and YOY long-toed salamanders.

Two other components that may contribute to adult orientation patterns, documented in spotted salamanders, are previous knowledge of the landscape (memory of favorable movement paths) by individuals, and differential survival within the population caused by availability of resources or threats/risks on the landscape (Regosin et al. 2003; Homan et al. 2008). Displaced ambystomatid salamanders usually attempt to return to breeding sites, olfaction being the homing mechanism with the most support (spotted salamanders: McGregor and Teska 1989), but a host of other sensory cues reviewed by Sinsch (2006) may play a role in amphibian navigation. Once in the terrestrial environment, survival of individuals is linked in part to the quality of available resources. Ambystomatid salamanders spend most of their time underground or under cover when in the terrestrial environment. Generally, mammal burrows provide access to sub-surface foraging and refuge from predators and desiccation. Some ambystomatids (eastern tiger and mole salamanders) can actively burrow (Semlitsch 1983), and from June–October we encountered several foraging long-toed salamanders < 5 cm deep in leaf litter, under moss, or in rotten logs and stumps (M. Atkinson-Adams, personal observation). Sub-surface refugia can be limited at the landscape level and/or confined to specific areas (Trenham 2001), and there is some evidence that individual ambystomatids (spotted and marbled salamanders)

will compete for these resources (Smyers et al. 2002), a trait well known in strictly terrestrial forest salamanders in the genus *Plethodon* (Jaeger et al. 1982). In northern populations of long-toed salamander, studies have found groups ranging from two to 14 individuals occupying the same overwintering refuge (Sheppard 1977, Atkinson-Adams et al. 2018), and when conditions were hot and dry in California, Anderson (1967) also observed the long-toed salamander sharing refuges and forming tight “balls” to reduce desiccation. In northern populations, if access to overwintering refuges of sufficient depth (below the frost line) is limited at a breeding site, differential survival of dispersing YOY may drive the orientation patterns seen in adults. This would explain why the two age classes often exhibit different orientation patterns, YOY being more variable. Interestingly, of six overwintering sites located at Stable Pond in 2014 (Atkinson-Adams et al. 2018), all occurred within a similar substrate (rotting wood of decaying root systems). Sites were in the predominant direction of adult emigration 3 – 181 m from the pond margin, and two contained juvenile salamanders; one was a YOY from that season, and one likely from the previous season occupying a refuge with an adult. If orientation is linked to individual survival, the 10 – 12-year lifespan of the long-toed salamander (Russell et al. 1996) provides plenty of time to reinforce orientation patterns at the population level (Homan et al. 2008).

The similarity between YOY and adult orientation patterns at inner fences may be due to the existence of areas with terrestrial habitat optimal for movement adjacent to the pond, but may also reflect favorable aquatic characteristics along the east side of the pond. While we did not survey specifically for egg masses or larvae, we captured breeding adults in the pond much more often in traps along the east shore (130 individuals) than along the west shore (10 individuals). Little is known about how larval salamanders are spatially distributed within a water body, but larval tiger salamanders (*A. mavortium nebulosum*) compete with one another for food and space (Johnson et al. 2003), and large larvae of long-toed salamander cannibalize smaller larvae (Pagnucco et al. 2011; Anderson 1967). Anderson (1967) noted that once larval long-toed salamanders reached a certain size, they dispersed somewhat uniformly around the water body to

avoid each other, but would grow tolerant of each other and aggregate in favorable locations as they neared metamorphosis. It is reasonable to hypothesize that if aquatic resources (e.g., available prey or shelter from predators) are better on the east side of the Stable Pond, it could create YOY emergence “hotspots” in favorable areas where larger larvae may concentrate before metamorphosis. At Linnet Lake, both Fukumoto (1998) and Pagnucco (2011) observed most egg masses and larvae along the west shore, specifically where woody debris and emergent vegetation were prevalent. We captured the majority of migrating adults along the west side of Linnet Lake, especially the southwest corner, which contained a large accumulation of woody debris that could provide both egg-laying substrates for adults and shelter for larvae from predacious lake chub (*Couesius plumbeus*). Anderson (1967) observed a preference for woody debris as a site for oviposition and as a refuge from predation for long-toed salamander at a high altitude site in California.

CONCLUSION

Both adult and YOY long-toed salamanders oriented non-randomly when arriving (adults) and leaving (adults and YOY) breeding sites in Waterton Lakes National Park. Orientation patterns were similar between the two age classes at Stable Pond and for adults between years at Linnet Lake. In both cases, orientation patterns near breeding sites likely reflect a combination of suitable terrestrial habitat for movement and aquatic habitat for adult breeding and larval development. Patterns further from breeding sites likely reflect a combination of suitable terrestrial habitat for movement and the location and availability of “quality” summer and winter refugia and food resources. Knowledge of how the long-toed salamander uses the terrestrial habitat surrounding aquatic breeding sites allows managers to make more informed decisions regarding protection of movement corridors and of critical foraging and overwintering sites when development threatens upland areas around known breeding locations.

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Chapter 2

OCCURRENCE PATTERNS OF THE WESTERN TIGER SALAMANDER (*AMBYSTOMA MAVORTIUM* BAIRD) IN RELATION TO UPLAND-HABITAT FEATURES AT THE NORTHERN LIMIT OF ITS RANGE IN NORTH-CENTRAL ALBERTA, CANADA

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ABSTRACT

The western tiger salamander (*Ambystoma mavortium* Baird) is one of the largest species of mole salamanders (Ambystomatidae) in North America and is a top predator in fishless aquatic systems. In addition to aquatic breeding habitat, the species requires adjacent upland habitat for foraging and for overwintering in subterranean small mammal burrows. In Canada, the western tiger salamander is a federally designated “species of special concern” in the prairie provinces. Degradation of upland habitat is listed as a key reason for the species’ decline, yet patterns of habitat use have never been systematically documented for Canadian boreal or prairie populations. Further, this region lacks detailed distribution data for western tiger salamanders, which are needed to infer trends and make status assessments. Therefore, we conducted a survey of western tiger salamander populations in the Beaver Hills region of north-central Alberta by sampling for adults and larvae in 40 potential breeding wetlands across land cover types that ranged from almost pure grassland to continuous forest. Because of their known association with small mammal burrows, we also surveyed northern pocket gopher (*Thomomys talpoides* Richardson) density as a proxy for potential overwintering sites. We used generalized linear models (GLMs) to model salamander occurrence and abundance as a function of land cover type and pocket gopher density. We also assessed occupancy and detectability patterns in order to estimate the number of tiger salamander populations within the 1,572 km² study area. We captured western tiger salamanders in 50% of the surveyed wetlands. The proportions of upland grassland or forest adjacent to wetlands were not significant predictors of tiger salamander occurrence or relative abundance of individuals within populations. However, pocket gopher density was a significant predictor of salamander occurrence but not abundance. We estimated a minimum of 625 (420-811, 95% CI) tiger salamander populations occupying comparable wetlands in the study area. We suggest future actions to locate and conserve populations of this large, cryptic salamander should consider pocket gopher density, in addition to traditionally used coarse habitat proxies such as land cover.

INTRODUCTION

In Canada, the western tiger salamander (*Ambystoma mavortium* Baird) is federally designated by the Committee on the Status of

Endangered Wildlife in Canada (hereafter, COSEWIC) as “a species of special concern” in Alberta, Saskatchewan, and Manitoba (hereafter, the prairie provinces) because of evidence that habitat loss and fragmentation, emergent diseases (e.g., *Ambystoma tigrinum* virus and chytrid fungus [Chytridiomycota]), and in some cases, fish stocking may be leading to declines (COSEWIC 2012). However, declines in attributes used in assessing a species’ status, such as number of occurrences, are only projected and have not been verified because little baseline data exist regarding the distribution and abundance of western tiger salamanders in prairie Canada. Similarly, degradation of upland (terrestrial) habitat by agriculture and resource development is listed as one cause of decline for populations (COSEWIC 2012), but upland habitat has never been systematically described for Canadian boreal or prairie populations. The terrestrial habitat and distribution of western tiger salamanders needs further examination to identify potentially critical habitat elements for these populations and to provide baseline data to assess future trends.

Intensive small-scale studies of tiger salamanders and other ambystomatid species (mole salamanders) have demonstrated that terrestrial habitat for these salamanders is usually in the form of subterranean runways and tunnels of various small mammal species (*A. mavortium*: (Hamilton Jr. 1946; Lomolino and Smith 2003; Richardson, Klenner, and Shatford 2000; Shipley and Reading 2006; Vaughan 1961); *A. tigrinum* Green and *A. californiense* Gray: (Loredo, Van Vuren, and Morrison 1996; Madison and Farrand 1998; Pittman 2005; Steen et al. 2006; Trenham 2001); other species: (Faccio 2003; Graeter, Rothermel, and Gibbons 2008; Semlitsch 1981; Kleeberger and Werner 1983; Madison 1997; Johnston 2000)). However, the terrestrial ecology of ambystomatid salamanders also needs evaluation at a larger spatial scale in order to understand distribution patterns of multiple populations rather than individuals within a single population. We defined a population as the group of breeding and non-breeding adult, juvenile, and larval salamanders inhabiting a single breeding wetland and adjacent upland habitat. Many pond-breeding amphibian populations exist within highly variable metapopulations within which frequent extinction and recolonization of

single populations and associated breeding sites is natural (see Smith and Green (2005) for a review). Therefore, caution is warranted when scaling highly variable small-scale and single-population patterns up to a landscape level that encompasses many populations (Petranka, Smith, and Scott 2004).

The western tiger salamander, like other tiger salamanders, is generally considered a ‘grassland’ species (Petranka 1998). This relationship is evident in much of the prairie provinces where the species occurs in short-grass and mixed-grass prairie ecosystems (COSEWIC 2012). Other Canadian populations (which are listed as “endangered” by COSEWIC) occur in a region characterized by Great Basin shrub-steppe grasslands (bunchgrass-sagebrush) in south-central British Columbia. Some populations in the prairie provinces occur in aspen parkland and dry southern boreal mixedwood forest matrices that have vegetation characteristic of the transition zone between the prairies to the south and continuous boreal forests to the north. This transition zone is a heterogeneous landscape with elements of native prairie grassland, cattle pasture, and agriculture embedded within a largely forested matrix (see ‘Study Area’ for details).

The distribution of western tiger salamander populations within this transition zone might be limited by the availability of grassland patches. Alternatively, terrestrial vegetation patterns might not significantly influence the distribution of populations in this region, as the species occurs in a wide variety of ecosystems other than grasslands throughout its continental range including deserts and alpine conifer forests (Petranka 1998; Stebbins 2003). Gray, Smith, and Grenes (2004) demonstrated that agricultural development adjacent to breeding ponds did not significantly influence western tiger salamander occupancy of wetlands in Texas (*A. tigrinum* at the time of publication), suggesting that western tiger salamander choice of terrestrial vegetation may be flexible if adjacent to wetlands that are suitable for breeding. However, the likelihood of western tiger salamander presence in a wetland increased with distance from forests in Wyoming (Bartelt et al. 2011), suggesting that the species prefers specific vegetation types adjacent to wetlands, likely in the form of

grasslands. Inferences from related species are inconsistent, as California tiger salamanders (*A. californiense*) are strongly tied to grassland ecosystems (Trenham 2001; Orloff 2011; Searcy, Gabbai-Saldate, and Shaffer 2013) while eastern tiger salamander (*A. tigrinum*) distribution is not significantly related to any specific type of terrestrial vegetation (Brodman 2010; Herwig et al. 2013; Knutson et al. 2004). To our knowledge, no studies have examined the distribution of western tiger salamander populations in boreal/prairie Canada, particularly where terrestrial vegetation, which was historically shaped by wildfire and bison (*Bison bison* L.) grazing (Campbell et al. 2000), is highly variable and patchy within the transition zone between the northern prairies and southern boreal mixedwood forest.

The Beaver Hills, Alberta, (Figure 1) lie within a disjunct patch of dry southern boreal mixedwood forest and is an ideal location to examine the distribution of western tiger salamanders within the prairie/boreal transition zone because the effect of terrestrial habitat on the distribution of populations can be isolated from that of aquatic habitat. Much of the existing literature on tiger salamander distributions, and that of other ambystomatid salamanders, focuses on aquatic habitat characteristics rather than terrestrial characteristics. This research has repeatedly demonstrated the importance of fishless water bodies with long hydro-periods as a factor influencing ambystomatid occupancy and abundance patterns (Cosentino, Schooley, and Philips 2011; Drake et al. 2014; Goldberg and Waits 2009; Herwig et al. 2013; Maurer, Stewart, and Lorenz 2014; Semlitsch et al. 2015). The Beaver Hills lie within the prairie pothole region and harbor thousands of permanent and semi-permanent wetlands with a sufficiently long hydro-period for larval salamander development. These wetlands are typically fishless (excluding some larger lakes), likely because of poor connectivity and frequent winter hypoxia (Cosentino, Schooley, and Philips 2011; Peterka 1989). Salinity can be highly variable among wetlands in the Beaver Hills and ranges from 16-899 μS (Nicholson 1995). However, Klaver, Peterson, and Patla (2013); found that salinity did not significantly influence western tiger salamander occupancy of wetlands in Wyoming, where available wetlands showed an

even greater range of salinity (10-1,300 μ S). Among the pothole wetlands in the Beaver Hills, those that are semi-permanent or permanent and lack fish populations should provide suitable breeding habitat for the species, presumably when adjacent to suitable terrestrial habitat. Because of the prevalence of winter hypoxia in wetlands, fish populations naturally inhabiting potholes are limited to brook stickleback (*Culaea inconstans* Kirtland) and fathead minnow (*Pimephales promelas* Rafinesque), and even these hardy species are typically absent due to winterkill (Nelson and Paetz 1992). Given the high abundance of potential aquatic habitat, the distribution of these northern peripheral populations of western tiger salamander might be disproportionately driven by the availability of terrestrial winter refugia. This relationship would be expected of an ectothermic tetrapod living in an area at the edge of its physiological limitations versus southern populations near the species' core range (Micheletti and Storfer 2015; Soule 1973). The objectives of our study were (1) to characterize the occurrence and abundance of western tiger salamanders in the Beaver Hills in relation to terrestrial habitat features, and (2) to use occurrence data to estimate the number of populations in the Beaver Hills.

We addressed the first objective by sampling potentially suitable breeding ponds with highly variable adjacent terrestrial habitat composition in 2013 and relating salamander occurrence and abundance at a wetland to terrestrial habitat variables with generalized linear regression and occupancy modeling. We predicted that salamander presence and abundance would be negatively related to the proportion of forested habitat because: i) tiger salamanders are commonly characterized as 'grassland species' (Petranka 1998), ii) other Canadian populations in British Columbia occur in a semi-arid grassland system (COSEWIC 2012), and iii) the likelihood of western tiger salamanders' occupancy of wetlands in Wyoming was significantly positively related to distance from forests (Bartelt et al. 2011). We addressed the second objective by extrapolating observed occupancy patterns to potentially suitable wetlands across the study area. Information on distribution, abundance, and terrestrial habitat preferences of the western tiger salamander is important for future status

assessments (e.g., by COSEWIC), where the decline in area occupied by a species or a decline in the number of individuals serve as key criteria for determining conservation status.

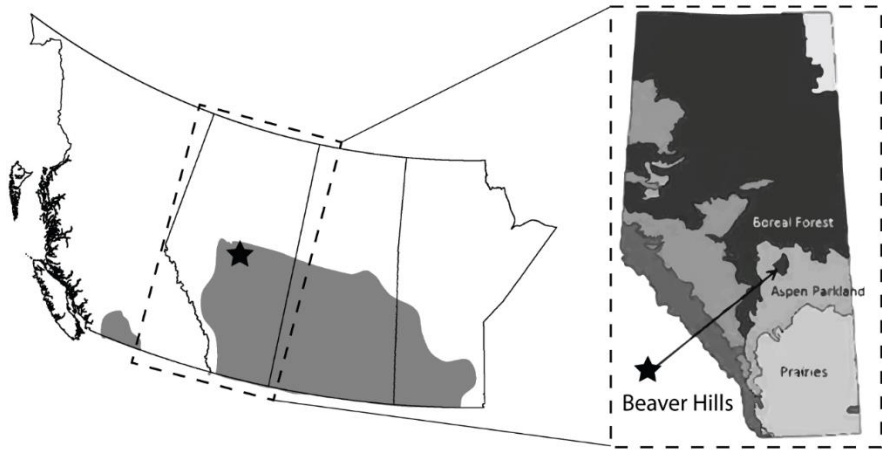


Figure 1. Study site location (black star) in relation to the Canadian distribution of the Western Tiger Salamander (*Ambystoma mavortium*). Inset: The Beaver Hills/Cooking Lake Moraine in Alberta, a disjunct patch of boreal forest surrounded by Aspen Parkland.

