THE OREGON SPOTTED FROG (*RANA PRETIOSA*) IN LOWLAND WESTERN WASHINGTON, USA: A POPULATION, PARENTAGE, & NON-BREEDING HABITAT ANALYSIS

by

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ABSTRACT

The Oregon spotted frog (*Rana pretiosa*) in lowland western Washington, USA:

A population, parentage, & non-breeding habitat analysis

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The at-risk Oregon spotted frog (OSF, *Rana pretiosa*) has disappeared from much of its geographic range. It is endangered in Washington State, and considered threatened under the US Endangered Species Act. Much research has been devoted to improving OSF breeding habitat management. Despite these important efforts, adult non-breeding habitat utilization in western Washington remains poorly known, and this is a significant gap in our understanding of this species. In western Washington, many OSF populations are small, genetically isolated, and embedded in a rapidly urbanizing matrix. Given these habitat limitations, determining the total habitat footprint of each OSF population is critical to their conservation. This study investigated the spatial relationship between breeding and non-breeding habitat utilization patterns of adult OSFs by using genetic sampling for one small population. This effort exploited the fact that a large fraction of egg masses (n=109) laid in 2014 (February-March) at the target study site, West Rocky Prairie (WRP), had already been genetically sampled. This effort sampled adult OSFs genetically in their non-breeding active-season habitat (July- September), and linked those adults (n=56) to breeding locations based on parentage of egg masses using CERVUS 3.0.7. Straight-line distance measurements of parent:offspring pairs (n=12) revealed that parents (n=2) traveled >2km and (n=1) >1km between breeding and non-breeding habitat. Based on microsatellites (n=12), *AR*=3.833, and COLONY analysis, N_e=25 (CI95: 15 to 43), 54% of sampled adults had ≥1 sibling within the sampled population, suggesting a recent bottlenecking for OSF at WRP. Most (83%) of all captured frogs were found in a small (10×6m) pond at WRP, indicating that non-breeding habitat may be limited. Management of OSF should consider all habitats that may contribute to its vulnerability. This study provides critical information about OSFs at WRP, and a basis of what to expect for non-breeding active-season habitat in other OSF populations.

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CHAPTER 1

INTRODUCTION

CURRENT STATUS OF THE OREGON SPOTTED FROG

The Oregon spotted frog (OSF, *Rana pretiosa*) is a highly aquatic ranid species, endemic to the Pacific Northwest (USFWS, 2015). The OSF has a historical range from Southwestern British Columbia to northern California (Hallock, 2013). However, due to impacts from human development, the species distribution has declined precipitously. The current distribution is much reduced, and historically widespread populations in the Willamette Valley, Oregon, and areas in California have been extirpated. However, isolated populations still exist in Oregon and Washington, and extend into southern British Columbia (Cushman & Pearl, 2007). Based on conservative historical distribution estimates, 79% of OSF populations have been lost; however, losses may actually reach 90% (Hayes, 1997). Although reduced, Washington has remaining populations located in North Puget Sound, South Puget Sound, and western Klickitat County (Hallock, 2013) (Figure 1.1).

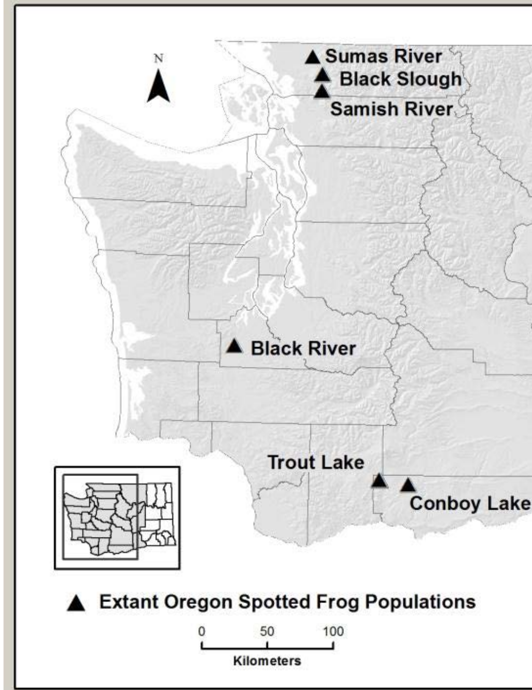

Figure 1.1. Current distribution of Oregon spotted frog populations in Washington State. Triangles do not represent single populations, but general areas of populations across the OSF's range in Washington State. Figure adapted from Hallock (2013).

LISTING STATUS

As of August 29th, 2014, the US Fish and Wildlife Service (USFWS) formally listed the OSF (*Rana pretiosa)* as a *Threatened Species* (USFWS, 2015). However, the OSF has been considered a *State Endangered Species* in Washington since 1997 (Hallock, 2013), and *Endangered* in Canada since 2000 (Haycock, 2000). These listings limit activities that could be deemed harmful to this species (Hallock, 2013). The OSF is classified in Oregon as a *Critically Sensitive Species,* and in California as a *Species of Special Concern*.

HABITAT REQUIREMENTS, LIFE HISTORY, & MANAGEMENT

Dependent on the stage of its life cycle, the OSF has varying seasonal hydrologic habitat requirements, which are partitioned into three temporal categories (Watson et al., 2003). Breeding season occurs between February and early April, the non-breeding season occurs between April and early October, and the overwintering season occurs between October and February of each year (Watson et al., 2003; M. Hayes, unpublished data). This life-history necessitates an understanding of what defines suitable habitat for each life stage as the season progresses. During the late non-breeding season in Washington, water availability may be limited due to lower amounts of precipitation during that time of year. Given the fact that this species is fully aquatic during all phases of its life cycle, multiple challenges may arise regarding its management if we do not fully understand its requirements for suitable habitat.

BREEDING SEASON

During the breeding season, between February and early April, adult males and females congregate in shallow, seasonal pools created by the seasonal expansion of their permanent water habitat from rain and snowmelt (McAllister & Leonard, 1997). Oregon spotted frogs require water depths less than 30 cm (average 18.5 cm) (Pearl & Adams, 2009) because these shallow waters warm quickly, which is important for embryonic development (Licht, 1971). OSF also prefers shallow waters with emergent and submerged "vegetation types (which) provide feeding areas, refuge from predators, and warmer water" (Pearl & Adams, 2009). The breeding season lasts approximately four weeks and the seasonal shallow habitat serves as oviposition (egg laying) sites

(McAllister & Leonard, 1997). Table 1.1 highlights the annual variation in oviposition times of OSF populations in British Columbia and Washington State across multiple years.

Table 1.1. Annual variation in oviposition start and end dates times, which has been linked to water temperature. [1] Depths & [2] Temperatures measured within 48 hours of egg-laying. _[3] Information from Licht (1971) where egg laying began at 6°C at the center of breeding ponds and 20.7°C adjacent to egg masses.

Location	Year	Oviposition Start Date	Oviposition End Date	Mean Water Depth @ Ovipos. sites[1]	Water Temp. @ Ovipos. sites[2]
Southwest B. C.	1968	March 1	March 10	5-12 cm.	-[3]
Southwest B. C.	1969	March 13	March 23	5-12 cm.	-[3]
Dempsey Creek	1995	February 23	March 10	11.5 cm (N=2)	47° F (8.5° C)
Dempsey Creek	1996	March 3	March 17	5.8 cm (N=7)	49° F (9.5° C)
Dempsey Creek	1997	February 21	March 20	10 cm (N=3)	52° F (11° C)
Trout Lake	1997	~March 26	--------------	--------------	--------------
Conboy Lake	1997	~March 16	~March 25	--------------	--------------

Table adapted from McAllister & Leonard (1997).

Breeding females in lowland sites breed every year (Licht, 1974), they lay a single egg clutch (mass) per year, and a single male generally fertilizes a single clutch (Phillipsen et al., 2009). In McAllister & Leonard (1997) adult females laid egg masses with an average of 643 eggs per mass in communal clusters of 10-75 masses, although higher numbers of masses (>100) have been reported (Tyson & Hayes, 2014). Figure 1.2 illustrates a communal egg mass.

Figure 1.2. Oregon spotted frogs communal egg mass. This mass includes >50 Oregon spotted frog egg clusters (blue circle) with a single clutch (red circle). Figure adapted from Kapust et al. (2012).

Oregon spotted frogs frequently breed in the same geographic locations each year, and depending on topography and seasonal water variation, will sometimes use the same oviposition site each year (Kapust et al., 2012; Watson et al., 2003). Figure 1.3 shows breeding habitat for the OSF.

Figure 1.3. High quality oviposition habitat for the OSF includes a shallow, seasonally flooded wetland, where Reed Canary Grass is infrequent. Figure adapted from Hallock (2013).