METHODS - STUDY AREA

The Beaver Hills/Cooking Lake moraine (Figure 1) encompasses 1,572 km² and lies roughly 25 km east of Edmonton, Alberta, Canada. The area is along the northern edge of the prairie pothole region and contains thousands of pothole wetlands and larger shallow lakes ($n = 3,057$; Natural Resources Canada 2007) that range in size from 0.006 to 2,749 ha. The moraine rises over 790 m above the surrounding aspen parkland and supports a patch of dry southern boreal mixed-wood forest. Dry southern boreal mixed-wood forests experience cooler average annual temperatures (1.1 degree Celsius) and higher average annual rainfall (461 mm) than the surrounding aspen parkland and prairie plateau (Natural Regions Committee 2006). The terrestrial landscape is heterogeneous and includes

natural grassland, shrubland, row-crop agriculture, and grazing land within a forested matrix. Forests are predominately deciduous poplar species (*Populus tremuloides* Michx. and *Populus balsamifera* L.) interspersed with spruce (*Picea mariana* [Mill.] Britton, Sterns and Poggenb. and *Picea glauca* [Moench] Voss (Natural Regions Committee 2006). The dominant understory is beaked hazel (*Corylus cornuta* Marshall), low-bush cranberry (*Viburnum edule* Michx.), and saskatoon (*Amelanchier alnifolia* Nutt). Native grasslands are fescue prairie (*Festuca* L. spp.). Open shrublands are dominated by willow (*Salix* L. spp.) and rose (*Rosa* L. spp.). The primary crops in cultivated areas include forage crops and grains, specifically barley, legumes, and canola. Poor soil and climate conditions have limited the extent of agricultural development to only 24% of the moraine proper, with the remainder comprised of 45% forests, 27% grassland/grazing land, and 4% urban development. Greater than 22% of the natural and grazing land is protected within parks: Elk Island National Park, Cooking Lake-Blackfoot Provincial Park and Recreation Area, Ministik Bird Sanctuary, and Miquelon Lake Provincial Park.

METHODS – DISTRIBUTION SURVEY

Site Selection and Terrestrial Habitat Variables

Each study site was a single wetland and the adjacent terrestrial habitat within 200 m of the wetland's edge. Terrestrial habitat was defined as the adjacent upland habitat within 200 m of the water's edge because this area likely encompasses the upland foraging and overwintering habitat of most breeding individuals within a population of pond-breeding amphibians, including ambystomatid salamanders (Brodman 2010; Porej, Micacchion, and Hetherington 2004; Semlitsch 1998). Wetlands with overlapping terrestrial habitat were not sampled. Habitat within each 200 m terrestrial zone was classified by percent land cover (Agriculture and Agri-foods Canada 2012) in ArcGIS10.1 (ESRI 2011). We selected wetlands in the study area with low heterogeneity in aquatic habitat and high heterogeneity

in terrestrial habitat so that we could isolate the effect of terrestrial habitat from aquatic habitat in relation to occupancy and abundance patterns.

We only selected sites with suitable aquatic habitat. Available wetlands in the study area were stratified to those within the size range of known local breeding wetlands and with a sufficiently long hydro-period for larval development, as characterized by semi-permanent and permanent wetlands (Zoltai and Vitt 1995) less than or equal to 7 ha. A wetland's permanence was assessed with aerial imagery and a site was included if on the first visit most of the wetland area was open water ringed by cattails (*Typha* L. spp.). A size limit of 7 ha was chosen based on the range of previously known western tiger salamander breeding wetlands ($n = 14$) in the study area, which ranged from 0.282 to 12.637 ha (incidental observations and surveys by previous students, Paszkowski lab).

Available sites in the study area were further stratified to maximize heterogeneity in terrestrial habitat and to limit variation from rare and anthropogenic variables. Sites were selected from those with greater than 90% adjacent forest and/or grassland terrestrial habitat so that we could test our fundamental question of whether occupancy and abundance are influenced by natural terrestrial features. Sites with rare (e.g., barren rock) and anthropogenic adjacent land cover (e.g., urban development, etc.) were also excluded to minimize the number of variables used in analysis. Forested land cover was defined as deciduous, coniferous, and mixed forest stands. Open land cover was defined as native grassland, shrubland, and pasture used for livestock grazing. Forested lands cleared for grazing were the only anthropogenic land cover included within this study because they resembled and were often visually indistinguishable from natural grasslands, and likely provide similar habitat for salamanders.

Study sites ($n = 40$) were randomly selected in ArcGIS (ESRI 2011) from available wetlands ($n = 1,137$) that met the above aquatic and terrestrial habitat criteria along a gradient of land cover composition ranging from 3 to 99% forest (Table 1). Average wetland size was 1.26 ha with a range of 0.04 to 6.09 ha.

Table 1. Capture totals (L = larva, A = adult) and terrestrial habitat characteristics (%F = % forest cover within 200 m of wetland edge, PGD = pocket gopher density within 30 m of wetland edge (mounds/ha)) for study sites (n = 40)

Site	May	June	July		Total		Comb. Total	Terrestrial Habitat		Wetland Area (ha)
			L	A	L	A		%F	PGD	
S3	1	7	1	12	1	20	21	58	200	0.42
P8	2	2	-	8	-	12	12	53	500	0.52
P16	4	1	3	3	3	8	11	33	2600	0.04
GS	6	3	-	1	-	10	10	53	4300	0.14
G43	-	3	4	1	4	4	8	99	150	1.78
P20	-	2	-	3	-	5	5	65	-	0.45
P2	1	1	-	2	-	4	4	3	240	0.39
S1	1	-	2	-	2	1	3	14	98	2.31
P15	-	-	2	1	2	1	3	48	-	0.18
P13	-	2	-	1	-	3	3	51	76	0.17
PT10	-	1	-	1	-	2	2	9	800	0.30
PT11	-	-	-	2	-	2	2	45	300	0.97
P6	-	2	-	-	-	2	2	71	1200	0.19
G22	-	-	2	-	2	-	2	88	73	1.05
G10	-	-	-	1	-	1	1	5	170	0.85
P23	1	-	-	-	-	1	1	19	-	0.18
G25	-	1	-	-	-	1	1	39	100	0.45
G46	-	-	-	1	-	1	1	86	50	2.28
G36	1	-	-	-	-	1	1	89	60	1.43
P19	-	-	1	-	1	-	1	91	-	4.05
P11	-	-	-	-	-	-	-	5	-	0.38
P18	-	-	-	-	-	-	-	28	43	0.30
G33	-	-	-	-	-	-	-	36	-	6.09
P22	-	-	-	-	-	-	-	47	-	0.46
PT13	-	-	-	-	-	-	-	51	-	5.51
P7	-	-	-	-	-	-	-	56	-	0.11
P24	-	-	-	-	-	-	-	63	-	1.90
G48	-	-	-	-	-	-	-	64	-	0.32
G19	-	-	-	-	-	-	-	64	-	0.86
P21	-	-	-	-	-	-	-	64	-	3.12
P4	-	-	-	-	-	-	-	65	-	0.50
G42	-	-	-	-	-	-	-	71	-	3.88
G31	-	-	-	-	-	-	-	77	-	3.23

Site	May	June	July		Total		Comb. Total	Terrestrial Habitat		Wetland Area (ha)
			L	A	L	A		%F	PGD	
G28	-	-	-	-	-	-	-	78	-	0.72
P10	-	-	-	-	-	-	-	79	34	0.41
G15	-	-	-	-	-	-	-	86	-	0.80
P12	-	-	-	-	-	-	-	88	-	1.14
G27	-	-	-	-	-	-	-	89	-	0.42
G13	-	-	-	-	-	-	-	90	-	2.05
G14	-	-	-	-	-	-	-	94	-	0.21
Total	17	25	15	37	15	79	94			

Captures for May and June included only adults. Dotted horizontal line separates sites with and without detections

Northern Pocket Gopher Activity Surveys

Northern pocket gopher (*Thomomys talpoides* Richardson) activity was also used to characterize western tiger salamander terrestrial habitat. Pocket gopher activity was quantified as the density of tunnel excavation mounds within 30 m of the wetland edge in 2014. Density surveys were limited to within 30 m because of logistic constraints. Multiple mounds were considered distinct if separated by undisturbed ground, with distinct mounds sometimes separated by less than 20 cm. Tunnel excavation mounds of pocket gophers provide good indicators of the extent of their subterranean tunnel systems because these mounds are created at the terminus of a subterranean tunnel, and are the excavated soil from those tunnels. Therefore the abundance of mounds should be directly related to the extent of tunneling. We tested this assumption by correlating the number of mounds with subsurface foraging tunnel density at a subset of study sites in 2014. Horizontal foraging tunnels were probed with a rebar stake to delineate the length of tunneling within the study areas. The number of mounds was strongly correlated with tunnel density (Pearson $r = 0.72$) and were considered a suitable proxy for subterranean pocket gopher tunnels potentially used as terrestrial habitat by western tiger salamanders. Western tiger salamanders are known to use these burrows, likely to forage, overwinter, and/or avoid desiccation (Hamilton Jr. 1946; Lomolino and Smith 2003; Richardson, Klenner, and Shatford 2000;

Shipley and Reading 2006; Vaughan 1961). Terrestrial juveniles and adults were observed in the excavation mounds themselves (Welsh 2015). Other larger burrowing mammals present on the landscape were Richardson's ground squirrel (*Urocitellus richardsonii* Sabine) and American badger (*Taxidea taxus* Schreber), neither of which was encountered at study sites. Burrow networks created by smaller tunneling mammals (family: Cricetidae (Fischer)) were not surveyed because they were difficult to detect in dense summer growth across large areas, although ambystomatid salamanders have been documented using them (Faccio 2003; Graeter, Rothermel, and Gibbons 2008; Semlitsch 1981; Kleeberger and Werner 1983; Madison 1997; Johnston 2000).

Aquatic Sampling for Salamanders

Each of the 40 wetlands was sampled for salamanders with aquatic drift fences and minnow trap arrays in each of three months, May, June, and July 2013. This sampling period encompasses peak salamander activity in the Beaver Hills when adults, larvae, or both are using aquatic habitats, and multiple samplings increased detection probability. Trapping began in the first week of May when pond ice receded enough to allow traps to be set in the water. An array consisting of 10 Gee minnow traps (length = 42 cm, diameter = 22.9 cm, mesh size = 6.35 mm) and a single, larger modified minnow trap (height = 76.2 cm, width = 45.7 cm, depth = 50.8 cm, mesh size = 6.35 mm) (see Welsh 2015 for details of trap design) placed along an aquatic drift fence (synthetic fiber silt fence, height = 0.9 m, length = 10 m) was placed in the wetland during each trapping session. The drift fence was positioned perpendicular to the shoreline in shallow water with the modified trap set at the deepest end and two Gee traps fastened horizontally along either side of the fence. The shoreline location for trap placement was selected haphazardly and changed with each visit. The remaining six Gee traps were spread evenly around the pond margin and attached to stakes in shallow water with airspace in the top of the traps. Traps were placed among emergent

vegetation (*Typha* spp.) when possible. The design of the modified minnow trap and aquatic drift fence allowed sampling of the entire water column at depths up to 30 cm while maintaining airspace for captured salamanders and air breathing animals (frogs, muskrats [*Ondatra zibethicus* L.], ducks, garter snakes [*Thamnophis* Fitzinger spp.], etc.) trapped incidentally. Traps were set in the afternoon and checked the following morning. Trapping effort was constant among sites and captures (count data) reflected catch per unit effort (CPUE), a measure of relative abundance. Individual salamanders were classified as adults or larvae based on the presence or absence of external gills.

Statistical Analysis – Generalized Linear Regression

Salamander detection (presence/absence) and relative abundance (counts for adults) for 2013 were modeled as a function of terrestrial habitat variables with generalized linear regression (R Core Team 2013). Salamander detection was defined as the presence of post-metamorphic individuals (hereafter, adults) and/or larvae during the sampling season. Salamander relative abundance was defined as the season's total CPUE of adults when present (counts). We used Firth's penalized maximum-likelihood logistic regression for presence/absence models because it is suitable for small sample sizes and is robust to model fitting errors associated with data separation (Albert and Anderson 1984; Heinze and Schemper 2002). Data separation occurred when one highly predictive variable (pocket gopher density) prevented model convergence by completely separating the response variable and thus prevented the creation of a descriptive model for the data. Salamander relative abundance was modeled with zero-truncated negative binomial regression. Three candidate models with different combinations of the two habitat variables were fitted to presence/absence and count data: (1) land cover; (2) pocket gopher density; (3) land cover + pocket gopher density. These were assessed for model fit with a likelihood ratio test (LRT), where a model was considered a good fit if it explained significantly more variation than a

model with no habitat covariates at $\alpha = 0.05$. Percent forest was selected as the land cover variable for use in each model and was strongly negatively correlated with percent open habitat within the 200 m buffer (Pearson $r = -0.94$). Besides open habitat, another land-cover variable excluded from analysis was temporary wetlands. Preliminary analysis using percent open habitat as the land cover variable in place of percent forest yielded consistent and complementary results. Missing northern pocket gopher data for two sites were estimated with Bayesian multiple imputation (Honaker, King, and Blackwell 2011) after preliminary analysis revealed no difference in results with different imputation methods or record removal.

Statistical Analysis - Occupancy Models

Western tiger salamander detection (presence/absence) was also modeled with terrestrial habitat variables using single-season occupancy models in program PRESENCE to account for potential bias resulting from imperfect detectability. Species detection during a survey is a product of both the species being present (occupancy = ψ) and the investigator's ability to detect it (detectability = p). Failure to detect a species when it is present can bias or underestimate occupancy by cryptic organisms with detectability less than one, like salamanders (MacKenzie et al. 2002).

Occupancy was modeled with terrestrial habitat variables and detectability was modeled based on larval presence/absence, as overall salamander detection likely increased among surveys when larvae were present and catchable in the pond (July) compared to detectability during surveys when only post-metamorphic individuals were present (May and June). The 'larval presence/absence variable' was quantified by dividing the sampling period into a binary variable based on the time period that larvae were captured in the pond (July) or were not yet detected (May - June). Three candidate models with site (ψ) and survey (p) predictor variables, and one without, were fitted to the data and ranked with AIC. The saturated model containing all predictor variables was assessed for

goodness of fit (GOF) with a Pearson's chi-square test and a parametric bootstrap procedure (MacKenzie and Bailey 2004). The coefficient standard errors (S.E.) were adjusted in all candidate models for overdispersion ($\hat{c} = 1.27$) with the following equation:

$$\text{S.E.}_{\text{adj}} = \text{S.E.} * \sqrt{\hat{c}}.$$

Models were then ranked by QAIC to account for overdispersion:

$$\text{QAIC} = (-2\log\text{lik}/\hat{c}) + k$$

where loglik is the maximized log-likelihood and k is the number of parameters, including \hat{c} (Burnham and Anderson 2002). The best model was selected as that with the lowest QAIC and site variables interpreted as significant at $\alpha = 0.05$. Any model less than $\Delta 2$ QAIC of the best model was deemed plausible and also interpreted (Burnham and Anderson 2002).

METHODS – OCCURRENCE ESTIMATES FOR BEAVER HILLS AND COOKING LAKE REGION

The minimum number of wetlands occupied by western tiger salamanders in the Beaver Hills was estimated by applying the 95% confidence interval of the site occupancy estimate (ψ) in the best occupancy model to the total number of available wetlands within the study area. For this exercise, available wetlands were those within the same criteria as previously described for study site selection. These were limited to wetlands with areas less than 7 ha and with greater than 90% forested and/or open adjacent land cover within 200 m of the wetland edge ($n = 1,137$).

RESULTS

Northern Pocket Gopher Activity Surveys

Northern pocket gopher mounds were detected at 45% of sites ($n = 18$) with an average density of 600 mounds/ha, ranging from 30 to 4,300 mounds/ha (Table 1). The high densities observed at some sites (see P16, GS, and P6) were likely a reflection of the relatively limited suitable habitat that was heavily used by pocket gophers at those particular sites, and is probably not a realistic reflection of actual pocket gopher abundance at those sites (Smallwood and Morrison 1999). Mounds were typically in open habitat or along the ecotone between open and forested habitats. No pocket gopher mounds were detected in forested habitat. However, although the relationship with forest cover was negative, pocket gopher mound density within 30 m of the wetland edge was not strongly correlated with land cover (percent forest) within 200 m of the wetland edge (Pearson's $r = -0.32$).

Aquatic Sampling for Salamanders

Salamanders were detected at 50% of sites for all three months combined, with detection at 20%, 28%, and 40% of sites in May, June and July, respectively. Eighteen percent, 27%, and 55% of the total number of individuals captured, were trapped in May, June, and July, respectively. All larvae were caught in July and represented 29% of the monthly capture and 16% of total captures (Table 1). Both larvae and adults were captured at 12.5% of the sites, adults only at 32.5%, and larvae only at 5%. Ninety-four percent of all salamanders were captured in modified traps or Gee traps along the aquatic drift fence and the remaining 6% were captured in "satellite" Gee traps without a drift fence.

Statistical Analysis: Generalized Linear Regression

Salamander detection (presence/absence) and relative abundance were not significantly related to adjacent land-cover composition in any suitable model (Table 2). Firth's logistic regression indicated that salamander detection was influenced by pocket gopher density adjacent to wetlands and this terrestrial variable was the only significant factor in either of the two presence/absence models that provided reasonable model fit (Table 2). In contrast, generalized linear regression of count data indicated that pocket gopher density did not explain salamander relative abundance, and no terrestrial habitat model described these count data accurately.

Table 2. Generalized linear models for salamander presence/absence (Firth's logistic regression) and relative abundance of adults (zero-truncated negative binomial regression on count data)

Presence/Absence (logistic regression)					
Model	Variables in model	p	Likelihood Ratio Test		
			χ^2	df	p
1	PGD (+)	<0.001*	19.99	1	<0.001*
2	%F (-)	0.059	3.58	1	0.059
3	PGD (+)	<0.001*	19.37	2	<0.001*
	%F (-)	0.58			
Abundance when present (zero-truncated negative binomial regression)					
1	PGD (+)	0.27	1.49	1	0.22
2	%F (+)	0.61	0.119	1	0.73
3	PGD (+)	0.15	1.91	2	0.38
	%F (+)	0.44			

Table shows the independent variables and the Likelihood Ratio Test for each model (to evaluate the fit of the model with a chi-square statistic, χ^2). Significant models are in bold and the likelihood ratio test is denoted with an asterisk (*). Significant independent variables within a model are also denoted with an asterisk and the direction of influence on the response variable is denoted as positive (+) or negative (-). PGD = pocket gopher density (mounds/ha), %F = % forest cover within 200 m of wetland edge

Table 3. Model results for occupancy and detectability of western tiger salamanders in program PRESENCE

Model	QAIC	ΔQAIC	Habitat variable	coefficient	S.E.	95% CI	detectability variable	Coefficient	S.E.	95% CI
$\psi(\text{PGD}), p(\text{lar})$	86.85	0.00	PGD *	2.43	0.86	0.74 – 4.12	lar*	1.38	1.37	0.01 – 2.75
$\psi(\text{PGD}+\%F), p(\text{lar})$	88.41	1.56	PGD *	2.44	0.93	0.62 – 4.26	lar*	1.37	1.36	0.01 – 2.73
			%F	-0.01	0.03	-0.03 – 0.09				
$\psi(\%F), p(\text{lar})$	103.92	17.07	%F	-0.02	0.01	-0.01 – 0.03	lar	1.27	1.31	-0.04 – 2.58
$\psi(\cdot), p(\text{lar})$	104.94	18.09					lar	1.27	1.31	-0.04 – 2.58

ψ is the probability that a species is present and p is the probability that a species is detected at an occupied site. Best models ($\alpha = 0.05$) are in bold and their significant habitat variables are denoted with an asterisk (*). Explanatory variables for each parameter in a model are in parentheses. PGD = pocket gopher density (mounds/ha); %F = % forest cover within 200 m of wetland edge; $p(\text{lar})$ = detectability is binomial variable for time period where overall species detectability varies between pre-larvae season (May-June) and larvae season (July). S.E. = adjusted standard error (s.e. * $\sqrt{\hat{c}}$). $\Delta\text{QAIC} = \text{QAIC}_i - \text{QAIC}_{\min}$

Statistical Analysis: Occupancy Models

Occupancy analysis results were consistent with those of generalized linear regression, as the best models indicated that salamander occupancy was not significantly influenced by adjacent land cover, but it was significantly related to pocket gopher density within 30 m of the wetland edge (Table 3). The best models also indicated that the likelihood of salamander detection increased significantly in July when catchable larvae were present in the wetland (Table 3). Imperfect and variable detectability did not significantly influence the observed relationship between site occupancy and adjacent terrestrial habitat.

RESULTS - OCCURRENCE ESTIMATES FOR BEAVER HILLS AND COOKING LAKE REGION

While the best occupancy model for describing salamander occurrence based on our 40 sampled sites included pocket gopher density as a predictor, we did not select it to predict the minimum number of occupied wetlands in the Beaver Hills because pocket gopher density data were unavailable for non-surveyed wetlands. Instead, we selected the occupancy model that contained no terrestrial habitat variables but included larval presence/absence as the detectability variable because it provided an estimate of the proportion of occupied wetlands after accounting for variable detectability for the species. This increased the occupancy estimate among all wetlands to 0.55 (0.37 - 0.71, 95% CI) compared to the naïve estimate of 0.50 based on our trapping data. The total number of semi-permanent and permanent wetlands with an area less than 7 ha and with greater than 90% adjacent forest/open habitat in the Beaver Hills was 1,137, therefore the minimum estimated number of wetlands occupied by western tiger salamanders in the Beaver Hills was 625 (420 – 811, 95% CI).

DISCUSSION

The distribution of the western tiger salamander in the Beaver Hills was affected by the nature of terrestrial habitat surrounding breeding wetlands. Our models indicated that the occurrence of western tiger salamander populations was best predicted by the density of northern pocket gopher excavation mounds adjacent to wetlands but was not significantly correlated with the composition of adjacent land cover. However, neither pocket gopher activity nor adjacent land cover explained salamander relative abundance at breeding wetlands. The positive relationship between western tiger salamander presence and northern pocket gopher activity in north-central Alberta was not surprising given the extensive evidence that ambystomatid salamanders use mammal burrows (see Hamilton Jr. 1946; Lomolino and Smith 2003; Richardson, Klenner, and Shatford 2000; Shipley and Reading 2006; Vaughan 1961; Loredó, Van Vuren, and Morrison 1996; Madison and Farrand 1998; Pittman 2005; Steen et al. 2006; Trenham 2001; Faccio 2003; Graeter, Rothermel, and Gibbons 2008; Semlitsch 1981; Madison 1997; Johnston 2000). In addition to a statistical association, all tiger salamanders encountered in terrestrial habitat (18 total, adults and juveniles) during our study were found in or immediately adjacent to northern pocket gopher tunneling activity (see Welsh 2015). One adult was actually captured in a minnow trap that was buried approximately 10-20 cm deep in a pocket gopher tunnel. These observations, combined with the significant positive relationship between salamander distribution and pocket gopher density suggest that western tiger salamanders in the prairie provinces, and perhaps elsewhere in Canada, are using habitats created by pocket gophers and that this habitat feature might be required for many populations to persist. This relationship is further supported by the sympatric distribution of the northern pocket gopher (Patterson 2003) and the western tiger salamander. The individual salamander found in a pocket gopher tunnel was trapped in June and the 17 other salamanders detected in excavation mounds were found in September, suggesting that at least some proportion of salamanders use the gopher tunnels as terrestrial foraging habitat in the summer and, that

perhaps, a larger proportion use them as winter refugia beginning in the early fall.

Pocket gopher activity and land cover were not strongly correlated, which is surprising because pocket gophers typically inhabit open areas like fields and meadows, and no pocket gopher activity was found in forests. This mismatch could be because the two variables were measured at different scales, with pocket gopher activity assessed within 30 m of the wetland edge and land cover characterized within 200 m. Also, pocket gopher activity varied among occupied sites with extensive open grasslands around wetlands having similar pocket gopher abundances to those of much smaller meadows originating from beaver (*Castor canadensis* Kuhl) activity or drought in otherwise forested habitats. It is unclear to what degree individual salamanders or populations are reliant on habitat features created by pocket gophers. Sixteen of 20 sites where tiger salamanders were present had northern pocket gopher activity whereas only two of the 20 sites without salamanders had pocket gopher activity. We recognize that habitat within 30 m and 200 m of the wetland edge does not encompass the entire area available to salamanders. Individual western tiger salamanders have been observed up to 3 km from the nearest wetland (Sarell and Robertson 1994), which suggests that at least some proportion of populations may be using terrestrial habitat outside of areas surveyed in our study. Root holes and other small mammal burrows might also provide habitat for western tiger salamanders in the Beaver Hills and across the prairie provinces, as they do for other, forest-dwelling ambystomatid salamanders (Faccio 2003; Madison 1997; Johnston 2000).

Occupancy estimates of western tiger salamanders in the Beaver Hills (55% of appropriate wetlands [37% - 71%, 95%CI]) were higher overall than occupancy of western tiger salamanders in Grand Teton and Yellowstone National Parks; here wetland occupancy ranged from 0 to less than 30% between 2004 and 2011, but was comparable to estimates for beaver-influenced ponds in 2002 (Hossack et al. 2015). In other studies from Yellowstone, western tiger salamander occupancy ranged between 7.3% – 15% of sites (Klaver, Peterson, and Patla 2013; Gould et al. 2012), which is much lower than occupancy estimates in our study. Occupancy of

western tiger salamanders in the Beaver Hills is also higher than that of eastern tiger salamanders in Minnesota, where estimated occupancy varied between 27% - 35% among candidate models (MacKenzie et al. 2003). Assuming similar effectiveness in surveys with regard to sampling effort and methods, these patterns suggest that a higher proportion of wetlands in the prairie/boreal transition zone are suitable for western tiger salamanders than elsewhere in the range of the species and the related eastern tiger salamander. It is also possible that other studies sampled a wider range of wetland types of which a higher proportion might have been unsuitable for salamanders.

The most recent COSEWIC status assessment (2012) elevated western tiger salamander populations in the prairie provinces to 'special concern' status from 'not at risk.' Declines were largely inferred based on recreational stocking of fish, and loss of wetlands and fragmentation of terrestrial habitat due to urbanization, the energy industry, and agriculture (COSEWIC 2012). At the time the status report was written, there were no estimates for western tiger salamander population occurrence patterns and the index of area occupied was limited to known sites, where the species was usually recorded due to incidental observations or encountered during general surveys for amphibians, fish, or other wetland biota. Our surveys, which were targeted to detect western tiger salamander, indicate that the species might be more widely distributed across wetlands in the prairie pothole regions of western Canada within the species' estimated 567,437 km² extent of occurrence. It is of note that the minimum number of wetlands inhabited by western tiger salamanders in the Beaver Hills was estimated based on occupancy patterns observed within wetlands fitting a specified size range and land-cover criteria, and cannot be meaningfully applied to the hundreds of wetlands lacking these attributes. Thus, our estimate should be viewed as conservative because known populations exist at sites that fall outside of these criteria. For example, we recorded western tiger salamanders in a 20 ha lake, demonstrating that salamanders do occur in wetlands much larger than sampled in our study (K. Welsh, personal observation). Further north in Alberta, western tiger salamanders have also been documented in lakes where small-bodied fishes occur (C.

Paszkowski, personal observation). Western tiger salamanders have also been recorded in natural and constructed wetlands within the city of Edmonton, suggesting that they will also use sites surrounded by little natural vegetation (Scheffers 2010; K. Welsh, personal observation). Previously, the number of occupied wetlands across the prairie provinces was estimated as greater than 500 (COSEWIC 2012). Based on these data, the projected number of occupied wetlands in the Beaver Hills alone was 625 (420 – 811, 95% CI), suggesting that the overall number of occupied sites in the prairie provinces was grossly underestimated and must be far greater.

Management recommendations and future management guidelines for western tiger salamanders in Alberta should ensure the preservation of northern pocket gophers in upland habitat adjacent to wetlands rather than infer salamander habitat based on adjacent land cover composition. This study suggests that land cover is a poor proxy for tiger salamander terrestrial habitat. This likely also applies to other areas within the prairie provinces where northern pocket gophers and tiger salamanders coexist. Other burrowing mammals, notably Richardson's and thirteen-lined (*Ictidomys tridecemlineatus* Mitchill) ground squirrels inhabiting these provinces should also be considered as potentially providing suitable habitat to western tiger salamanders. Western tiger salamanders in Saskatchewan have even been observed in holes made by black-tailed prairie dog (*Cynomys ludovicianus* Ord; Parks Canada, personal communication).

Future surveys for western tiger salamanders in Canada should be conducted to establish occupancy and abundance patterns in regions outside of the prairie/boreal transition zone in the prairie provinces, particularly the mixed-grass and short-grass prairie. Long-term datasets are crucial for understanding trends in highly variable amphibian populations (Pechmann et al. 1991), therefore multiple annual surveys are needed to determine real declines in the number of occupied sites or the number of individuals within sites. Accounting for imperfect detection did not significantly influence the interpretation of site variables in relation to salamander occurrence or abundance. This indicates that future surveys for

western tiger salamanders should allocate sampling effort towards encompassing more sites rather than repeat visits to fewer sites within a season. Any future surveys for western tiger salamanders and other ambystomatid salamanders should use aquatic drift fences when trapping. Aquatic traps along drift fences were much more effective than free-floating minnow traps, with 94% of salamanders in 2013 captured along the drift fence. Finally we propose that our approach of establishing an *a priori* survey design, based on available local or region information, could prove to be an efficient way of assessing and predicting the distribution and relative abundance of other ambystomatid species and other species of cryptic, pond-breeding salamanders throughout North America.

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Chapter 3

**IS THERE AN ECOLOGICAL BARRIER
BETWEEN THE TWO SPECIES
(*STRAUCHII* AND *CROCATUS*) OF
ALLOPATRIC SPOTTED NEWTS OF THE
GENUS *NEURERGUS* IN TURKEY?
AN OVERVIEW OF THE EFFECT OF THE
GLACIER MOUNTAINS OF HAKKARI**

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ABSTRACT

Neurergus strauchii is an endemic species, narrowly distributed in eastern Anatolia, with an elevation range from 1000 m to 2100 m asl.

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Neurergus crocatus inhabits both southeastern Anatolia and northwestern Iran, and prefers lower altitudes than *N. strauchii*. *N. strauchii* and *N. crocatus* are allopatric salamander species. Although many phylogenetic studies have been carried out on these species, niche conservatism, which has gained wide attention in recent literature, has not been examined in any studies. I address here whether any ecological barriers exist in the distribution range of the two newt species *N. strauchii* and *N. crocatus*. According to the findings of this study, an identity test was found to be significantly different from the null distribution, whereas a background test was not significantly different from the null distribution. In addition, both linear and blob range-break tests showed that the environmental divergence between *N. strauchii* and *N. crocatus* was not significantly different from the null distribution. These tests can generally be interpreted only in one way: there is no niche conservatism between *N. strauchii* and *N. crocatus*. These allopatric species have similar niche characteristics in the geographic areas they occupy. Therefore, these two species have been separated by non-climatic geographic barriers. Thus, the glacier mountains of Hakkari appear to be an important barrier resulting in the speciation of *N. strauchii* and *N. crocatus*.