Oregon spotted frog eggs develop between 14 and 30 days, the eggs hatch and tadpoles find open water in order to consume bacteria, algae and detritus (McAllister & Leonard, 1997). After 13 to 16 weeks, the OSF tadpoles metamorphose into juvenile frogs (McAllister & Leonard, 1997). In some OSF sites, juvenile frogs move into ponds along with the adult frogs for the summer months (Hallock, 2013); however, they have also been known to use shallower habitats (M. Hayes, personal communication).

OVERWINTERING SEASON

Overwintering for this species is considered the time between October and February of each year, based on studies conducted on overwintering characteristics at Conboy Lake National Wildlife Refuge (Hayes et al., 2001) and Trout Lake (Hallock &

Pearson, 2001) in Eastern Washington; and Dempsey Creek in western Washington (Risenhoover et al., 2001).

In a study of 11 adult female radio-telemetered OSFs at Dempsey Creek in lowland western Washington, 5 adults remained relatively active, while 6 others were generally sedentary, with a total average movement of 6.7 m per day (Risenhoover et al., 2001). Ninety-five percent of the habitat utilized by OSFs in this study was palustrine wetland, with varying levels of vegetation and cover Table 1.2 (Risenhoover et al., 2001). For 90% of the observations made in this study, ice was not encountered (Risenhoover et al., 2001).

Table 1.2. Frequency of OSF habitat locations during overwintering in lowland western Washington

Classification	Count	Percent
Palustrine:	(3564)	(95)
Persistent emergent:	2773	77
Sedge-rush	580	21
Reed canary grass	840	30
Other:	1353	49
Open water	1151	86
No open water	202	14
Scrub shrub	791	23
Upland:	(186)	(5)
Pasture	186	5

Table adapted from Risenhoover et al. (2001)

In comparison, a study of overwintering habitat was conducted at Conboy NWR (Hayes et al., 2001), which is a higher elevation (550-561 m [1,804-1,840 ft.]) OSF site (Hallock, 2013). In this study, 10 individual male and female adult OSFs were pit tagged, ice was prevalent, and significantly more movement was observed before ice occurred

(Hayes et al., 2001). Furthermore, frogs were found to utilize several vegetation types including floating vegetation, upland vegetation, and OSFs were observed in open water or ice (Hayes et al., 2001).

Based on the results of Risenhoover et al. (2001), in lowland western Washington, it appears that temperature may play an important role in the movement of OSF across the landscape, although these results were not significant. Hayes et al. (2001) did find a significant difference in movement where frogs tended to move more during pre-ice conditions than during icy conditions. These results warrant further investigation into the movement and habitat utilization during the overwintering period. Furthermore, both of these studies had small sample sizes (10-11 individuals) and may not adequately represent the overwintering habitat utilization of the OSF.

NON-BREEDING SEASON & MANAGEMENT

Multiple studies, some using radio telemetry, have described the home ranges and habitat utilization of various OSF adult populations during the breeding and overwintering seasons. They also demonstrated what habitat regions individual populations are utilizing at metamorphosed and juvenile life stages. However, adult non-breeding habitat requirements remain widely unknown, as much of the research into Oregon spotted frog habitat utilization has focused on breeding. This emphasis on breeding habitat has also been the primary focus of management objectives for the species in western Washington. However, breeding only occurs during the late winter/early spring months (Watson et al., 2003), which leaves the rest of the OSF annual cycle less understood.

In an attempt to understand home range of the OSF throughout the year, Watson, et al. (2003) attached radio telemetry devices to individual frogs at the previously discussed Dempsey creek, a single site in lowland western Washington. Watson et al. (2003) tracked a total of 60 adult OSF at varying times intervals between 1997-1999. However, only 18 of these individual frogs were tracked during the non-breeding, active dry season. This work revealed that OSFs move to small, deep remnant pools during the dry season in June-August (Watson et al., 2003). During the dry season, these wetlands often decrease in area or dry out due to sun exposure and reduced precipitation, and remnant pools are typically some of the few remaining aquatic habitats. These results indicate that the home range of this species drastically decreases (2 to 4 times) during the dry summer months (Watson et al., 2003; Hallock, 2013).

This study was valuable in indicating locations where OSF reside during the non-breeding season. However, these results may not adequately represent the non-breeding habitat utilization of the OSF across its geographic range, as it was conducted at a single study site. Furthermore, given the highly aquatic nature of the OSF, it is possible that these remnant pools may be a limiting factor as related to habitat requirements for this species, a possibility that warrants further investigation. Research on OSF adult non-breeding, active season habitat is lacking for multiple sites and populations, and warrants further investigation. This thesis study will fill some of these gaps by characterizing the habitat used by adults during the non-breeding summer season at a different site in western Washington.

PRIMARY HABITAT REQUIREMENTS & MANAGEMENT

Remnant OSF populations in Washington typically require palustrine wetlands, which are connected to stream networks (Hallock, 2013). "The perennial creeks and associated network of intermittent tributaries provide aquatic connectivity between breeding sites, active season habitat and overwintering habitat" (Hallock, 2013). These systems also provide a constant flow of oxygenated water. This may be especially important for the species during hot summer months where water tends to become stagnant (Hallock, 2013). The wetlands where OSFs live include a mix of aquatic bed, emergent, scrub-shrub, and forested areas (Hallock, 2013). The dynamic habitat requirements of the OSF, and the heavy alteration to the majority of sites make management of this species and their habitat difficult. "Proper management of the remaining isolated frog populations requires site-specific knowledge of vegetation characteristics, home range, and seasonal changes in hydrology that may affect movements" (Watson et al., 2003). In order to successfully manage this species, it is vital that its habitat-range requirements are understood, and that management is adapted accordingly.

HABITAT THREATS & MANAGEMENT

A number of factors contribute to the decline in OSF populations, many of which are caused by habitat alterations from anthropogenic effects. Isolation, low effective population sizes, exotic flora and fauna, altered landscape and hydrology, and changes in water chemistry are just a few contributors (Watson et al., 2003; Hallock, 2013). The primary concern, which will likely produce the best results for this species' population, is in preserving and restoring quality habitat. Since resilient habitats and ecosystems tend to have resilient species populations, it is important to focus on preserving and restoring quality habitat for the benefit of all species in OSF associated wetlands.

Reed Canary Grass has greatly altered the majority of primary habitat for this species, and has become a primary focus in breeding habitat restoration for the OSF in Washington State (Kapust et al., 2012). Introductions of invasive American Bullfrogs in OSF habitat have altered the predatory dynamics concerning the OSF, and have contributed to their decline (Pearl et al., 2004). Finally, contaminant levels and low water conductivity in their habitat may have contributed to high mortality in both embryonic and adult life phases (Marco et al., 1999).

While these issues have major management implications, restoration of OSF habitat must first be guided by well-researched documentation of what habitats OSFs are using. Furthermore, before altering their habitat for restoration, each population's genetic health must be assessed.

STUDY RATIONALE

Research on OSF genetics has provided information about genetic variation across their range (Blouin et al., 2010), setting the stage for further analyses. That research was range-wide, but OSF management is often focused on small isolated populations. Therefore, conservation requires site-specific understanding of OSF habitat, population genetics, and local landscape processes. Understanding habitat utilization patterns and population structure is critical to developing appropriate strategies to manage these small populations.

The feasibility with which OSF egg masses can be detected has led to a focus on breeding surveys to gain general knowledge about OSF population sizes, and track trends. As a consequence, in Washington State, the nature of breeding habitat is reasonably well understood. However, the habitats where OSFs breed are largely ephemeral, as in lowland western Washington, they breed at high water during late winter. When the water recedes from breeding sites, adult OSFs are thought to move to different habitats for the non-breeding season. Furthermore, since adult OSFs are often cryptic (Hallock, 2013), the nature of non-breeding habitat and their linkage to breeding sites is largely unknown for most sites in western Washington.

This study examines the non-breeding habitat utilization of OSF adults during the 2014 active season (summer to early fall), by using genetics to spatially link these adults to eggs laid earlier in 2014 at specific oviposition sites (breeding habitat) at West Rocky Prairie (WRP). WRP is a known, Washington Department of Fish and Wildlife (WDFW) managed, OSF site in the upper Black River drainage in western Washington. Genetic

data was used to infer parentage and characterize this population. Both the genetic and habitat components of this study will help guide management activities at WRP and other sites with similar habitat characteristics. In particular, information obtained from this study characterizes OSF non-breeding active-season habitat in a manner useful to habitat managers responsible for ensuring survival of this population in the long-term. If non-breeding active season habitat somehow limits the OSF population size at WRP, this research can inform the appropriate direction for management of this population, and potentially other populations. Furthermore, linking seasonal habitats utilized via parentage analysis can identify the habitat footprint of the biologically effective, rather than the total OSF population at the site. This study includes two key components, breeding and non-breeding habitat utilization across temporal and spatial scales, and genetic linkage between those habitats.