Keywords: Anatolia, speciation, salamander, niche, ecological niche modelling

INTRODUCTION

Environmental conditions (i.e., barriers) that keep organisms from dispersing have been extensively affecting the organization of biodiversity on Earth. Barriers lead to diversification of both animals and plants, and tend to limit the distribution of populations or species (Holt et al., 2013). Moreover, barriers cause numerous important ecological processes such as speciation (Caplat et al., 2016). Turkey has four mountain belts including the Northern Anatolian Mountains, Western Anatolian Mountains, Toros (Taurus) mountain ranges in the south, and Anatolian diagonal range spanning from the northeastern corner of the Mediterranean Sea to the southeastern corner of the Black Sea. The highest mountain peaks in Turkey are Ağrı (5137 m) and Cilo and Süphan mountains, all of which are higher than 4000 m (see Sekercioğlu et al., 2011 for details). Therefore,

due to the high mountain barriers (Kosswig, 1955), Turkey is one such country that has high species richness in the Palearctic region (Ambarlı et al., 2016). The varied topography and microclimates created throughout the many altitudinal gradients have resulted in high diversity among the plants (Atalay, 2006) and vertebrates (Sekercioğlu et al., 2011). The mountains often present refugia to organisms, particularly amphibians. Amphibians are ideal model organisms for the reconstruction of colonization (Gonzalez et al., 2014). Mountain ridges, roads, and open or dry habitats reduce gene flow between populations or species (Beebee, 2013; Crawford et al., 2007; Gül, 2013).

The genus *Neurergus* includes four mountain brook newt species including *N. strauchii*, *N. crocatus*, *N. derjugini*, and *N. kaiseri* (AmphibiaWeb, 2019; Barabanov and Litvinchuk, 2015; Hendrix et al., 2014; Olgun et al., 2016; Özdemir et al., 2009; Schneider and Schneider, 2010). The geographic distribution of this genus ranges from the Turkey–Iraq and Iraq–Iran borders to the southern Zagros Mountains of Iran (Steinfartz et al., 2002). Turkey is home of two species of this genus both of which are globally vulnerable (IUCN, 2019): *N. strauchii* inhabits a small geographic area encompassing the area from Kökpınar in the province of Malatya in the west to Aşağınarlıca in the province of Van in the east. *N. crocatus* has a larger distribution range extending from the south and west of the Lake Van up to the south of Hazar Lake (Pasmans et al., 2006; Olgun et al., 2015). In addition, *N. strauchii* represents three subspecies, namely, *N. s. strauchii*, *N. s. barani*, and *N. s. munzurensis* (Olgun et al., 2016). In their recent research, Rancilhac et al. (2019) suggested that *N. s. strauchii* and *N. s. barani* should be considered distinct species; however, they did not include the subspecies *N. s. munzurensis*, first described by Olgun et al. (2016). Thus, the systematics of the genus *Neurergus* in Turkey is still unclear. Therefore, it was evaluated *N. strauchii* as a single species in this study for investigating whether there exists a geographic barrier between the two species *N. strauchii* and *N. crocatus*. I used niche identity and background similarity tests to assess whether ecological niche modeling generated from *N. strauchii* and *N. crocatus* are identical or not much similar than expected. In addition, I

used linear and blob range-breaking tests to determine whether the biogeographic boundaries of glacier mountains pose to be a significant environmental barrier.

MATERIAL AND METHODS

Species Occurrence Data

Barabanov and Litvinchuk (2015) studied model-predicted potential geographic distributions for *Neurergus* species and actual species occurrence data for *N. strauchii* and *N. crocatus* in detail. According to this as well as several other studies, there are 43 known locality records for *N. strauchii* (Baran and Öz, 1986; Bogaerts et al., 2006; Hendrix et al., 2014; Koyun et al., 2013; Lanza et al., 2010; Olgun et al., 2015, 2016; Öz, 1994; Özdemir et al., 2009; Pasmans et al., 2006; Ragghianti et al., 1987; Schmidtler, 1994; Schmidtler and Schmidtler, 1970; Schneider and Schneider, 2010; Steindacher, 1887) and 24 for *N. crocatus* (Al-Adhami and Hameed, 1988; Al-Sheikhly et al., 2013; Baran and Öz, 1986; Biricik, 2009; Gorgees et al., 1977; Nader, 1969; Najafi-Majd and Kaya, 2010, 2013; Schneider and Schneider, 2010, 2011; Uğurtaş et al., 2015; Villwock, 1961). All analyses related to species occurrence data were handled using Wallace v1.0.6 (Kass et al., 2018), which is a flexible application written in R v3.5.2 (R Core Development Team) for reproducible ecological niche modeling. Thus, as most of the species occurrence data indicated duplicate records, they were removed from the dataset. To refrain from spatial sampling biases, a spatial thinning method was implemented using the “spThin” (Aiello-Lammens et al., 2015) library under Wallace v1.0.6 using the R package. After duplicates were deleted from the data and they were spatially thinned, a total of 23 and 9 records for *N. strauchii* and *N. crocatus* remained, respectively, and were used in this study (Figure 1).

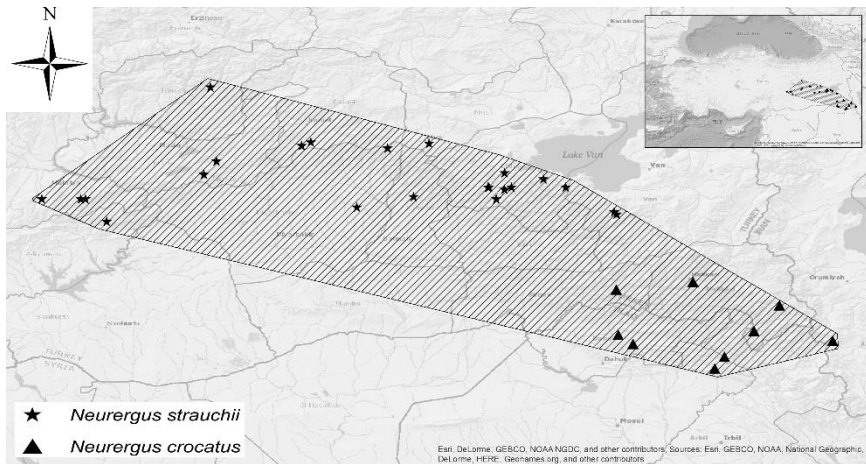


Figure 1. The geographic distribution patterns of *N. strauchii* and *N. crocatus* within minimum convex polygon based on literature data.

Environmental Variables and Ecological Niche Modeling

All bioclim layers were extracted from the WorldClim dataset at a resolution of 30 arc-s (~ 1 km) (Hijmans et al., 2005). According to Barabanov and Litvinchuk (2015), bioclim variables Bio2 (mean diurnal range; °C), Bio3 (isothermality), Bio4 (temperature seasonality), Bio5 (maximal temperature of the warmest month; °C), Bio8 (mean temperature of the wettest quarter; °C), Bio14 (precipitation of the driest month; mm), Bio15 (precipitation seasonality; CV), and Bio19 (precipitation of the coldest quarter; mm) are important based on known preferences of *Neurergus* species, and were selected under Wallace v1.0.6 in R using the package “raster” (Hijmans et al., 2019). To process environmental variables, the minimum convex polygon was chosen as the background extent, and the study region buffer distance was set at 0.05 degree. Then, predictor rasters by background extent and sample background points were masked as 10.000 background points. All these processes were performed under Wallace v1.0.6 in R package using the “sp” tool (Pebesma et al., 2018) and “rgeos” (Bivand et al., 2018).

Table 1. Performance of 48 models created during the evaluation process for *N. strauchii* and *N. crocatus*. Grey backgrounds show settings using for the models.

Settings		<i>Neurergus strauchii</i>			<i>Neurergus crocatus</i>		
Feature classes	Reg. multipliers	MTP omission rates	10pct omission rates	AICc	MTP omission rates	10pct omission rates	AICc
L	0.5	0.083333333	0.291666667	499.1606501	0.25	0.5	178.0112108
LQ	0.5	0.083333333	0.341666667	506.9906075	0.25	0.5	177.9050043
H	0.5	0.408333333	0.616666667	NA	0.708333333	0.791666667	NA
LQH	0.5	0.366666667	0.616666667	NA	0.708333333	0.791666667	NA
LQHP	0.5	0.408333333	0.616666667	NA	0.708333333	0.791666667	NA
L	1	0.083333333	0.25	497.5907513	0.25	0.5	178.7558537
LQ	1	0.083333333	0.333333333	504.5450522	0.25	0.5	178.4773745
H	1	0.166666667	0.658333333	NA	0.125	0.5	200.6133533
LQH	1	0.125	0.608333333	NA	0.125	0.5	200.6121001
LQHP	1	0.166666667	0.658333333	NA	0.125	0.5	200.6133533
L	1.5	0.083333333	0.25	492.8980267	0.125	0.125	173.8031593
LQ	1.5	0.083333333	0.291666667	498.8166262	0.125	0.25	175.6193521
H	1.5	0.083333333	0.583333333	NA	0	0.25	182.3881043
LQH	1.5	0.083333333	0.416666667	NA	0	0.25	182.3881043
LQHP	1.5	0.083333333	0.583333333	NA	0	0.25	182.3881043
L	2	0.041666667	0.25	493.5870025	0.125	0.125	173.8031593
LQ	2	0.083333333	0.25	497.2281827	0	0.125	175.92609
H	2	0.083333333	0.291666667	601.8190687	0	0.25	183.4218374
LQH	2	0.083333333	0.333333333	576.552775	0	0.25	183.4218374
LQHP	2	0.083333333	0.291666667	686.1516977	0	0.25	183.4218374
L	2.5	0.041666667	0.25	494.5200124	0	0	173.8031593
LQ	2.5	0.041666667	0.25	495.5245211	0	0	176.2646573
H	2.5	0.083333333	0.291666667	537.5030376	0	0	175.9357873

Settings		<i>Neurergus strauchii</i>			<i>Neurergus crocatus</i>		
Feature classes	Reg. multipliers	MTP omission rates	10pct omission rates	AICc	MTP omission rates	10pct omission rates	AICc
LQH	2.5	0.083333333	0.333333333	514.677016	0	0	175.9357873
LQHP	2.5	0.083333333	0.291666667	548.7446898	0	0	175.9357873
L	3	0.041666667	0.25	493.1038545	0	0	173.8031593
LQ	3	0.041666667	0.25	496.3072743	0	0	173.8031593
H	3	0.083333333	0.291666667	525.2403913	0	0	173.8031593
LQH	3	0.083333333	0.333333333	508.6105599	0	0	173.8031593
LQHP	3	0.083333333	0.291666667	525.2685254	0	0	173.8031593
L	3.5	0.041666667	0.25	493.6125771	0	0	173.8031593
LQ	3.5	0.041666667	0.25	500.2332757	0	0	173.8031593
H	3.5	0.083333333	0.291666667	511.4306884	0	0	173.8031593
LQH	3.5	0.041666667	0.25	502.6944156	0	0	173.8031593
LQHP	3.5	0.083333333	0.291666667	511.4306884	0	0	173.8031593
L	4	0.041666667	0.25	494.2132427	0	0	173.8031593
LQ	4	0.041666667	0.25	498.1916215	0	0	173.8031593
H	4	0.083333333	0.25	513.7213788	0	0	173.8031593
LQH	4	0.041666667	0.25	504.2924293	0	0	173.8031593
LQHP	4	0.041666667	0.25	509.4897586	0	0	173.8031593

The R package “ENMeval” (Muscarella et al., 2014) under Wallace v1.0.6 in R was used to evaluate all Maxent modules with k -fold cross-validation, partitioning into four bins by block method and assessing all combinations of models containing up to five feature classes (L, LQ, H, LQH, and LQHP), and regularization multipliers from 0.5 to 4 by step 0.5 (Merow et al., 2013). All ecological niche models were constructed using Maxent (Phillips et al., 2006) function in the ‘dismo’ package (Hijmans et al., 2017) under Wallace v1.0.6 in R. The models with the lowest AICc for *N. strauchii* and *N. crocatus* were selected as optimal among candidate models. However, as the number of parameters is higher than the number of species occurrence data of *N. strauchii* and *N. crocatus*, errors occurred while calculating some of the AICc scores. Therefore, the lowest AICc score with a correct value was selected as the best model (Table 1).

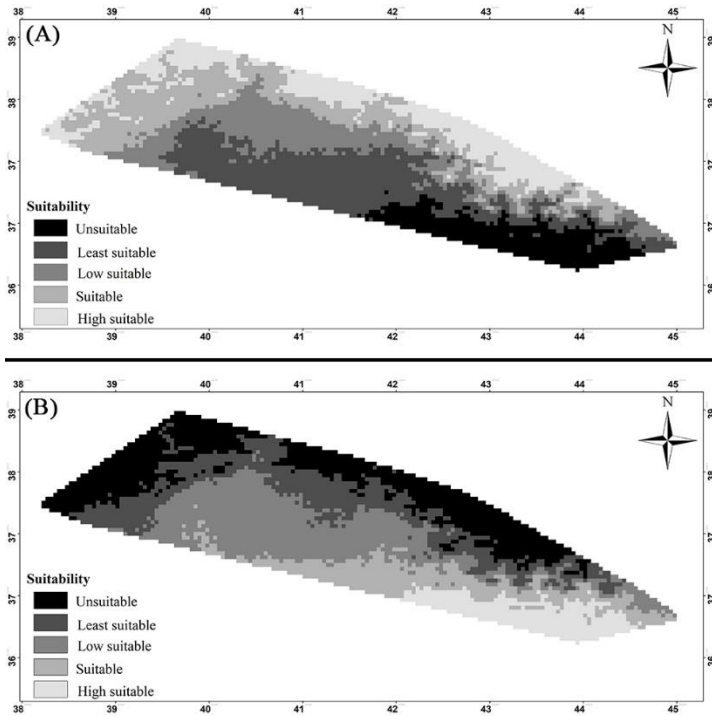


Figure 2. Predicted distribution patterns within minimum convex polygon for *N. strauchii* (A) and *N. crocatus* (B).

I used the R package “ENMTools” to assess the hypothesis whether the best-fit ENMs for *N. strauchii* and *N. crocatus* were quantitatively different from each other, and hence likely to represent differences in niche use between the two species (Warren et al., 2010). First, I used an identity test to compare whether the empirical values measuring the niche overlap for the two species significantly differed from the expected distribution (Warren et al., 2014). Thereafter, I conducted an asymmetrical background “similarity test” to determine whether the overlap in habitat use for both species was primarily due to the overlap in the available habitat (Warren et al., 2008). Finally, I ran a linear range-break test to determine whether the climates of the geographic areas occupied by *N. strauchii* and *N. crocatus* were significantly different from each other (Glor and Warren, 2011). For each test, two empirical measures of niche similarities, Schoener’s *D* and Hellinger distance (*I*), were compared. Further, 100 replicate analyses were run for each test to estimate the probabilities associated with the null hypothesis of niche similarity (Warren et al., 2008).

RESULTS

Ecological Niche Models

On the basis of the 10th percentile training presence logistic threshold, ecological niche modeling was separately conducted for both *N. strauchii* and *N. crocatus* with high AUC values (0.77 ± 0.165 for *N. strauchii* and 0.82 ± 0.163 for *N. crocatus*). According to the modeling of *N. strauchii*, the most suitable habitats emerged within almost the entire study area; the southeastern most portion of the study area was not suitable in terms of bioclimatic conditions (Figure 2A). On the contrary, suitable habitats for *N. crocatus* appeared in the southeast part of the study area, whereas the northern part was found to be unsuitable (Figure 2B). For both species, the variable with the highest percent contribution was precipitation seasonality (Bio15).