CHAPTER 2

HABITAT UTILIZATION

METHODS

FIELD METHODS

WEST ROCKY PRAIRIE STUDY AREA

West Rocky Prairie (WRP) is located in Thurston County in lowland western Washington (Figure 2.1.1), and includes a wetland complex with two areas of focus, hereafter referred to as East Side and West Side marshes (Tyson & Hayes, 2014). WRP, a verified OSF site since 1999, has been under state ownership (WDFW) since 2006, and is one of approximately 50 locations where the OSF resides across its geographic range (Tyson & Hayes, 2014). The site has been the focus of numerous studies for the OSF, including a series of controlled studies on the response of OSF oviposition to mowing of the invasive Reed Canary Grass (Kapust et al., 2012; Tyson & Hayes, 2014).

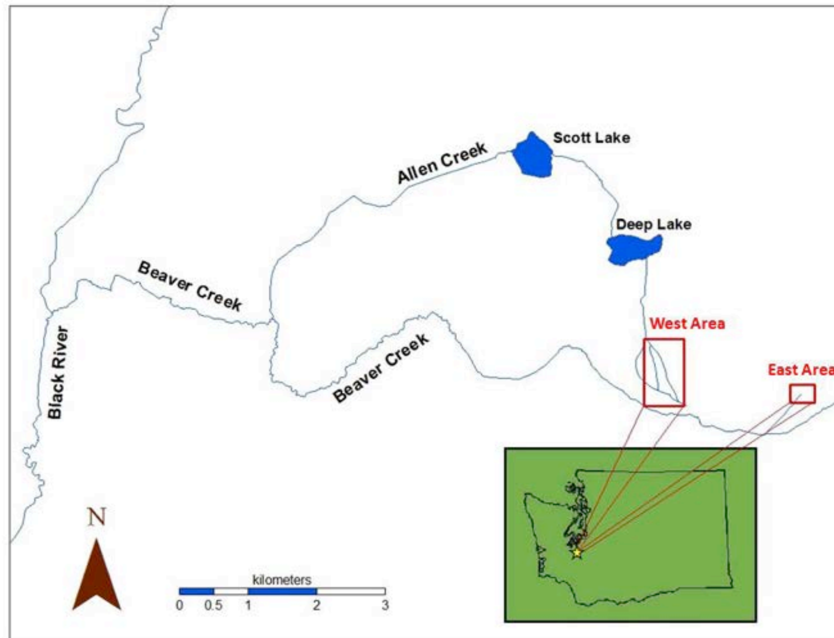

Figure 2.1.1. Map of Washington, and the location of West Rocky Prairie. Headwaters include Allen Creek (West Side) and Beaver Creek (East Side), which are connected to the Black River. Red boxes indicate the geographic locations of the West Area and East Area where surveys in this study were conducted. Figure adapted from Tyson & Hayes (2014).

West Rocky Prairie Wildlife Area encompasses multiple habitat types including prairie, forested area, and wetlands. Figure 2.1.2 includes the area of WRP ownership by WDFW, and the locations of known OSF oviposition sites (Tyson & Hayes, 2014). The West and East side marshes include study sites for Reed Canary Grass management, while the Central West and Central East sites are not currently a part of those ongoing studies. These sites are surveyed annually for OSF egg masses.

Figure 2.1.2. Aerial photograph of the WRP land ownership. Orange boxes indicate locations of detected OSF egg masses in recent years. Figure adapted from Tyson & Hayes (2014)

At the west side of the West Rocky Prairie site, there are two small ponds in relatively close proximity to this population's oviposition habitat. Based on preliminary observations, adults appear to use one of these ponds (South Pond) (M. Hayes, unpublished data), and hence, may utilize the second pond (Tilley Pond). Figure 2.1.3 is a detailed map of the West Side Survey Area.

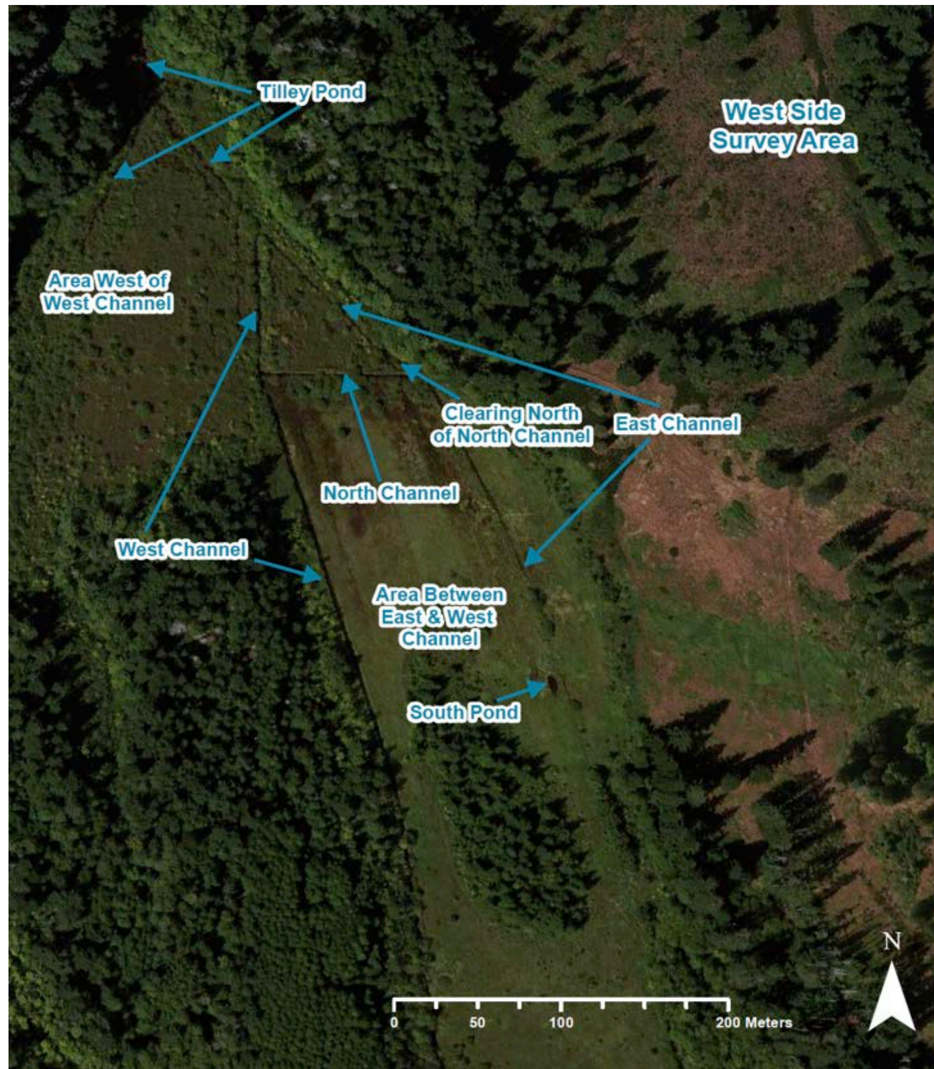

Figure 2.1.3. Aerial photograph of the West Side Survey Area and the locations of the South Pond where OSF adults have been previously observed, and the location of Tilley Pond where adult OSFs may be present.

**World Imagery Base Map by ESRI 2015. Map developed by Chelsea Waddell, 2015.*

This study included multiple components, which began with WDFW directed annual egg-mass surveys during the 2014 breeding season (February to March). During these egg-mass surveys, in which I was a major participant, a total of 218 eggs were collected from 109 egg clusters throughout the WRP site for a separate study of gene flow. Availability of this genetic data was instrumental to my work, and the methods used

in this study are described below. In order to determine adult post-breeding (July-September 2014) habitat use, myself, with the assistance of volunteers and WDFW employees, surveyed both ponds, the intervening marsh, and the surrounding marsh footprint, to capture adults. I used these surveys to gather both habitat and genetic data for adults. With the help of the Molecular Genetics Laboratory (MGL) at WDFW, I subsequently performed a genetic parentage analysis to determine the locations and habitat preferences of the parents of offspring sampled during breeding season (See Genetics Chapter).

BREEDING HABITAT SURVEYS

At the West Rocky Prairie site, OSFs began laying their eggs in the West Side marsh on March 1st, 2014 (Tyson & Hayes, 2014). Surveys for egg masses are typically done using Visual Encounter Surveys (VES), where surveyors walk parallel to each other, 1-2 meters apart, scanning in front and to the left and right of the observer. Once an egg mass is detected, it is marked with a pin flag. Egg mass fidelity, number of clusters and masses, and habitat measurements are taken, and entered into a PDA (Personal Digital Assistant) Excel spreadsheet (Tyson & Hayes, 2014). Measurements include GPS points in Decimal Degrees, number of egg masses at the location, air and water temperature in C°, water depth in *cm*, developmental stage (Gosner, 1960), and percent mortality (Tyson & Hayes, 2014). The sites were typically revisited, depending on the accessibility of the site, until the OSF tadpoles hatched.

Eggs were collected for genetic analysis from egg mass clusters located in six representative locations. The West Side was partitioned into 3 locations: West North, West Central, and West South. The other 3 were the East Side, Central West, and Central East areas. See Figure 2.1.2 for site locations. Two eggs were collected randomly from randomly selected egg masses at each site for genetic analysis, and stored at room temperature in cryogenic tubes filled with DNA-grade ethanol (M. Hayes, unpublished data).

NON-BREEDING HABITAT SURVEYS

Field surveys were always conducted by two or more people, which included myself with the help of volunteers and/or WDFW employees. Field surveys for adult OSFs during the non-breeding season were conducted in two seperate sessions. The first session began on July 22nd, 2014 and continued through August 10th, 2014. The second session began on September 5th, 2014 and continued until September 19th, 2014. Three standard survey methods were used to conduct this research. Each of the three methods described here were conducted throughout the field survey component of the study.

A study-specific PDA (Personal Digital Assistant) with an excel spreadsheet was used for all data collection throughout the study. Equipment for these survey methods included a Garmin GPS, digital thermometer, large dip nets for each surveyor, chest-waders for each surveyor, a waterproof digital camera, an iPhone 4S, and equipment vests. Additional equipment was required for occasions where adults were captured and included a bendable ruler, a digital scale, Ziploc bags, Sharpie markers, ethanol resistant pens, and sterile Epicentre® Catch-All™ Buccal Swabs.

Visual Encounter Surveys

Visual encounter surveys (VES) were conducted by slowly walking the entire marsh and pond areas (Figure 2.1.4), or by using an inflatable fishing tube where areas were too deep to walk. These VES involve one or more individuals walking parallel to each other, 1-2 meters apart, scanning the area to the left, right and in front of them for post-metamorphic OSF. When an individual was seen, a dip net or hand capture was used to capture the animal, depending on the configuration of the habitat and accessibility to the animal (Figure 2.1.4).

Figure 2.1.4. Visual encounter surveys were conducted to capture OSF adults. On the left is an image of a walking visual encounter survey in a narrow channel. The middle image shows the capture of a frog using the dip-netting technique. The image on the right is an adult male OSF captured using the aforementioned dip net technique. Photo Credits: Sierra Blakeley & Cameron Smith.

Surveys conducted in deep water channels and deep ponds included visual encounter surveys using an inflatable fishing tube (Figure 2.1.5).

Figure 2.1.5. Floating Visual Encounter Surveys were conducted in areas where water was too deep to survey with Walking Visual Encounter Surveys. Photo Credit: Julie A. Tyson.

The floating method was used to survey both the centers and edges of each pond, as the ponds and some channels were too deep to survey by walking, even with chest waders. Similar to the walking VES surveys, when adult OSFs were observed, they were captured by hand or with a dip net depending on animal positioning and nearby vegetation structure. Captured individuals were processed (see Animal Processing section) in situ.

All adult OSFs observed were recorded and an attempt was made to capture all observed adults. Captured OSF adults were processed according to the approach described below (see Animal Processing section). Once each animal was processed, individuals were immediately released behind the individual who captured it when

surveys were conducted in channels and the marsh. When animals were captured in the ponds, they were placed in inflated zip lock bags with water and kept in the shade. This helped decrease the likelihood of recapturing the same individual during the same survey session. These individuals were then released back into the ponds and marsh unharmed when the capture session was complete.

Additionally, habitat measurements (see Habitat Characteristic Measurements below) were taken from random locations across the surveyed landscape (see Habitat Results Figures 2.2.2. & 2.2.3) where adults were not detected. This information was collected to determine OSF habitat preference during the non-breeding season in 2014.

Capture of adult OSFs was also done using float-enhanced minnow traps. The traps were set up with short (2-3 meter) aquatic drift fences to increase the probability of captures (Olson et al., 1997) (Figure 2.1.6).

Figure 2.1.6. Floating minnow traps. On the left is an image of multiple minnow traps placed in sections of Tilley Pond where they were connected with drift nets. On the right is an image of float enhanced minnow traps connected to a drift net in the East Channel of WRP, and an individual reaching into the trap through a zipper opening to retrieve the species within it. Photo credit: Sierra Blakely.

Traps were left open overnight and were checked within 12 hours (on the following day). Minnow traps were also left open while surveyors were present and conducting walking and floating surveys. Minnow traps were set up at intervals greater than 10 meters apart across the different aquatic habitats (marsh, ponds) at West Rocky Prairie. Trapped animals were then processed (see Animal Processing) in situ, and released 2-3 meters away from the trap to minimize the likelihood of recapturing the same individual during the same survey session. Minnow traps were discontinued during the study, as they did not capture more than 2 adults over the entire study, and the monitoring effort they required was substantial. Although they were discontinued, they were particularly useful for finding other species that are present in the wetland. These species included the Olympic Mudminnow (*Novumbra hubbsi*), Three-Spined Stickleback (*Gasterosteus aculeatus*), and larval stage northwestern salamander (*Ambystoma gracile*), which is shown in a minnow trap in Figure 2.1.7. Other species encountered across the wetland include the Common Garter Snake (*Thamnophis sirtalis*), Northern red-legged frog (*Rana aurora*), and leech (Hirudinea). Juvenile OSF, metamorphosed juvenile OSF, and OSF tadpoles were also observed. Appendix A contains the common and scientific names of observed flora and fauna at WRP during OSF adult non-breeding habitat surveys.

Figure 2.1.7. Incidental Species in minnow traps: Larval Northwestern Salamander. Photo Credit: Sierra Blakeley.

Animal Processing

Animals were processed according to standard WDFW protocols approved for handling amphibians in the field (Beaupre et al., 2004). Once OSF adults were captured using the aforementioned survey methods, they were processed using the following template. For display mapping purposes, a GPS coordinate was taken for the location of each animal captured in Decimal Degrees to the 6th decimal place. Air and water temperature, and weather condition (e.g., mist, rain, sun, cloud cover) were also immediately recorded at the location of observed or captured OSF adults. Temperature is an important covariate for understanding habitat utilization and also influences the likelihood of detection (M. Hayes, Personal Communication). Additionally, the general location of the individual was noted. These categorical notes included whether the animal was on land, in the water, or on the bank, whether they were in full sun or shade, and if they were on or near vegetation. For each captured adult, I also measured snout-vent

length (SVL) in millimeters where the animal was gently pressed on the ruler with the snout at 0mm, and measured to the vent (or tail). Animals with SVL greater than 50mm (M. Hayes, personal communication) were included in the study, as lengths >45mm indicate that males have reached sexual maturity (Hallock, 2013 via C. Pearl, personal communication) (Figure 2.1.8).

Figure 2.1.8. Snout-to-vent measurements: conducted for each animal and was measured in millimeters. Photo Credit: Chelsea Waddell.

Additionally, shank length (knee to heel) was measured in millimeters (Figure 2.1.9), and mass was measured for each animal in grams (Figure 2.1.10) to determine their body condition (Yahnke et al., 2013).

Figure 2.1.9. Shank measurement: taken in millimeters (mm). Photo Credit: Chelsea Waddell

Figure 2.1.10. Mass was measured in grams for each captured animal. Photo Credit: Chelsea Waddell

In addition to the measurements described above, I determined each captured animal's gender (Figure 2.1.11). For the Oregon spotted frog, gender is typically identified by looking at the presence or absence of nuptial pads, which are only present on males (Hallock, 2013). Males use these nuptial pads to latch on to females (ampelxus) during oviposition.

Figure 2.1.11. Male & Female Identification. Male (left) has a nuptial pad in the location of the thumb, and female (right) does not have the nuptial pad present. Photo Credit: Chelsea Waddell.