Identity and Background Tests between *N. strauchii* and *N. crocatus*

The null hypothesis of niche identity between *N. strauchii* and *N. crocatus* was rejected because empirical values for Schoener's D (0.504, $P < 0.01$) and Hellinger's-based I test statistics (0.806, $P < 0.01$) were significantly different from the null distribution for the identity test. These results indicate that the distributions of the two species are not random with respect to the available bioclimate characteristics (Figure 3A-B). In contrast, to the asymmetrical background test, empirical values for Schoener's D (0.49, $P > 0.05$) and I test statistics (0.79, $P > 0.05$) were not significantly different from those of the null distribution (Figure 3C-D). According to these results, there is no evidence for niche conservatism between *N. strauchii* and *N. crocatus*, because the difference between their climatic niches is similar in magnitude to the climatic differences between the geographic regions they are found in.

Range-Break Test between *N. strauchii* and *N. crocatus*

In a linear range-break test, empirical values for Schoener's D (0.506, $P > 0.05$) and Hellinger's-based I test statistics (0.808, $P > 0.05$) were not significantly different from the null distribution, indicating that the bioclimatic characteristics do not differ significantly between these two geographical areas. Therefore, I reject the hypothesis that a particularly abrupt environmental transition occurs across the distribution of the two species being studied (Figure 4A-B). In the blob range-break test, empirical values for Schoener's D (0.503, $P > 0.05$) and Hellinger's-based I test statistics (0.805, $P > 0.05$) also indicated that there was no significant difference in the bioclimatic conditions between the areas occupied by *N. strauchii* and *N. crocatus* (Figure 4C-D).

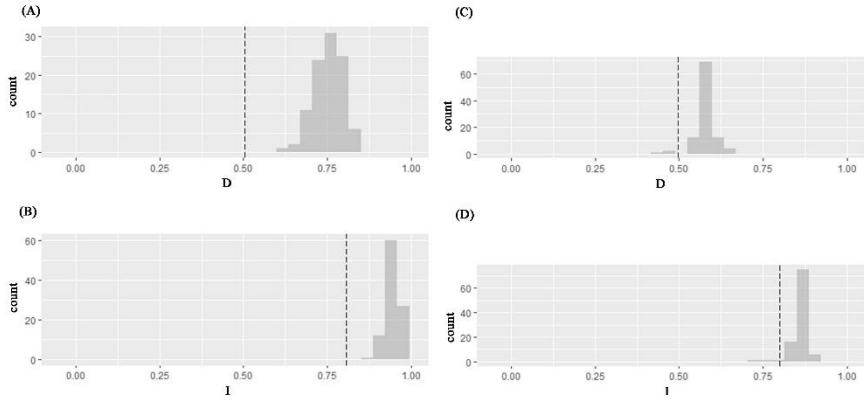


Figure 3. Results of an identity test (A and B) and asymmetric background similarity test (C and D) for *N. strauchii* vs. *N. crocatus*. The dashed line indicates the empirical measure for each test statistic and the grey curve shows the null distribution.

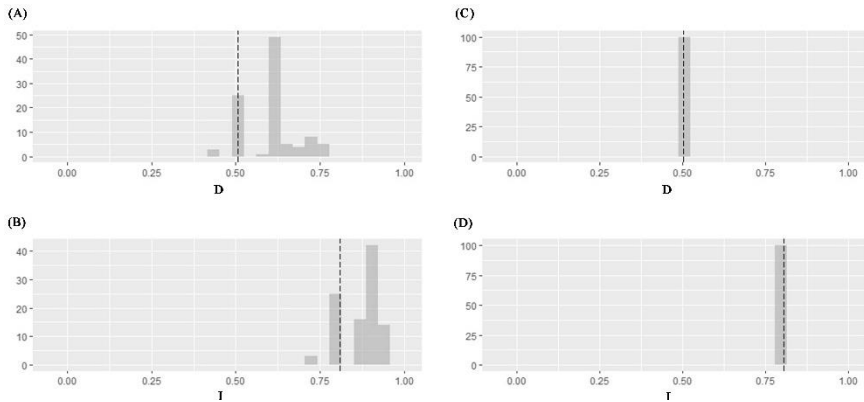


Figure 4. Linear (A and B) and blob (C and D) range-break test results for *N. strauchii* vs. *N. crocatus*. The dashed line represents the empirical measure for each statistic and the grey curve shows the null distribution.

DISCUSSION

Climatic niche helps determine how species are distributed geographically, and it has important consequences for speciation (Jezkova and Wiens, 2018) because the climatic niche strongly affects the area

where a species occurs. Therefore, the climatic niche may drive either the formation of new and distinct species or the termination of a species due to climate change (Castro-Insua et al., 2018). Allopatric speciation, which generally occurs through the formation of geographic barriers such as the movement of continents, and the formation of mountains, islands, water bodies, or glaciers, is widely known to be the most common geographic mode of speciation (Wiens, 2004; Harrison, 2012). Niche conservatism refers to the tendency of closely related species to maintain similar niches over a long period of time, and this term is generally associated with allopatric speciation (Wiens and Graham, 2005). In this study, I investigated whether niche conservatism existed between *N. strauchii* and *N. crocatus*. According to my results, the comparisons of both *N. strauchii* and *N. crocatus* revealed that although their niches are not much different from that expected (Figure 3C-D), they are neither equivalent (Figure 3A-B). Moreover, the allopatric range boundaries of *N. strauchii* and *N. crocatus* were not associated with environmental gradients in both the linear and blob range-breaking tests (Figure 4). Therefore, the fragmentation between these two species did not occur across an abrupt environmental gradient along glacier mountains of Hakkari. My results support a suggestion put forward by Wiens and Graham (2005). The glacier mountains that separate allopatric sister species is within their climatic niche envelope; therefore, I can reject the pattern that niche conservatism may or may not create (Wiens, 2004; Wiens and Graham, 2005).

What could then explain the biogeographic pattern of these two species? Vicariance is the formation of some kind of barrier such as mountain ridges or an ocean basin or a land barrier occurring as a result of various historical events (Li et al., 2009). The geographic patterns of this study area indicate that vicariant events due to geographical barriers are likely to have led to the formation of these species. The separation of *N. strauchii* and *N. crocatus* based on mitochondrial genes is estimated to be 8.5–13.9 mya (Steinfartz et al., 2002). Probably, this separation between *N. strauchii* and *N. crocatus* started with the formation of the glacier mountains up to the Iranian border, the northwestern Zagros Mountains, 9–

10 mya (Steinfartz et al., 2002), because the paleogeography of Anatolia began to form in the Early Miocene (20.5–19 mya; Popov et al., 2004). In that period, the northern, eastern, and southern regions of Anatolia were surrounded by shallow seas (Akgün et al., 2007). During the mid-middle-Miocene (14–13 mya) period, the northwest of Iran and southeast of Anatolia have become close to each other, and in the late-middle-Miocene (12–11 mya) period, the Sarmatian Sea between these lands closed (Popov et al., 2004). Thus, these tectonic events gave rise to the Taurus mountain range of Anatolia (Çıplak, 2003, 2004). The southeastern part of the Taurus Mountains is the most important recently glaciated region in Turkey, especially Cilo glacier mountains, which has a high mountain peak (4135 m; Çiner, 2004). It is likely that these mountain ridges between *N. strauchii* and *N. crocatus* resulted in a barrier effect because *N. crocatus* prefers a lower altitude than *N. strauchii* according to the species occurrence data (Figures 1–2). A similar pattern in the case of the lizard genus *Apathya* further revealed the importance of the Taurus mountains in the southeastern Anatolia. In this pattern, the lineages of *Apathya* between the Anatolian and Iranian groups diverged into two clusters around 13.66 mya during the middle-Miocene, and a vicariant event creating an east and west sea barrier between the southeastern Anatolia and Iran led to the division of the genus *Apathya* in this area (Kaplı et al., 2013). This vicariant event not only affected the speciation of animals in this region, but also the diversification in the plant genus *Grammosciadium* began in approximately the Miocene (7.5 mya) period, and resultantly *G. cornutum* and *G. scabridium* are restricted to the areas along the glacier mountains of Hakkari (Koch et al., 2017).

Consequently, the southeastern Taurus mountains including the glacier mountains of Hakkari found in the Irano-Anatolian Biodiversity Hotspot are home to many endemic plant and animal species (Noroozi et al., 2018). The genus *Neurergus* includes the most well-known amphibian species in this hotspot, and it seems that the formation of high mountains, which accelerated during the middle-to-late Miocene (15–5 mya) period, resulted in allopatric speciation between *N. strauchii* and *N. crocatus*. Given this

observation, it is likely that many other species such as those of the genus *Neurergus* may have been isolated similarly by these high mountains.

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Chapter 4

PHOTORECEPTORS OF THE SALAMANDER

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ABSTRACT

Rod and cone photoreceptor cells capture photons of light, transforming light signals into neural signals. Many of the key insights into the structure and function of rod and cone photoreceptors were first obtained by studying retinas of salamanders, especially *Ambystoma tigrinum*. This chapter summarizes the processes by which light is converted into neural signals by rods and cones and then transmitted to downstream neurons in the retina, with a focus on salamander photoreceptors. We also discuss the different types of photoreceptor cells and other light-sensitive cells in salamanders and their role in vision.

Keywords: retina, rod, cone, photoreceptor, vision, neurotransmission

1. INTRODUCTION

Since the turn of the twentieth century, researchers have used various species of salamander (order Urodela) to study the retina, the neuronal tissue that converts light stimuli to neuronal signals. The retina consists of three fundamental layers: 1) the outermost layer, which contains the cell bodies of photoreceptors, 2) a middle layer, which contains the cell bodies of bipolar cells (BCs) and other interneurons, and 3) the innermost layer, which contains retinal ganglion cell (RGCs). Light is focused onto the retina by the cornea and lens. Light entering the retina passes through the various retinal layers before it reaches the photoreceptor cells that lie at the back of the retina. Photoreceptor cells absorb photons, transforming them into changes in transmembrane voltage. Light-evoked voltage changes spread to the synaptic terminals of photoreceptors where they are transmitted to BCs by changes in release of the neurotransmitter glutamate. Signals in BCs are then transmitted at their synapses to RGCs. RGC axons form the optic nerve, which carries light-driven neuronal signals to the brain. Elucidating the properties of synaptic signaling between these cell types and others (e.g., horizontal and amacrine cells) within the retina has been the subject of extensive investigation. This chapter focuses on the physiology of salamander rod and cone photoreceptors, exploring the key role that salamanders have played in studying photoreceptor function.

Perhaps no organism other than the mouse has been more integral to our understanding of photoreceptor neurotransmission than the salamander. The most commonly used salamander species in retinal research have been the mudpuppy (*Necturus* species) and tiger salamander (*Ambystoma tigrinum*). Axolotl (*Ambystoma mexicanum*), various newt species, and *Salamandra* species have also been studied. One advantage of using these animals is that they are cold-blooded, so their retinas are easier to keep alive *in vitro* than those of warm-blooded animals. Retinas of salamanders and other amphibians also have larger retinal neurons than mammals, which facilitated early anatomical studies [1, 2] and allowed some of the first descriptions of photoreceptor light-driven responses using single-cell electrophysiology techniques [3-7]. Indeed, rods in the mudpuppy are

among the largest—if not the very largest—rods known to science [8]. One of the earliest anatomical studies using electron microscopy to examine photoreceptor ultrastructure was also performed in mudpuppy [9].

While studies on photoreceptors from other species have also contributed, the salamander retina has proven especially useful in the study of photoreceptor biology. While the following sections focus on salamander, given the remarkably conserved evolution of the vertebrate retina [10], most characteristics hold true for other vertebrates as well.

2. ROD AND CONE LIGHT RESPONSES

Vertebrate retinas contain two major types of photoreceptor cells: rods and cones (Figure 1). While both rods and cones convert light to neuronal signals, they are specialized to function best at different light levels. Rods are exquisitely sensitive at low (scotopic) light levels but their signaling saturates at higher light levels. Cones are less sensitive than rods and incapable of signaling in dim light but instead provide visual signals at bright (photopic) light levels at which rods are saturated.

Both the anatomy and physiology of photoreceptors are specialized for efficient phototransduction, the conversion of light into neuronal signals. Rods have outer segments containing stacks of membrane-enclosed organelles known as discs. The major protein in the disc membrane is the light-sensitive protein rhodopsin, an isoform of opsin. Cones exhibit a similar structure as rods, but the cone discs are not fully enclosed within the plasma membrane, instead forming a series of invaginating membrane folds. Like rod discs, cone discs are packed with light-sensitive opsin proteins. Passage of a photon through this dense stack of discs increases the likelihood that it will be absorbed by a light-sensitive opsin [11].

Salamander rods are much larger than their cones (Figure 1). The volume of a salamander rod is approximately $2000 \mu\text{m}^3$ [12, 13] while a single cone is only approximately $70 \mu\text{m}^3$ [13, 14]. Most of this is due to the larger size of rod outer segments [11, 15]. In both rods and cones, the outer segment is connected to the inner segment by a narrow connecting

cilium. In salamander photoreceptors, the photoreceptor inner segment and nucleus-containing cell body (soma) are continuous with one another (Figure 1). The ellipsoid region of the inner segment just beneath the outer segment is densely packed with mitochondria to supply the massive energy needs of the cell. In rods, a short axon connects one and occasionally two or three synaptic terminals to the cell body, while cone synapses are located in a compartment at the base of the cell body (Figure 1). As we discuss later, the only type of ion channel in the outer segment is the cyclic nucleotide-gated (CNG) channel involved in phototransduction, but the inner segment, soma, and synaptic terminal possess a variety of other ion channels that modify light-evoked changes in membrane voltage (V_m) generated by phototransduction in the outer segment [16].

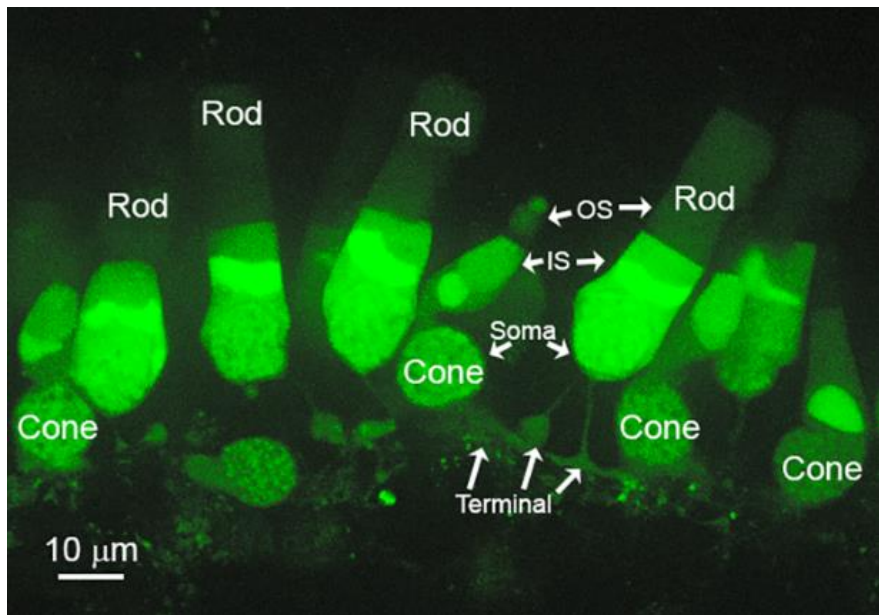


Figure 1. Rod and cone photoreceptors of the salamander retina. Outer segments (OS) contain the phototransduction apparatus and are much larger in rods than cones of salamander retina. Inner segments (IS) have a dense cluster of mitochondria in the ellipsoid region just beneath the outer segment. The soma is continuous with the inner segment and contains the nucleus. In cones, the synaptic terminal is also continuous with the soma, whereas rod terminals are connected to the soma by a thin axon. Some rods, such as the one denoted by the rightmost two arrows, have two terminals. Rods and cones were loaded with the Ca^{2+} -sensitive dye Fluo4.