Photographs of the dorsal pattern for each captured adult were taken and used in a study-specific photo-book, in the form of a power point, based on the protocol established by WDFW. Adult OSF dorsal patterns remain similar across years and serve as a useful tool for rapid identification of individual OSF adults (M. Hayes, unpublished data). See Figure 2.1.12 for an example of dorsal pattern identification of an individual captured on July 14, 2014 and August 10, 2014. These photographs can be used to identify whether animals have been captured during previous years or previous survey sessions, and will be integrated into a master photo-book, which has already been

established for this site. The study-specific Power-Point photo-book was particularly useful for identifying individuals who had already been sampled for genetics in this study. The photo-book was updated prior to each field survey session.

Figure 2.1.12. Adult Oregon spotted frog dorsal patterns is a useful tool for rapid identification of individuals. The photograph (left) is a male captured on July 14, 2014; the photograph (right) is the same male recaptured on August 10, 2014. Photo Credit: Chelsea Waddell.

For each captured animal, all of the above measurements were taken before determining whether the animal had previously been captured during the study. The Power Point photo-book consisted of each individual captured at their first location of capture, their SVL, shank length, mass, gender, general location of capture, unique identifier code, and date of initial capture and sampling. See Figure 2.1.13 for an example of the photo-book.

Figure 2.1.13. For each sampled individual, a power point slide was developed. This included (from left to right) their Snout Vent Length (SV), Shank length, Mass, general location of capture, unique identifier code, gender, date of initial capture and sampling, and image of the animal. Photo Credit: Chelsea Waddell.

The captured animal would be compared, based on dorsal pattern, to the images within the photo-book, and would either be deemed a recapture (an animal that had previously been captured) or a new individual. If the animal were deemed a recaptured individual, it would not be sampled for buccal cells. However, if the individual were deemed a unique new individual, it would then have its mouth swabbed for buccal cells.

Field Sampling For Genetic Material

All captured and processed OSF adults, as described above, were also sampled for genetics by conducting the buccal sampling technique outlined in Pidancier et al. (2003),

Poschadel & Moller (2004), and Gallardo et al. (2012). Buccal sampling is a less invasive alternative to the more commonly used toe-clipping method for collecting tissue samples from amphibians. Tissue sampling included swabbing each captured unique individual's mouth with duplicate buccal swabs (Epicentre® Catch-All™ Sample Collection Swab). Mouth swabs were done only when the animal was deemed a unique individual based on In-Field dorsal pattern recognition, as describe above. Swabs were dried immediately, and stored at -20°C. See Appendix B for full protocol for buccal swabbing using the Epicentre© Catch-All™ Sample collection Swabs.

Habitat Characteristic Measurements

Multiple covariates were measured for locations where adults were captured and for locations where adults were not detected. In order to determine what habitat adults prefer based on their detectability during the survey, vegetation type, temperature, cloud cover, OSF juvenile abundance, and incidental amphibian and fish species presence were collected for all areas surveyed to determine which areas the adults preferred over others.

Survey Area by Region & Organisms Observed

During field surveys, areas were partitioned by geographic location. The locations included: Small (South) Pond, East Channel, Clearing North of East Channel, North Channel, West Channel, Area West of West Channel, Tilley Pond, Pond Adjacent to Tilley Road, and the East Side Survey Area. In order to quantify the number of times each area was visited, the master Excel spreadsheet was filtered based on these locations, and the sum of the number of days each site was visited was quantified. Additionally, the types of other species observed in these areas were listed.

ANALYSIS OF EFFORT METHODS

TOTAL EFFORT

Calculation of total effort during the detection surveys was based on start and end times from each survey, and summary statistics were calculated using Microsoft Excel. Specifically, the average number of hours for each survey was calculated by dividing the sum of hours for each survey by the number of survey days. The sum of the product of the number of hours a single survey took, and the number of surveyors present on that day, was used to calculate the *Total Survey Hours*. *Effort per Animal* was calculated by dividing *Total Survey Hours* by the number of adult Oregon spotted frogs captured, which included new captures and recaptures. This *Effort per Animal* calculation is based on the effort of each surveyor, and not based on the number of frogs captured within each survey.

CATCH PER UNIT TIME

Additionally, a calculation of *catch per unit time* was calculated by partitioning all of the survey observations into two-hour time intervals. For each time interval, the percentage of times an animal was captured during that time interval was calculated based on the total number of observations that occurred at that time interval. The total number of days animal observations made for each time interval was then summed.

SPATIAL ANALYSIS METHODS

All habitat distribution maps of captured and sampled adults were done using ArcGIS 10.2 (ESRI, 2015). The same World Imagery base map (ESRI, 2015) was used for every map developed in this study. All attributes were compiled from collected field data. Methods used for making these maps include point selection, and polygon formation based on GPS points collected in the field. The maps included in the Habitat Results section show the entire surveyed area, locations where adults were captured and sampled, locations where egg masses were observed and sampled, and vegetation types throughout the surveyed area. All points were based on GPS data collected with a Garmin handheld GPS unit; the average error for each point was 11.86 feet, based on the total number of points taken with the error noted (n=184).

SURVEY AREA & VEGETATION TYPE

Based on survey points, polygons were generated using the Create Features tool in ArcMAP 10.2. Polygons of the survey area were generated based first on viewing the ESRI World Imagery base map (ESRI, 2015), and then based on survey points gathered during field collection. Survey Area polygons were then broken up into 5 land cover types based on the dominant species observed; open water, reed canary grass, sedge, scrub/shrub/willow, and other (See Appendix A for scientific names). Using the Snapping Tool, each adjacent polygon was snapped to the one next to it to ensure the area of each land cover type equaled the sum of the total survey area. For each land cover type, multiple polygons were made adjacent to each other, depending on the orientation of other nearby polygons. To remove excess lines between polygons, the Dissolve tool in

ArcMAP 10.2 was used. To calculate the ‘Other’ land cover type, the polygons generated for open water, reed canary grass, sedge, and scrub/shrub/willow were merged, using the Merge tool in ArcMAP 10.2. The Erase tool was then used to erase the merged area from the total survey area; this generated the polygon for “other” in ArcMAP 10.2.

The areas of each land cover type, and the total survey area, were then calculated by using the Editing Tool and the Calculate Geometry tool in the attributes table of ArcMAP 10.2 (ESRI, 2015). Area was calculated in both acres (ac) and square meters (m^2). The difference between the survey area and the sum of all land-cover-type polygons was then calculated to demonstrate the accuracy of the snapping tool and polygon formulation.

ADULT LOCATIONS

With the total number of adults observed or captured during the study, the Select by Location tool in ArcMAP 10.2 (ESRI, 2015) was used to determine which land cover type the animals were observed in. These were then separated into captured and observed, and a map was generated to convey the locations of each observed or captured adult, and the land-cover types within the survey area.

HABITAT UTILZATION

RESULTS

EGG-MASS SURVEYS

Egg-mass surveys were conducted between February and March 2014, and eggs (n=218) for genetic analysis were collected from 109 egg masses at six locations. Figure 2.2.1 shows the locations where egg masses were observed. A total of 336 egg masses were observed among these sites, with the highest amount observed (n_{total}=288) at the west side survey area (west north, west central, west south). For the east side survey area (n_{total}=15), central east (n_{total}=24), central west (n_{total}=9).