2.1. Phototransduction

Absorption of photons by light-sensitive opsin photopigment molecules initiates a cellular signaling cascade that ultimately produces a light-evoked change in trans-membrane voltage. Opsins comprise 85% of the outer segment protein content. Localized to outer segment disc membranes, opsins are membrane-bound G-protein coupled receptors (GPCRs) that are covalently bound to a photon-absorbing chromophore cofactor. In aquatic phase salamanders, this light-sensitive chromophore is principally 3-dehydroretinal (vitamin A2-aldehyde). During metamorphosis to the land phase, axolotl and tiger salamander rods begin to use retinal (vitamin A1-aldehyde) [17], the same chromophore used by mammals (including humans). Shifting from the A2 to A1 chromophore shifts the absorbance spectra to shorter wavelength. This adaptation appears well-suited to the shift in spectral environments from muddy or tannin-stained water that absorbs longer wavelengths to air.

Rods and different cone subtypes possess different opsin proteins. Structural differences among opsins imparted by differences in only a handful of amino acids alter the propensity of the chromophore to absorb photons of a particular wavelength. In most animals, a photoreceptor expresses only a single opsin isoform and, because different opsins vary in their spectral sensitivity, each type of photoreceptor cell has a “preferred” color to which it responds most efficiently [18]. As we shall see, certain salamander cones violate this general rule by expressing multiple opsins.

Many details of the phototransduction process were discovered in amphibian rods. The transduction cascade is similar in rods and cones, with key differences being the identity of the opsin and some of the transduction protein isoforms [19]. The opsin in rods is known as rhodopsin because of its reddish color in its unbleached, light-sensitive state. When the chromophore (retinal or 3-dehydroretinal) absorbs energy from a photon, it isomerizes from its 11-*cis* configuration to an all-*trans* configuration. The GPCR activity of rhodopsin bound to the *cis* form of the chromophore is negligible, but its isomerization to the all-*trans* form induces a conformational change in the opsin protein, forming a highly-

active configuration known as Metarhodopsin II. The active GPCR binds to and activates a G protein, transducin. Like all G proteins, transducin activity is dictated by the phosphorylation state of its bound guanine nucleotide. Transducin is in an inactive “off” state when bound to GDP, but in a highly active “on” state when bound to GTP; Metarhodopsin II activates transducin by catalyzing exchange of GDP for GTP. Salamander rods and cones, like those of mammals, contain different transducin isoforms that contribute to rod/cone differences in sensitivity and kinetics [20].

Active, GTP-bound transducin has a high affinity for phosphodiesterase 6 (PDE6), and when the two interact, PDE6 activity is stimulated to operate orders of magnitude above its basal rate [21]. PDE6 hydrolyzes cyclic GMP (cGMP) to 5'-GMP, precipitating closure of CNG cation channels in the outer segment cell membrane. As their name implies, CNG channels are opened by an elevation in the concentration of a cyclic nucleotide, cGMP. The cGMP level is determined by the balance between its formation by the enzyme guanylyl cyclase (GC) and its destruction to 5'-GMP by PDE6. In darkness, PDE6 activity is low and therefore cGMP levels are high. The high concentration of cGMP keeps CNG channels in an open and active state, allowing a constant influx of cations, principally Na^+ . This influx is balanced by an outward flux of K^+ ions through K^+ channels located in the inner segment [22], generating a current that circulates between the inner and outer segments in darkness (the “dark current”).

When CNG channels are active, the photoreceptor membrane is in a relatively depolarized state in darkness with a trans-membrane voltage of approximately -40 mV. Following photon absorption and activation of PDE6, the decline in cGMP leads to closure of cation channels in the outer segment, which in turn inhibits the dark current and results in membrane hyperpolarization. Figure 2 illustrates responses of a salamander rod (Figure 2A) and cone (Figure 2B) to increasingly bright flashes of light. Some of the first intracellular recordings showing that light causes rods and cones to hyperpolarize were performed in mudpuppy retina [3-6, 23, 24]. One of the first studies demonstrating the existence of the

circulating dark current and its inhibition by light was also carried out in the mudpuppy retina [7].

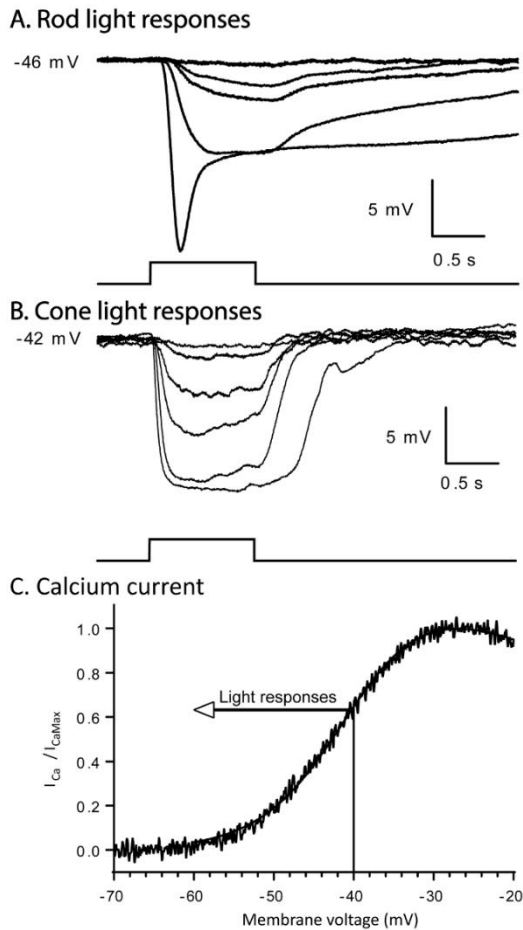


Figure 2. A.: Rod light responses evoked by a series of increasingly bright flashes (~ 0.5 log unit intensity steps). The brightest flash evoked a large hyperpolarizing response that activated I_h channels, which then depolarized the rod to make the peak response quite transient. The resting potential in darkness for this rod was -46 mV. B.: Cone responses were evoked in the presence of a dim blue background by a series of light flashes. C.: Cone calcium current (I_{Ca}) evoked by a ramp voltage protocol (0.5 mV/ms). At a typical resting potential of -40 mV, I_{Ca} is near its activation midpoint. When a cone hyperpolarizes to light, the reduction in I_{Ca} reduces glutamate release. Cones can hyperpolarize by up to 20 mV, with larger responses causing progressively smaller reductions in I_{Ca} . Rod I_{Ca} shows similar features to cone I_{Ca} .

Other studies in amphibians using salamander, frog and toad retina were also crucial in understanding molecular details of the dark current and its inhibition [25]. Amplification by the phototransduction cascade is remarkable, allowing rods of salamander and other species to signal the absorption of only a single photon [26]. Virtually every step in the process is amplified stoichiometrically or biochemically. For example, in frog rods, a single Metarhodopsin II molecule activates ~60 transducin molecules and a single activated PDE6 enzyme hydrolyzes many molecules of cGMP. Taken together, roughly 72,000 cGMP molecules are hydrolyzed when a photon activates a single rhodopsin molecule in amphibian rods [26]. By contrast, the amplification in mouse rods is much weaker, with activation of only about a dozen transducin molecules and hydrolysis of only ~2,000 cGMP molecules [26, 27].

2.2. Shutoff of Phototransduction

The phototransduction cascade triggered by photon absorption must be turned off and this termination process is tightly regulated. A short time after activation, Metarhodopsin II is phosphorylated by rhodopsin kinase. Phosphorylated rhodopsin binds the inhibitory protein arrestin, which blocks the ability of rhodopsin to activate transducin. Transducin must also be inactivated to halt the cascade. Inactivation of transducin by hydrolysis of its bound GTP to GDP is the rate-limiting step in the phototransduction shutoff process [28]. Once this hydrolysis occurs, PDE6 activity drops precipitously, cGMP levels rise, and CNG channels can reopen.

2.3. Visual Pigment Regeneration

Light converts 11-*cis*-retinal (or 11-*cis*-3-dehydroretinal) to an all-*trans* configuration to activate opsin. To restore light-sensitivity, the chromophore must be converted from its all-*trans* form back to its *cis*

form. The first step in this process is that the all-*trans* bleached chromophore must dissociate from the opsin protein [29]. This frees up the opsin molecule to bind a new molecule of 11-*cis*-retinal, thereby regaining its ability to be activated by a photon. The conversion of all-*trans*-retinal to its *cis* form involves a regenerative process known as the retinoid cycle. In the vertebrate retina, rods accomplish this by exporting “spent” chromophore from the rod outer segment to adjacent retinal pigment epithelium (RPE) cells where all-*trans*-retinal is converted to 11-*cis*-retinal through an enzymatic cascade, and the *cis* form is then transported back to rods. As demonstrated originally in salamander retina, cones use a different method to regenerate pigment that involves transport into neighboring Müller glia cells [29, 30].

2.4. Light Adaptation

Because the cessation of CNG channel conductance is the means by which light is translated into voltage, it is advantageous for photoreceptors to be able to expand the range of light intensities over which CNG channels remain open [31]. Both rods and cones are capable of adjusting the magnitude of their responses as the mean level of illumination is varied, a process known as adaptation, but cones do so much more efficiently. Rod signals are easily saturated by ambient light and so even modest background illumination renders rods incapable of signaling further luminance increases. By contrast, efficient adaptation allows cones to operate over a vast range of ambient luminance and they almost never saturate [32, 33]. Light adaptation accelerates the decay of photoreceptor light responses, producing a shorter photon integration time and reducing sensitivity. However, accelerated responses also permit detection of contrast and motion with higher temporal precision in brighter conditions [34].

Early studies in tiger salamander established an essential role for Ca^{2+} as an internal messenger regulating adaptation in rods and cones [14, 35-37]. The closure of CNG channels during phototransduction

reduces the influx of Ca^{2+} but extrusion of Ca^{2+} from the outer segment by $\text{Na}^+/\text{Ca}^{2+}$ exchangers continues unimpeded, causing a decline in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The decline in $[\text{Ca}^{2+}]_i$ activates guanylate cyclase, stimulating the production of cGMP to re-open CNG channels. The decline in $[\text{Ca}^{2+}]_i$ also slows the hydrolysis of cGMP by PDE6. Together, these effects cause a reduction in response sensitivity because more phototransduction must occur to produce an equivalent amount of CNG channel closure [31]. The combination of reduced PDE6 activity and increased guanylate cyclase activity by decreased $[\text{Ca}^{2+}]_i$ are the chief mechanisms of photoreceptor adaptation in salamander [38, 39] as well as other species [31].

2.5. Transformation of Light Responses by Ion Channels in the Inner Segment and Terminal

As described above, the process of phototransduction converts light stimuli into changes in V_m . Whereas the outer segment has only CNG channels involved in phototransduction, there are various voltage- and calcium-dependent ion channels that modify light-evoked V_m changes once they reach the inner segment and synaptic terminal. Electrophysiological recordings from rods and cones of salamander retina have identified five types of ion channels in the inner segment and synaptic terminal [40-43]: 1) the hyperpolarization-activated current (I_h), an inward (depolarizing) cation current mediated by HCN1 channels in the inner segment [16, 44], minimal above -50 mV and maximal below -90 mV; 2) outward (hyperpolarizing) voltage-dependent K^+ currents (I_{Kx}), active at potentials above -60 mV; 3) L-type I_{Ca} , an inward current active above -50 mV, 4) outward Ca^{2+} -activated K^+ currents ($I_{\text{K}(\text{Ca})}$), and 5) inward Ca^{2+} -activated Cl^- currents ($I_{\text{Cl}(\text{Ca})}$) [16, 40, 45].

When photoreceptors are depolarized in darkness, I_{Kx} mediates the outward flux of K^+ ions that completes the dark current while I_h remains minimally active [22, 46]. When light closes CNG channels, the outward I_{Kx} hyperpolarizes the membrane. As the membrane hyperpolarizes I_h is

activated, which in turn tends to drive the V_m more positive. I_h therefore limits the amplitude of hyperpolarizing rod and cone light responses and makes the hyperpolarizing responses of rods to bright light more transient (Figure 2). By limiting the magnitude and duration of light-evoked hyperpolarization, I_h also acts as a high-pass filter, thereby improving the temporal resolution of photoreceptor signaling.

3. PHOTORECEPTOR SUBTYPES

3.1. Rod Subtypes

In tiger salamander retina, photoreceptors are comprised of approximately 60% rods and 40% cones [47, 48]. Most species have only a single type of rod, but anurans and many urodele amphibians have two rod subtypes: large “red” rods and small “green” rods that can be distinguished by their size, spectral sensitivities, and relative abundance [47, 49, 50]. Franz Boll identified red and green rods based on their colored appearance under the light microscope in the late 19th century [51]. Large red rods are the dominant subtype in salamander retina, comprising 98% of all rods, while small green rods represent only 2%. Large rods contain rhodopsin with a peak sensitivity at ~500 nm when using the A1 chromophore and ~525 nm when using the A2 chromophore [17]. Small “green” rods contain an opsin more sensitive to short (S) wavelength light with a peak sensitivity of 433 nm [52-55]. Small S rods are thought to be unique to tiger salamanders and tailless amphibians such as toads and frogs [8, 56], as newt species do not have S rods [57].

3.2. Cone Subtypes

Like most vertebrates, salamanders have multiple subtypes of cones with distinct spectral sensitivities. Salamander cones can also be distinguished morphologically as double cones possessing both a principal

and smaller accessory member, large single cones, and small single cones [47, 49]. Large single cones are the most abundant, comprising two-thirds of the cone population. The second most abundant are double cones that (counted as a single unit) make up 23% of the salamander cone population. A double cone is a single cell with two morphologically distinct cones fused together at the level of the inner portion of their inner segments, a region known as the myoid [8]. Most vertebrates excluding placental mammals have double cones [58]. Small single cones comprise the remaining ~10% of salamander cones [50].

Large single cones and double cones in tiger salamander retina are both maximally sensitive to long wavelength (610 nm) light [52, 59, 60]. Small single cones have multiple spectral subtypes that can be maximally sensitive to red (L), green (M), blue (S), or UV light [47, 61]. Salamander S cones and “green” rods express the same opsin [62]. Although photoreceptors are canonically understood to each express only a single opsin [18], studies of salamander cones show that salamander cones can express more than one opsin pigment [52, 60]. However, the spectral sensitivity of each cone is strongly dominated by the most highly-expressed pigment [60]. For example, UV cones of salamander contain three opsins, but expression of the UV-sensitive opsin is highest by two orders of magnitude [52]. Red-sensitive L cones in tiger salamander also possess low levels of S pigment [50, 60]. Other exceptions to the “one pigment rule” exist throughout the animal kingdom, including cones in mice that express both S and M opsins [63-65]. In addition to shifting from A2 to A1 chromophore during metamorphosis from the aquatic to the land-phase, S cones disappear from salamander retina while S rods increase in abundance [60, 66].

3.3. Dimensionality of Salamander Color Vision

While a given photoreceptor absorbs photons more efficiently at certain wavelengths, the activity of a single photoreceptor in isolation from its neighbors is effectively achromatic. The broad absorption spectra of

photopigment molecules means that photoreceptors are potentially capable of absorbing photons across the entire visual spectrum, although certain wavelengths are absorbed far more readily than others. Thus, a red-sensitive cone can respond identically to red and green light if the green light is bright enough for its opsin molecules to capture an equal number of photons. The output of a photoreceptor reports only its total quantal capture, not the intrinsic wavelength of the photons that it captures. This is known as the principle of univariance [67].

Because the activities of individual photoreceptors are achromatic, output from multiple photoreceptors of differing spectral sensitivities must be combined and compared to produce color perception. This comparison begins at the first synapse in the retina between cones and horizontal cells [68]. Horizontal cells (HCs) are second-order neurons that receive excitatory synaptic inputs from rods and cones, but also make inhibitory connections back onto rods and cones. HCs extend dendrites laterally over a wide area of retina to collect inputs from many photoreceptors. HCs are also strongly coupled to one another by gap junctions, allowing signals from one HC to travel to its neighbors. This further expands the collecting area, or receptive field, of the cell. Inhibitory feedback from HCs back to individual cones allows the average response from many cones over a wide region of retina to be subtracted from the response of an individual cone. By subtracting spatially averaged light levels from local changes in illumination measured by individual cones, inhibitory feedback from HCs to cones improves the detection of edges, spots and lines. In addition, HC spectral sensitivity is shaped by the inputs from multiple spectrally distinct photoreceptors and therefore differs from the narrower spectral sensitivity of an individual cone. Feedback inhibition from HCs to cones therefore also allows spectral comparisons between an individual cone and its surrounding background.

Feedback inhibition from HCs to cones operates by regulating the activity of cone Ca^{2+} channels [69]. As first shown by studies in salamander, HCs also provide feedback inhibition to rod Ca^{2+} channels by a similar mechanism [70]. When depolarized, as occurs in darkness, HCs inhibit I_{Ca} in presynaptic photoreceptors, thereby reducing their synaptic

output [71-74]. The mechanistic details of this inhibition remain incompletely resolved [75], but studies in newt, salamander, and other species have found that most of this feedback is due to horizontal cell-mediated pH changes in the synaptic cleft [70-73, 76, 77]. When HCs are depolarized, the synaptic cleft pH becomes more acidic. This in turn reduces photoreceptor I_{Ca} magnitude and shifts its voltage dependence in the positive direction, diminishing Ca^{2+} influx into the photoreceptor terminal. Grassmeyer and Thoreson [78] found that depolarization of a single HC reduced Ca^{2+} influx only at cone ribbon sites that were directly contacted by that particular HC. The insulation of HC-photoreceptor contacts within invaginations may help to isolate feedback to only the subset of presynaptic ribbons contacted by a given HC.

Predictions relating color dimensionality (e.g., dichromatic or trichromatic) to cone spectral classes were first confirmed in humans, where psychophysical and behavioral experiments can be more easily used to investigate color vision. Humans have three spectral classes of cones that are maximally sensitive to long, medium or short wavelengths, and the dimensionality of human vision is trichromatic: all colors that can be perceived by humans can be reconstituted by combining light of the primary colors red, green, and blue. The dimensionality of color vision may match, but not exceed, the number of spectral classes of photoreceptors, and dimensionality must be determined behaviorally [64, 79]. Such behavioral experiments are difficult to conduct in lower organisms. Nevertheless, many studies have shown that a wide variety of species perceive color and use color to direct behavior, though the exact dimensionality of color vision in most species is speculative at best [64].

Evidence suggests that various species of salamanders are capable of perceiving color [80-83], and studies of retinal ganglion cell responses to colored stimuli suggested that the tiger salamander retina adapts optimally to accentuate the natural chromatic properties of the natural salamander habitat [84]. Przyrembel and colleagues [83] used prey catching behavior in the fire salamander (*Salamandra salamandra*) to show the capability for trichromatic photopic vision, consistent with three spectral classes of cones in this land phase salamander. The land phase tiger salamander, which

loses S cones during metamorphosis, has UV, M, and L cones, suggesting it may also be capable of trichromatic vision. The aquatic phase tiger salamander has all four cone types, raising the possibility of tetrachromatic vision, but this has not yet been tested empirically .

In anuran amphibians (toads and frogs) that possess both S and M rods, researchers found that at light levels near absolute visual threshold where only rods operate, frog rods could relay color signals for phototaxis but not other behaviors [85]. This finding was consistent with the hypothesis first posed by Denton and Wyllie [56] that rod-mediated vision could be dichromatic in species that possess rods of multiple spectral subtypes. Rods also appear to influence phototaxis behavior in salamanders [86]. However, in contrast to frogs, toad rods did not appear capable of signaling color for any behavior [85], indicating that the presence of two spectrally distinct types of rods does not provide a sufficient basis to assume dichromatic vision at scotopic light levels.

Unlike the univariant responses of individual cones, certain types of second-order bipolar and horizontal cells in the retina can depolarize to one wavelength and hyperpolarize to another. The presence of such “color-opponent” responses allows cells to signal differences in the wavelength of light. Interactions between rods and cones that can potentially support color vision have been demonstrated in amphibian retina. Stimulation of rhodopsin-containing “red” rods evokes a depolarizing response whereas stimulation of long wavelength-sensitive cones evokes a hyperpolarizing response in color opponent bipolar cells and ganglion cells of *Xenopus* and color opponent horizontal cells of mudpuppies (*Necturus*) [87]. A second type of horizontal cell in mudpuppy revealed color opponent interactions only under light-adapted conditions, where they hyperpolarized in response to short wavelength stimulation of rods and depolarized to long wavelength stimulation of cones [88]. Blue/yellow opponent responses have also been recorded in retinal ganglion cells, thalamus and tectum from *Salamandra salamandra* [80, 81].

3.4. Photoreceptor Interactions

Salamander photoreceptors are strongly coupled to one another through connexin36 gap junctions [89]. Each rod is directly coupled to at least four neighboring rods [46] with an average coupling coefficient of 0.07 [89]. In other words, 7% of the light response evoked in one rod will also appear in its neighbor. By attenuating low frequency responses, electrical coupling contributes to band-pass filtering of rod responses. Rods also couple to cones in salamander retina, albeit weakly. A subpopulation of rods (~10%) show especially strong coupling to cones, but their coupling conductance is still only about half of that between rods. The strongest rod-cone coupling is between rods and large single cones [89]. Coupling between photoreceptors with differing spectral sensitivities can degrade the capability for color discrimination [90], but cone-cone coupling in salamander is quite weak, unlike mammals where rod-rod coupling is weak and cone-cone coupling is strong [59, 91]. Another difference with mammals is that coupling strength in salamanders does not appear to vary with circadian changes [89].

4. SYNAPTIC TRANSMISSION BY SALAMANDER RODS AND CONES

Because of their large size, salamander rods and cones have been a valuable model system for studying how light responses that originate in the outer segment are transmitted to second-order horizontal and bipolar cells in the retina. Membrane voltage changes that reach the synaptic terminal of rods and cones are transmitted to second-order bipolar and horizontal cells by altering the rate at which photoreceptors release the neurotransmitter L-glutamate. As in other excitatory neurons, release of glutamate occurs by the Ca^{2+} -dependent fusion of small, glutamate-filled synaptic vesicles with the plasma membrane and the associated release of vesicular contents into the synaptic cleft.

4.1. Regulation of Synaptic Release by Voltage-Gated Ca^{2+} Channels in Rods and Cones

Voltage-gated Ca^{2+} channels in photoreceptors were first characterized in salamander retina and shown to be L-type calcium channels, similar in many ways to Ca^{2+} channels in muscle cells. L-type channels are characterized by their long-lasting (i.e., sustained) activation and sensitivity to dihydropyridines [45, 92-95]. Photoreceptor I_{Ca} activates and deactivates quickly, allowing it to precisely track light-dependent voltage changes. I_{Ca} begins to activate above -50 mV and is maximally active near -20 mV (Figure 2C). The resting V_m of rods and cones in darkness of about -40 mV is close to the midpoint for the activation voltage of I_{Ca} in salamander cones, which averages -38 mV [78]. Because photoreceptors are generally incapable of depolarizing much beyond -40 mV, they do not use the entire dynamic range of I_{Ca} available to them to control release rate [96]. However, this arrangement means that in darkness the I_{Ca} voltage activation function is at its steepest and most linear, so I_{Ca} is optimally positioned to encode small increments in light (Figure 2C). Photoreceptors possess a particular subtype of L-type channels, $\text{Ca}_v1.4$, that exhibits limited Ca^{2+} -dependent and voltage-dependent inactivation [97]. This important property allows these channels to remain active and support exocytosis indefinitely in darkness.

Ca^{2+} ions that enter the terminal through $\text{Ca}_v1.4$ bind to Ca^{2+} sensor proteins on the vesicle surface to trigger vesicle fusion and the exocytosis of glutamate. Because I_{Ca} is activated by depolarization, vesicles are released continuously in darkness. Hyperpolarization by light leads to the closure of Ca^{2+} channels, slowing the rate of vesicle release. Changes in release rate track changes in I_{Ca} nearly linearly to accurately encode light-dependent changes in V_m . In this way, photoreceptor V_m is transformed to a synaptic signal that continuously fluctuates depending on the amount of light being absorbed by the cell at any moment.

Because I_{Ca} (and therefore the modulation of the rate of exocytosis) is inactive much below -50 mV, photoreceptors are incapable of signaling light stimuli if the cell becomes too strongly hyperpolarized. Salamander

rods have a slightly more negative resting V_m than cones, closer to -45 mV [98], so saturation occurs more easily in rods. As discussed above, the re-opening of CNG channels during light adaptation and the activation of I_h at hyperpolarized potentials both help prevent saturation.

Photoreceptors also possess mechanisms to prevent excessive depolarization. If sufficient numbers of voltage-gated Ca^{2+} channels are active in darkness, the resulting influx of Ca^{2+} will further depolarize the cell which can activate additional Ca^{2+} channels, leading to regenerative depolarization and produce action potentials similar to those observed in the heart. One mechanism that limits this is the activity of I_{Kx} , the K^+ current that is strongly activated by depolarization. Ca^{2+} influx also activates Cl^- ($I_{Cl(Ca)}$) and K^+ ($I_{K(Ca)}$) currents [45, 99]. $I_{Cl(Ca)}$ is mediated by chloride channels in the synaptic terminal [94]. E_{Cl} is near the dark membrane potential in cones, so $I_{Cl(Ca)}$ tends to boost depolarization at more negative potentials but restrain depolarization from exceeding the dark potential. E_{Cl} is slightly more positive in rods, so $I_{Cl(Ca)}$ is more likely to exert a depolarizing influence [100, 101]. At the same time, the efflux of Cl^- anions that accompanies $I_{Cl(Ca)}$ activity below E_{Cl} can directly inhibit Ca^{2+} channels to act as a brake on excessive activation [102, 103]. Finally, although Ca^{2+} -dependent inactivation of Ca^{2+} channels is more limited in salamander photoreceptors than in many other neurons, there is still some Ca^{2+} -dependent inactivation that limits sustained activity in darkness [92, 104]. Furthermore, the sustained influx of Ca^{2+} ions in to the synaptic terminal in darkness is sufficient to deplete extracellular Ca^{2+} ions from the limited volume of the synaptic cleft, limiting I_{Ca} amplitude [104].

4.2. Role of the Synaptic Ribbon in Photoreceptor Exocytosis

The properties of communication between photoreceptors and postsynaptic neurons are shaped by a specialized ensemble of presynaptic molecules with a unique architecture [105-107]. The graded changes in membrane voltage and continuous vesicle release exhibited by photoreceptors is unique to only a few cell types in the CNS, including

photoreceptors, retinal BCs, and hair cells of the cochlea and vestibular system [105]. Most neurons depolarize briefly using action potentials and require a refractory period of hyperpolarization between spikes. To accommodate the voluminous and continuous release of vesicles needed to track continuous changes in membrane voltage, photoreceptors contain a huge depot of vesicles within their terminals and synaptic ribbon-style active zones help deliver vesicles to release sites where they undergo exocytosis (Figure 3). Synaptic ribbons are plate-like protein structures in the terminals of neurons that specialize in graded neurotransmission. When examined using electron microscopy, ribbons typically look like electron-dense bars lined with synaptic vesicles (Figure 3). Along with many cytoplasmic vesicles, a pool of vesicles is tethered to each ribbon. These vesicles then descend toward the membrane surface where release sites are located. Vesicle movement does not require ATP but seems to involve passive diffusion along the ribbon surface [108, 109]. A number of recent reviews describe the molecular architecture and physiology of ribbon synapses [95, 105-107].

Salamander rods and cones contain an average of 7 and 13 ribbons apiece, respectively [110-113]. Figure 3 shows an example of four ribbons in a putative salamander cone contacting three processes of post-synaptic neurons. Individual rod terminals contain 4-5 ribbons apiece, but many rods have two terminals and occasionally three. By comparison, mammalian rods contain only a terminal with a single large ribbon and mammalian cones have 10-50 small ribbons [105]. Ribbons tether vesicles and deliver them to release sites. This task is especially important in photoreceptors, which can contain hundreds of thousands of highly mobile vesicles in their terminals: salamander rods and cones contain an average of 215,000 and 89,000 vesicles, respectively [114, 115]. Of these, approximately 3550 (~700/ribbon) vesicles are tethered to ribbons in a salamander rod with approximately 150 (~25/ribbon) of these vesicles docked at the membrane and thus available for immediate release [113, 116, 117]. Each salamander cone contains approximately 1400 total ribbon-associated vesicles (~110/ribbon) with approximately 250 (~15-20/ribbon) docked at the membrane [111, 118]. Therefore, while rod

ribbons tether far more total vesicles than cones, the number of docked, immediately-releasable vesicles is greater in cones [119].

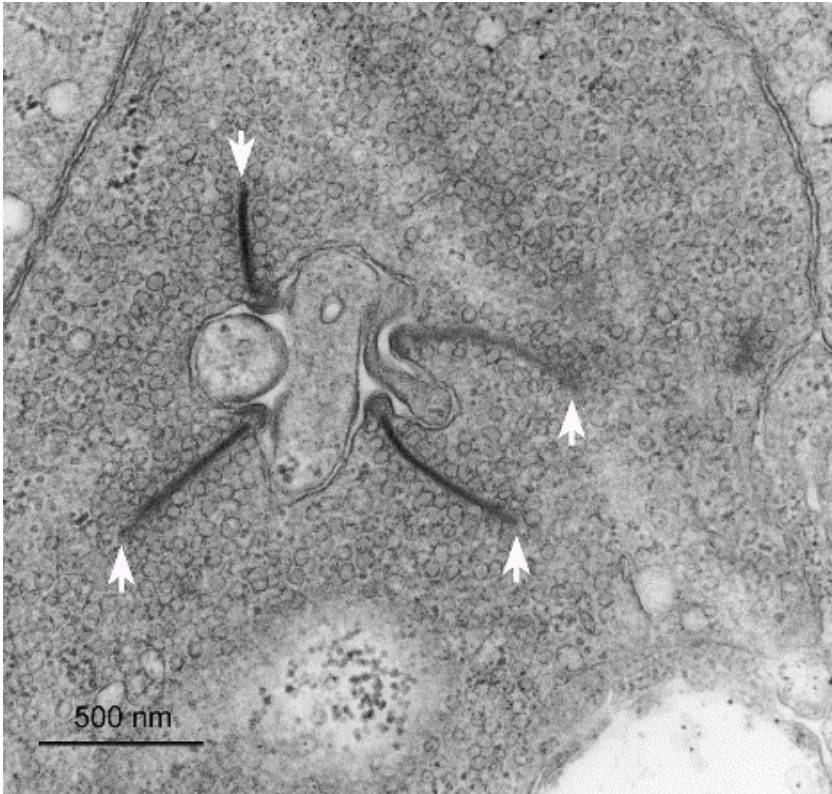


Figure 3. Electron micrograph of a synaptic terminal from a salamander photoreceptor showing four synaptic ribbons (arrows). The flat, plate-like structure of a ribbon forms an electron-dense bar when cut in cross-section. Small clear synaptic vesicles line the ribbons and populate the surrounding cytoplasm. Beneath the four ribbons are three post-synaptic processes. Typically, the two lateral processes are horizontal cell dendrites and the central process is from a bipolar cell. The size of these ribbons suggests this was probably a cone photoreceptor.

Salamander cones release vesicles only via their ribbons [113, 120]. By correlating anatomical vesicle pool sizes with electrophysiological measurements of excitatory postsynaptic currents (EPSCs), Bartoletti and colleagues [111] showed the size of the initial, fast component of the postsynaptic current was equal to the number of vesicles along the bottom

two rows of the ribbon that contact the cell membrane. These docked vesicles comprise a readily releasable pool (RRP) released within a few milliseconds of Ca^{2+} influx [119]. A second, slower component to the EPSC accompanies release of vesicles tethered higher up along the ribbon. These vesicles descend the ribbon to replenish sites vacated by the release of RRP vesicles with a time constant of ~ 100 ms [111, 121, 122]. The total releasable pool on a cone ribbon is almost six times the size of the RRP [111]. After depleting the ribbon-tethered releasable pool, release can be sustained almost indefinitely by the mobilization and attachment of cytosolic vesicles to the distal pole of the ribbon and subsequent delivery down the ribbon. The maximal sustained rate of release during strong, maintained depolarization at each salamander cone ribbon has been estimated to reach between 40-90 vesicles/s [111, 115]. The replenishment of vesicles to release sites becomes the rate-limiting step during sustained depolarization [121, 122].