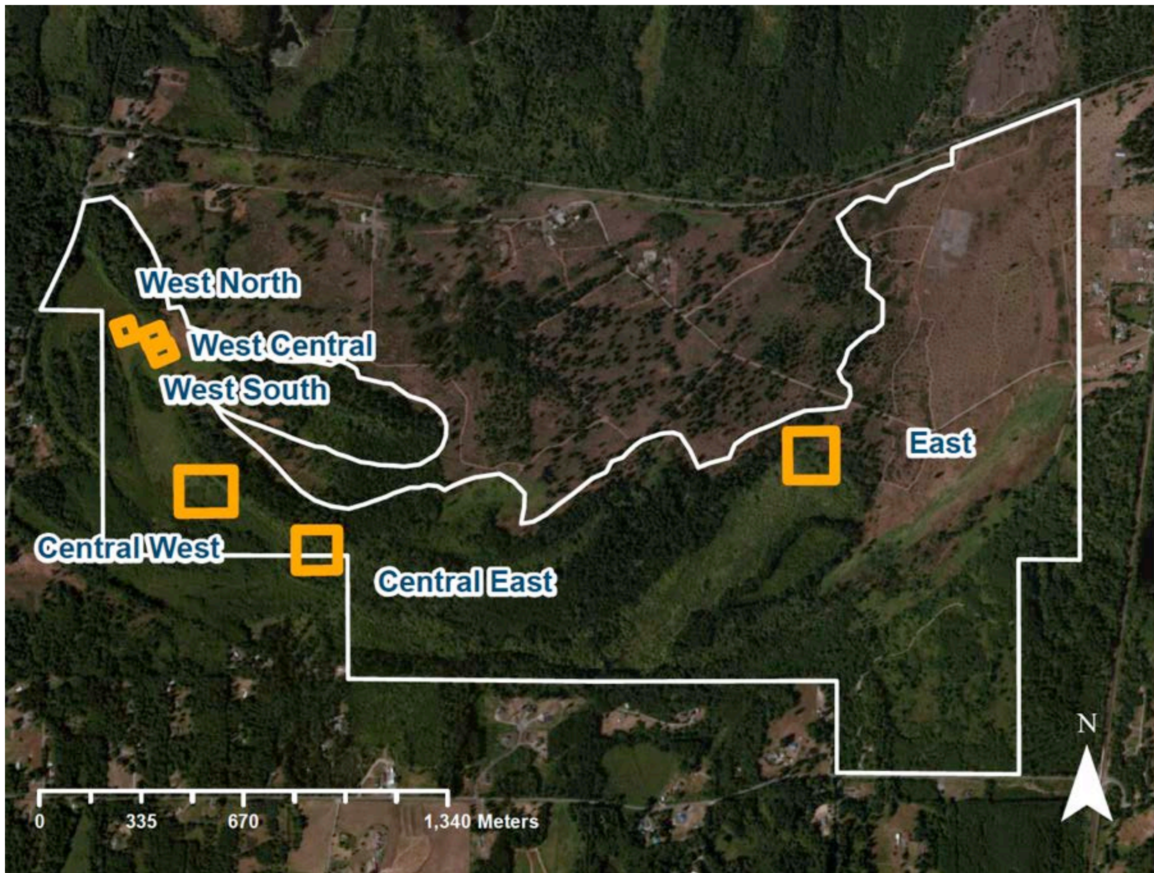

Figure 2.2.1. WDFW employees and volunteers at West Rocky Prairie collected egg masses between February and March 2014: egg masses were coded based on the six locations demarcated by orange boxes.

**World Imagery Base Map by ESRI 2015; West Rocky Prairie WDFW Management Resource Boundary; coordinates collected by WDFW employees and volunteers, 2014. Map developed by Chelsea Waddell (2015).*

NON-BREEDING HABITAT SURVEY AREA

A total of 29.1 acres were surveyed during the adult survey component of the study. Surveyed areas included the west and east sides of West Rocky prairie, and a small pond off Tilley road (Figure 2.2.3), which is southwest of Tilley pond (Figure 2.1.3 in Methods). These surveyed areas represent land types that are inundated with water during the wet season, in addition to areas of exploration where adult OSF surveys had not been done in the past. Figure 2.2.2 is a map of the overall survey area and the West Rocky

prairie state ownership polygon, while Figure 2.2.3 is zoomed in to the west and east side survey areas, respectively. Areas to the northwestern side of the west side survey area include exploratory survey expeditions for open water sources, where the hope was to find adult Oregon spotted frogs.

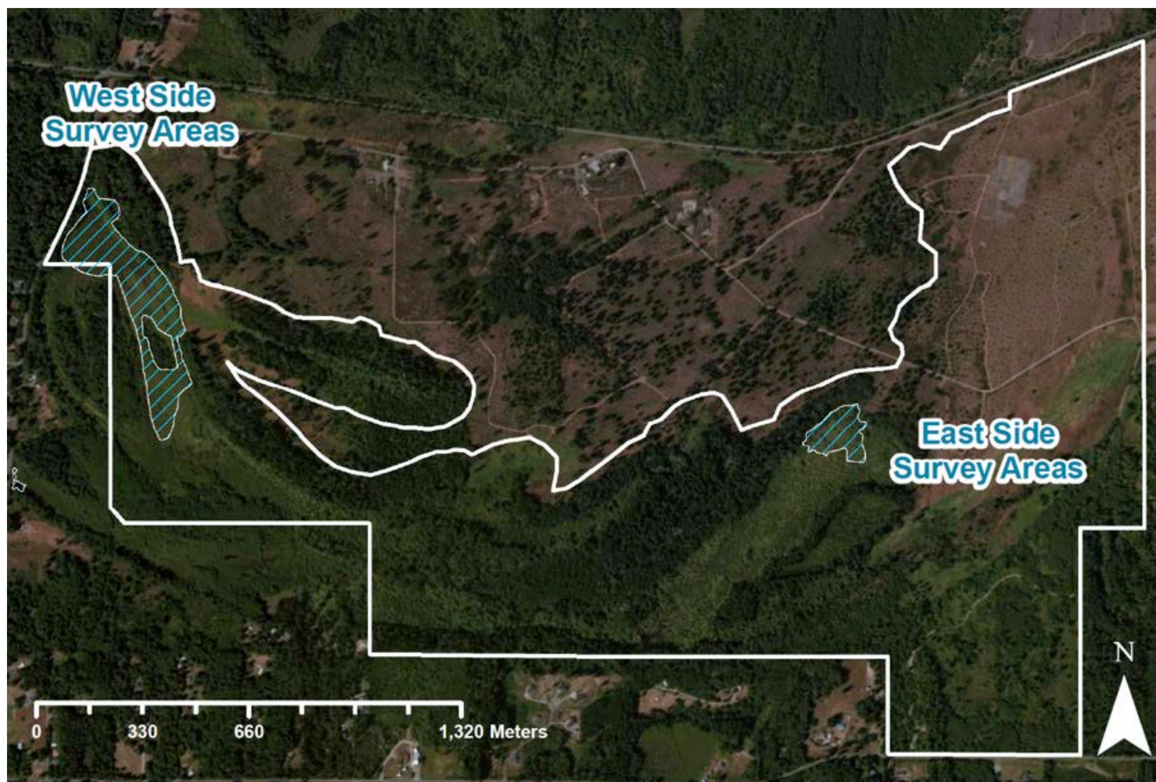

Figure 2.2.2. Map representative of the entire survey area within the Washington state owned West Rocky Prairie. Surveys were partitioned into two general areas: West Side and East Side, with particular attention to the West Side area.

**World Imagery Base map by ESRI 2015; West Rocky Prairie WDFW Management Resource Boundary; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map developed by Chelsea Waddell (2015).*

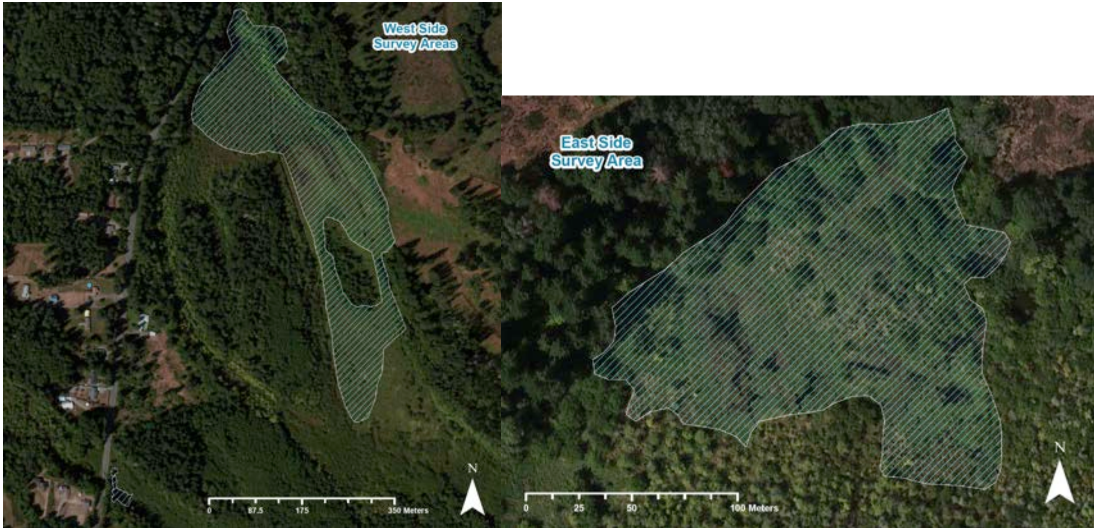

Figure 2.2.3. Map representative of the West Side Survey Area & Pond South of Tilley Pond (right) and the East Side Survey Area (left) area within the Washington state owned West Rocky Prairie. Surveys were partitioned into two general areas: West Side and East Side, with particular attention to the West Side area.

World Imagery Base Map by ESRI 2015; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map made by Chelsea Waddell (2015).