Ribbons also play a role in priming vesicles for release. Snellman et al., [120] found that acute disruption of synaptic ribbon function using fluorophore-assisted light inactivation (FALI), a technique in which ribbons are inactivated with oxidative photodamage, produced a reduction in the sustained but not immediate components of the exocytotic response in both retinal BCs and cones. This phenotype was not caused by anatomical changes: the number of vesicles attached to the ribbon in the presumed RRP and RP were unchanged after FALI, suggesting the ribbon itself promotes vesicle release competence. This priming step has been suggested to be ATP-dependent [108, 123].

Signal transmission in rods involves ribbon-related mechanisms analogous to those described for cones. However, rods are also capable of a significant amount of non-ribbon release [124, 125]. This non-ribbon release can be triggered by Ca^{2+} -mediated Ca^{2+} release (CICR) from endoplasmic reticulum (ER) within the rod terminal [125-130]. CICR and non-ribbon release occurs more slowly than Ca^{2+} channel-mediated ribbon release, producing a slower exocytotic response in rods than cones. This slower mode of exocytosis contributes to the slower release kinetics of rods compared to cones [119, 131, 132], matching the slower kinetics of

rod light responses [133]. Ca^{2+} ions that are released from intracellular stores are replenished by diffusion of Ca^{2+} within the ER from the soma and other parts of the cell [126, 134]. By continuously replenishing ER Ca^{2+} stores at the terminal, CICR-mediated release can be maintained continuously in long periods of darkness.

4.3. Exocytotic Proteins in Rods and Cones

Synaptic release involves a large assembly of specialized proteins. Central to the process of vesicle fusion are the SNARE proteins and associated Ca^{2+} sensor molecules. Like most synapses, salamander photoreceptors use the SNARE proteins SNAP25 and synaptobrevin, as well as syntaxin 3B, rather than the syntaxin 1 isoform used at most other synapses. Syntaxin 3B is also used at ribbon synapses of bipolar cells and photoreceptors in other species [135-137].

Exocytosis of synaptic vesicles in ribbon-bearing retinal bipolar cells and inner hair cells can be triggered by increasing $[\text{Ca}^{2+}]_i$ from ~ 100 nM in resting conditions to >8 - 10 μM [108, 138-140]. The relationship between $[\text{Ca}^{2+}]_i$ and release at these ribbon synapses accords with the properties of exocytosis at other CNS synapses [141, 142]. However, studies of tiger salamander revealed that synaptic vesicle exocytosis from rods and cones is unusually sensitive to Ca^{2+} , requiring less than 1 μM $[\text{Ca}^{2+}]_i$ to stimulate release [115, 117, 121, 143, 144]. In addition, the Ca^{2+} sensor in rods and cones shows a relatively linear relationship between Ca^{2+} and release [98, 117, 121, 144, 145]. By contrast, most synapses, including bipolar cells, show a highly cooperative relationship between $[\text{Ca}^{2+}]_i$ and exocytosis, such that release rises steeply with the 4th or 5th power of $[\text{Ca}^{2+}]_i$ [138, 141, 142, 146-151]. The mechanisms responsible for low cooperativity in photoreceptors are not yet clear. Salamander rods and cones do not possess synaptotagmin 1 [152], the Ca^{2+} sensor used at many CNS synapses, and it is possible that biochemical characteristics of the Ca^{2+} sensor imparts these properties to release. However, if so, this would imply that mammalian rods and cones, which possess Syt1 [137, 153, 154],

would not exhibit a low cooperativity and high affinity release mechanism. If low cooperativity/high affinity exocytosis is a universal property of photoreceptors, it would suggest that the Ca^{2+} dependence of rods and cones is modified by other properties of the synaptic release apparatus.

The high cooperativity ($n = 4-5$) between Ca^{2+} and exocytosis that has been described at many synapses is thought to be advantageous because increases in active zone $[\text{Ca}^{2+}]$ can be translated into release rates orders of magnitude above the basal rate [155], maximizing signal over noise when binary signals are conveyed by action potentials. Low cooperativity may instead be more advantageous at photoreceptor synapses that do not experience action potentials. A shallow relationship between $[\text{Ca}^{2+}]_i$ and exocytosis may prevent dramatic changes in release rate over the physiologic range of $[\text{Ca}^{2+}]$, and in so doing, improve the signal to noise ratio of continuous release. Such a system might allow for superior analog encoding.

4.4. Synaptic Contacts of Rods and Cones

Photoreceptor ribbons sit atop membrane invaginations in rod and cone terminals. Postsynaptic dendrites from multiple neurons enter these invaginations to receive synaptic input from photoreceptors [105]. Postsynaptic cells can also make basal contacts with rod and cone terminals at non-ribbon sites outside these invaginations. Despite being located further from the site of ribbon-mediated release, basal contacts appear to receive input from vesicles released at the ribbon. The time required for glutamate to diffuse to basal contact sites produces slightly slower responses in cells that make basal versus invaginating contacts [156]. HCs, ON BCs, and OFF BCs can all make both invaginating and basal contacts with presynaptic photoreceptors in salamander retina [157]. In the invagination beneath each ribbon, a pair of horizontal cell dendrites typically flanks a central BC dendrite (Figure 3). In mammals, ON BCs make invaginating contacts while OFF BCs make flat contacts. By

contrast, most invaginating contacts in salamander retina are made by OFF BCs and most flat contacts are made by ON BCs [157].

Multiple HCs and BCs that all receive synaptic input from the same cell can respond differently to the same stimulus based on multiple factors. One of the most critical response determinants is the type of postsynaptic glutamate receptor that is expressed. HCs and OFF BCs express ionotropic glutamate receptors, cation channels gated by glutamate that depolarize the cell with fast kinetics when activated. Thus, HCs and OFF BCs exhibit the same response polarity to illumination—hyperpolarization—as photoreceptors. Salamander HCs and OFF BCs express both kainate (KA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) type ionotropic receptors [158]. The different molecular variants of these ionotropic receptors have not been characterized in salamander retina. AMPA receptors mediate most of the postsynaptic current in salamander HCs and BCs [110, 132]. In amphibians and other quadrupeds, HCs and BCs do not express NMDA-type glutamate receptors that are often found in other neurons.

The ON BC is unique in the CNS in that it hyperpolarizes to glutamate rather than depolarizes. Thus, while ON BCs receive the same inputs from photoreceptors as HCs and OFF BCs, the polarity of their response is inverted: they depolarize in response to illumination and hyperpolarize in darkness. To generate this hyperpolarizing response to glutamate, ON BCs utilize a metabotropic glutamate receptor, mGluR6. Like other mGluRs, mGluR6 is a GPCR that requires intracellular signaling cascades to effect responses mediated by other ion channels. Acting through the G protein, G_{α} , activation of mGluR6 by glutamate closes TRPM1 cation channels [159]. The cell type-specific expression of different combinations of glutamate receptors contributes to an impressive functional diversity among salamander BCs, with almost a dozen subtypes identified [160].

The output of photoreceptors is amplified by synaptic transmission to HCs and BCs, so that small changes in presynaptic release rate produce comparatively large postsynaptic responses.

With dim flashes near threshold, synaptic gain from salamander rods to horizontal cells (i.e., the amplitude of the voltage response in a horizontal cell compared to the rod response) averages 5x. The gain from cones measured near threshold in darkness is only 1.5x, but increases to 5x after 3 min of light adaptation and approaches 8x after 15 min [161]. This voltage gain is a function of both the number of synapses and the strength of each individual synaptic connection. The increase in voltage gain suggests that synaptic strength is increased during light adaptation.

Under light-adapted conditions, a small change in contrast (<10%) that evokes barely detectable responses in a cone can evoke much larger responses in salamander BCs [162]. Contrast gain provides a metric that is independent of the number of synapses. It measures the percent change in response amplitude as a function of the change in percent Michelson contrast:

$$\text{Michelson contrast (\%)} = \left(\frac{\text{stimulus} - \text{background}}{\text{stimulus} + \text{background}} \right) \times 100$$

(Equation 1)

The contrast gain in bipolar cells under light-adapted conditions is 12.5x greater than the contrast gain in cones, but contrast gain diminishes with higher contrasts in bipolar cells [163]. In other words, bipolar cells are exquisitely sensitive to small changes in contrast but become increasingly less sensitive to large contrast changes. Current and voltage changes in bipolar cells show similarly high contrast gains, indicating that this gain occurs at the synapse and is not a result of voltage-dependent channels in bipolar cells [164]. The contrast gain in HCs is similar to cones and lower than that of bipolar cells. Both HCs and cones show a similar contrast gain to small and large contrast changes [165, 166].

While the average contrast gain for bipolar cells is quite high, there is considerable variability among cells. Recording from land-phase salamanders, Burkhardt et al., [167] found that under light-adapted conditions, the distribution of contrast gain values among individual bipolar cells closely matched the distribution of real-world contrasts

measured in 65 natural images. In the natural world, low contrasts far outnumber high contrasts. This distribution is matched by the dynamic range of contrast responses among bipolar cells, consistent with efficient coding of visual stimuli [167].

5. PHOTORECEPTOR METABOLISM

5.1. Creatinine Shuttle and Inner Segment Metabolism

To maintain a constant ionic balance across the membrane, Na^+ ions that enter the outer segment through open CNG channels in darkness must be extruded from the cell. As in other cells, the chief molecule in this process is the Na^+/K^+ -ATPase. This ionic pump uses energy provided by the hydrolysis of ATP to extrude 3 Na^+ ions and take up two K^+ ions. Photoreceptors express a large number of Na^+/K^+ -ATPase molecules on the inner segment membrane [168, 169]. The tonic activity of Na^+/K^+ -ATPase in darkness requires hydrolysis of prodigious amounts of ATP which in turn requires considerable amounts of glucose and oxygen; indeed, photoreceptors are among the most energy-intensive cells in the body [170].

Salamander retinas are avascular, so all of the oxygen and glucose delivered to the retina is supplied by the choroidal vascular plexus that lies beneath the retina and retinal pigment epithelium. One surprising feature of photoreceptor metabolism is that it relies heavily on aerobic glycolysis [171, 172]. The lactate produced by glycolysis in photoreceptors is exported to supply retinal pigment epithelial and Müller cells [173].

In addition to tremendous glycolytic capacity, the outermost regions of the inner segment are packed with mitochondria. Continuous Na^+/K^+ -ATPase activity and the ongoing release of vesicles in darkness both require energy [170]. Species with vascularized retinas have mitochondria in photoreceptor synaptic terminals that can use oxygen supplied by the intraretinal vasculature to produce the ATP needed to sustain synaptic release.

However, most of the oxygen delivered from the choroid is captured and utilized by mitochondria in photoreceptor inner segments [174], so little oxygen is available to photoreceptor synaptic terminals in avascular retinas. Furthermore, unlike rods in vascularized retina, salamander terminals do not contain mitochondria [112, 175]. The ATP generated by mitochondria in the inner segment of salamander rods and cones is readily consumed by the many Na^+/K^+ -ATPase molecules in the inner segment, allowing very little ATP to reach the synapse.

Linton et al., [175] showed that in darkness salamander photoreceptors circumvent this problem by protecting some of the hydrolyzable ATP produced in the inner segment by first converting it to phosphocreatine (PCr) and then transporting PCr to synaptic terminals. Photoreceptors in avascular retinas, including salamander, have a high concentration of the enzyme creatine kinase B (CK-B) in their terminals which can then convert PCr back to ATP. This PCr shuttle allows energy produced by central mitochondria to be transported to intracellular regions with high energy requirements without it being consumed *en route*.

5.2. Intracellular Ca^{2+} Regulation in Rods and Cones

Excessively high or low free $[\text{Ca}^{2+}]_i$ can both be toxic to photoreceptors [176]. Multiple mechanisms exist to stabilize Ca^{2+} levels in the photoreceptor cytosol [177]. The inner and outer segments of salamander rods regulate Ca^{2+} levels independently from one another [178, 179]. Outer segments extrude Ca^{2+} ions that enter through CNG channels using $\text{Na}^+/\text{Ca}^{2+}$ exchangers whereas inner segments rely on plasma membrane Ca^{2+} ATPase (PMCA) pumps to remove excess Ca^{2+} [178, 180, 181]. Inner segment Ca^{2+} can also be sequestered in the endoplasmic reticulum [128, 180] and mitochondria [182]. The presence of Ca^{2+} -binding proteins in the synaptic terminal and cell body further help buffer Ca^{2+} [131].

Among the various buffers, calbindin is especially prominent in double cones of salamander and mudpuppy retina [50, 183]. However, overall endogenous Ca^{2+} buffering is surprisingly weak in salamander rods and cones, equivalent to only 0.05 – 0.1 mM EGTA [113, 184].

6. INTRINSICALLY PHOTSENSITIVE RGCs AND PINEAL PHOTORECEPTORS

Though retinal rod and cone photoreceptors are the cells that initiate the primary stream of visual information, they are not the only cells that a salamander uses to sense light. Salamanders also contain both intrinsically photosensitive retinal ganglion cells (ipRGCs) and photosensitive cells within the pineal gland that can absorb photons and transmit light-dependent signals. While mammalian ipRGCs constitute only a small fraction of all RGCs, the only study to our knowledge that has examined salamander ipRGCs found that all salamander ON RGCs (which constitute approximately 10% of all RGCs) are intrinsically photosensitive [185]. Because they contain the visual pigment melanopsin, ipRGCs do not require photoreceptor input to be activated, though they also receive photoreceptor input via BCs. Mammalian ipRGCs project to many brain targets that are not directly involved in image formation [186], but the targets of salamander ipRGCs/ON RGCs are less clear.

The salamander pineal gland contains another kind of nontraditional photoreceptor that synthesizes melatonin and is morphologically similar to retinal photoreceptors, including the presence of synaptic ribbons [187]. As in higher vertebrates, the pineal gland is a critical regulator of circadian rhythms. In tiger salamanders, pineal photoreception has been shown to help orient the organisms directionally [188-191], which is thought to confer an ability to determine the solar azimuth angle even on overcast days [187]. In the eastern red-spotted newt, pineal photoreception appears to directly influence the internal magnetic compass [192]. Even the blind, cave-dwelling olm (*Proteus anguinus*), which is essentially eyeless,

contains opsin-expressing pineal photoreceptors, though these cells exhibit degenerated outer segments [193].

7. SALAMANDER RETINA AS A TRANSLATIONAL MODEL

The remarkable ability of Urodele amphibians to regenerate their limbs has made them a valuable model for studying tissue regeneration (see reviews [194, 195]). Urodele amphibians are also capable of regenerating retinal neurons and spinal cord tissue after metamorphosis into adulthood [196-200]. These properties have also sparked interest in urodeles as models for studying the loss of regenerative capability in aging or excessive cell growth in cancer [201, 202]. Salamanders can also serve as a useful model for aging retina. Urodele amphibians grow throughout their life and aquatic tiger salamanders longer than 26 cm are thought to be >10 years old. Large salamanders show age-related changes to their retina that parallel many changes in human retina [203]. Studies in salamander retina have identified intracellular signaling pathways, such as the RhoA pathway, that mediate plasticity and remodeling of photoreceptors and may someday prove useful for therapy retinal detachment and degeneration [204-209].

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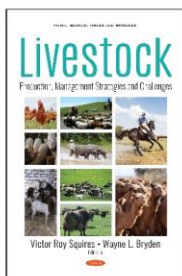
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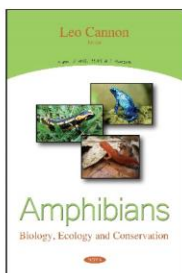
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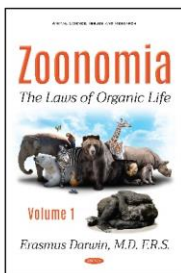
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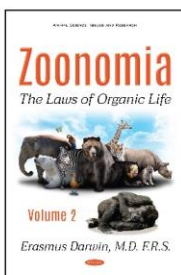
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