EFFORT

Adult capture surveys were conducted between July 22nd, 2014 and September 19th, 2014, with a total of 28 survey days. The surveys were partitioned into two sessions. The first session was between July 22nd and August 10th, which included 17 survey days. The second session was between September 4th, 2014 and September 19th, 2014, which included 11 survey days. Between two and four surveyors were present each survey day. A total of 13 surveyors participated in the adult capture component of the study, two of them were WDFW employees, and others included volunteers and myself. The average length of each survey was 5.76 ± 2.08 hours, which were typically performed during the mornings (Table 2.2.1). The total survey hours, calculated based on total person hours of effort, was 395.3 hours. Additionally, 194 new and recaptured adults were captured during the adult survey component of the study, each of which took an average of 2.04

hours to capture based on the total person hours. However, an average of 7.07 hours of effort was required to capture new adults, or those that had not yet been captured during the study. See Table 2.2.1 below for summary table of the outlined results.

Table 2.2.1. Summary of effort per adult captured

Surveyors (n)	Survey Days (n)	Average Survey Hours/ Day	Standard Dev.	Surveyor/Day (n)	Total Survey Hours	Adults Captured (n)	Person Hours/ adult captured	Person Hours/ new adult captured
13	28	5.76	±2.08	2 to 4	395.3	194	2.04	7.07

Additionally, observations per unit time were calculated in order to determine in which time interval the most captures occurred (Table 2.2.2.). Most observations occurred between 10:00 and 11:59 hours. This time period also had the most captures of adult Oregon spotted frogs. However, the highest percentages of adults observed per total observations were between 16:00 and 19:59 hours, indicating that these times may be best for capturing adult Oregon spotted frogs. Although, this result was likely confounded by the lower number of days (n=6) surveys were occurring during those time periods when compared to time intervals between 8:00 and 15:59. Additionally, those later times also had a lower number of survey locations where observations occurred.

Table 2.2.2. Summary of effort per adult partitioned by two-hour time intervals

Total Observations (n)	68	185	120	53	16	3
Days (n)	17	24	21	19	5	1
Animal Observations (n)	32	94	36	15	14	3
% Animal Observations	47.1%	50.8%	30.0%	28.3%	87.5%	100.0%

ADULT LOCATIONS BY LAND COVER TYPE

No adult Oregon spotted frogs were observed or captured in the East Side survey area or the pond next to Tilley Road. Adults were either observed or captured in the East Channel, the clearing North of the East Channel, the West Channel, the North Channel, Tilley Pond, and in the small pond in the West Side Survey Area (Figure 2.2.5). Although, the majority of adults captured were present in the small South Pond, which is 10×6 meters (Tyson & Hayes, 2014), and varied in depth over the course of the study (Figure 2.2.4). Of the 194 captured adults in this study, 161 or 82.99% of them were captured in the small pond.

Figure 2.2.4. Small Pond in the West Side Survey Area of West Rocky Prairie; Image taken on July 22nd, 2014.

The second most abundant location where adult Oregon spotted frogs were captured was the East Channel. A total of 25 adults (12.89%) were captured there, and many of those individuals were located in the part of the East Channel directly adjacent to the small pond, as can be seen in Figure 2.2.5. The other locations where adults were captured include the West Channel, where 5 adults (2.58%) were captured, the pond North of the East Channel, where 2 adults (1.03%) were captured, and the North Channel, where 1 adult (0.52%) was captured.

Figure 2.2.5 shows the geographic locations of the 194 captured adults, and the locations of observed, but missed adults. Adults were only observed in open-water land-cover types (Table 2.2.3), and the point within the small pond represents 161 captured adults and observations of missed adult Oregon spotted frogs.

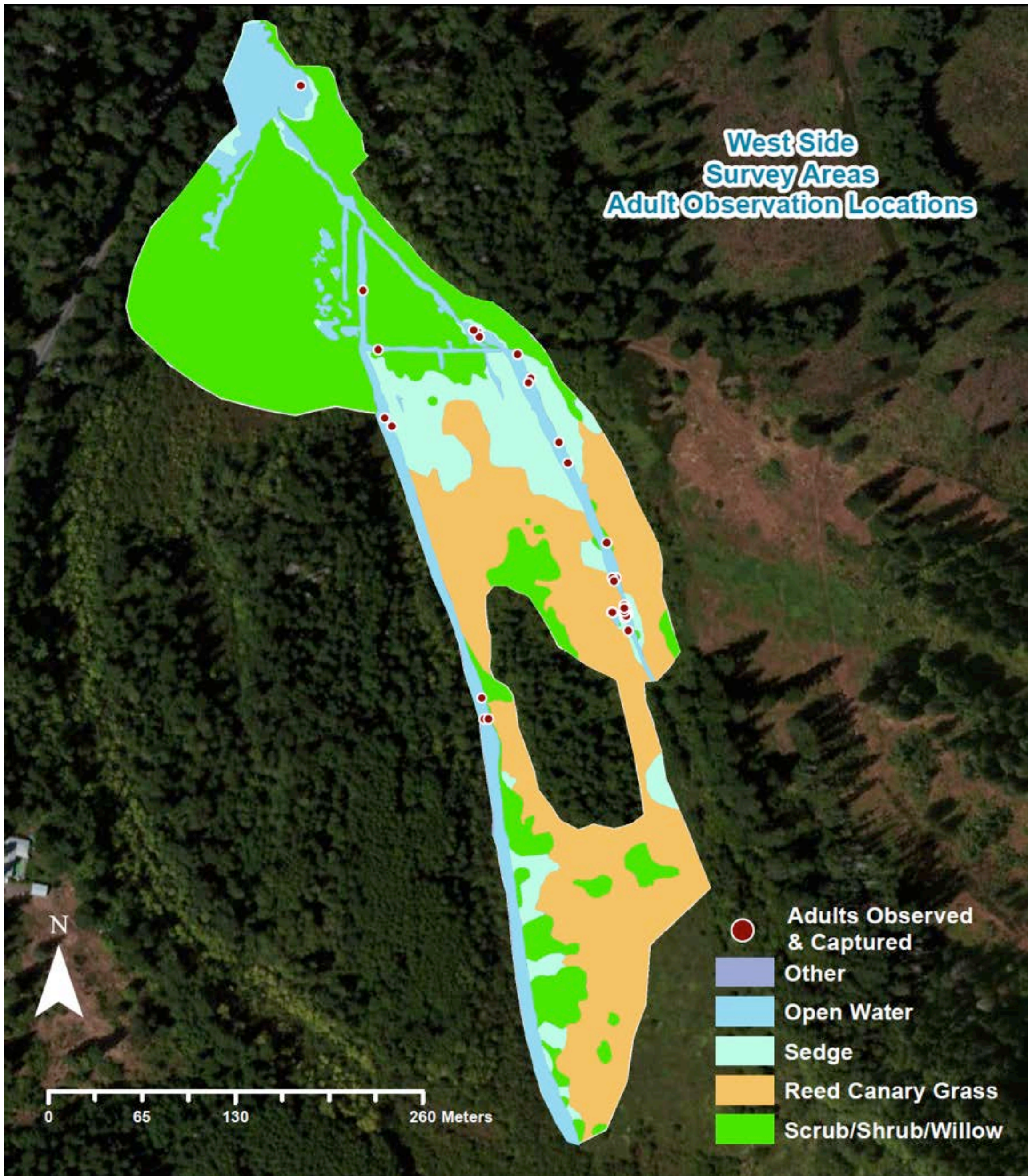

Figure 2.2.5. Locations of observed and captured adults, and the land cover types they were observed in.

**World Imagery Base Map by ESRI 2015; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map developed by Chelsea Waddell (2015).*

LAND COVER

Land cover type was partitioned into five distinct categories of dominant vegetation or land cover: *scrub/shrub/willow, sedge, reed canary grass, open water,* and *other* (See Appendix A for scientific names). The *other* land cover type generally represents an area that did not have survey points associated with it, or the land cover type was not distinguishable based on the 2015 ESRI World Imagery base map used for developing the land cover type polygons. The land cover types are depicted below (Figures 2.2.6, 2.2.14, & 2.2.16) in the form of three maps, each of which describes one of the three survey areas assessed in this study; they include the West Side Survey Area, East Side Survey Area, and the Pond/Clearing near Tilley Road.

ANIMAL PRESENCE

The area of each land cover type was calculated for all areas surveyed for this study. The total survey area was 29.1 acres based on the survey-area polygon, which included the West Side Survey Area, the East Side Survey Area, and the Pond adjacent to Tilley Road (Table 2.2.3). Each land-cover-type polygon was snapped to other adjacent land-cover types and the survey-area polygon, and encompassed a total of 28.77 acres, or 98.9% of the survey area. This result indicates that the snapping method used for describing the land cover types throughout the entire survey area was successful.

All 194-captured adult Oregon spotted frogs were observed in the open water land cover classification (Table 2.2.3).

Table 2.2.3. Area of land cover type and the number of captured adults within each.

Scrub/Shrub/Willow	0	11.0
Reed Canary Grass	0	7.8
Sedge	0	2.8
Open Water	194	5.6
Other	0	1.5
Total	194	29.1

SURVEY AREAS

While field surveys were being conducted, survey areas were partitioned into regions based on geographical features. These regions, and the number of times they were visited, are outlined in Table 2.2.4. The types of species detected within each area, and whether minnow traps were present, are also described in Table 2.2.4. Regions with higher abundance of adult OSF were visited more frequently than those where adult OSF were infrequently, or not detected, as the study objective was to determine where the parents of egg masses resided during the non-breeding season.

Table 2.2.4. Survey Regions: Number of surveys, use of minnow traps, and diversity of species present within each region.

Area West of West Channel	4	No	Juvenile OSF, Metamorphosed OSF
Pond South of Tilley Pond	2	No	Northern Red-legged Frog
East Channel	15	Yes	Adult OSF, Juvenile OSF, Metamorphosed OSF, OSF tadpole, Northern Red-legged Frog, Olympic Mudminnow, Three-Spined Stickleback, Common Garter Snake, Leech, Northwestern Salamander
East Side	2	No	Juvenile OSF, Northern Red-legged Frog
North Channel	6	No	Adult OSF, Juvenile OSF, Metamorphosed OSF
Clearing North of North Channel	7	Yes	Adult OSF, Juvenile OSF, Metamorphosed OSF, OSF tadpole, Olympic Mudminnow, Three-Spined Stickleback, Northwestern Salamander
Small Pond	13	Yes	Adult OSF, Juvenile OSF, Olympic Mudminnow, Common Garter Snake, Northwestern Salamander
Tilley Pond	8	Yes	OSF Adult (observed), Juvenile OSF, OSF Tadpole, Olympic Mudminnow, Three-Spined Stickleback, Northwestern Salamander, Common Garter Snake
West Channel	10	No	Adult OSF, Juvenile OSF, Metamorphosed OSF, Olympic Mudminnow, Northern Red-legged Frog
Area Between West Channel and East Channel	>2	No	No animals observed

See Appendix A for scientific names.

WEST SIDE SURVEY AREA

Figure 2.2.6 is a map of the land-cover types found throughout the West Side survey area. Each area was surveyed a minimum of 3 times on the West side, with the most frequent surveys occurring at the small pond. Since these surveys were conducted during the dry season in western Washington, many of the areas that are inundated within the wetland during oviposition time did not have water present. However, areas in the northwestern side of the West Side survey area, which is dominated by scrub/shrub/willow, was frequently inundated with water. The clearings west of the diagonal portion of the West Channel were areas of particular exploration for adult Oregon spotted frogs, and had not been surveyed for adults prior to this study. These areas were chosen through examination of aerial photographs that indicated the presence of water, which was confirmed by our surveys. Observationally, the amount of water within the open water land cover type decreased substantially between the survey session conducted from July to August, and the session conducted from August to September, 2014.

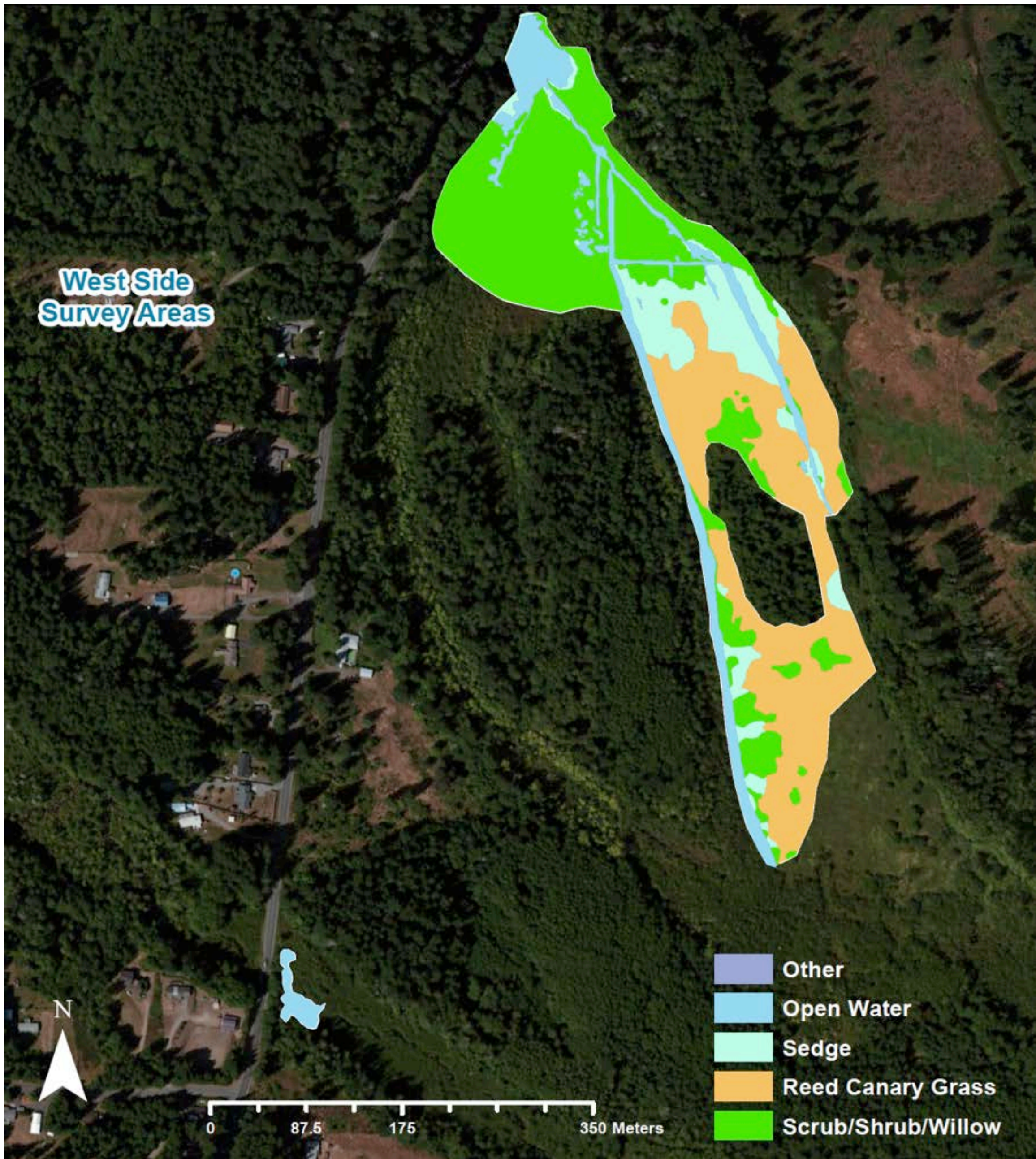

Figure 2.2.6. West Side Survey Area: Land Cover Type.

**World Imagery Base map by ESRI 2015; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map developed by Chelsea Waddell (2015).*

Small Pond

The Small Pond was the second most surveyed area (n=13), as this area had the highest abundance of detected adult OSF. Many species were present in this area, as demonstrated in Table 2.2.4. Additionally, minnow traps were set up in the small pond, but they yielded very few adult OSF. The small pond was surveyed predominantly by walking VES, but Floating VES was also used. Observationally, water levels decreased in the small pond between the time of the first survey session (July to August) and the second survey session (August to September) (Figure 2.2.7).

Figure 2.2.7. Images of the water level change in the Small Pond. The photograph on the left was taken on Aug. 10th, 2014. The photograph on the right was taken on Sept. 19th, 2014. Photo Credit: Chelsea Waddell & Cameron Smith.

East Channel

Portions of the East channel were surveyed most (n=15). This is, in part, due to the fact that the East Channel area directly adjacent to the Small Pond was surveyed at the same time as the small pond. Additionally, minnow traps were set out in this area. However, they yielded very few adult OSF and a high abundance of other species, as well as juvenile, metamorphosed, and tadpole OSF. All species were also detected within the

East Channel, including the Common Garter Snake (*Thamnophis sirtalis*). According to M. Hayes (personal communication), Common Garter Snakes prey upon young OSF. This area typically had water present (Figure 2.2.8), and was traversed using the walking Visual Encounter Surveys (VES). The northern extent was narrow with heavy vegetation, typically had water present, and was generally more difficult to traverse (Figure 2.2.8). During the second survey session (August to September 2014), water had disappeared from large portions of the East Channel.

Figure 2.2.8. East Channel: Image on the left is a portion of the southern extent of the East Channel taken on July 25th, 2015 (Photo Credit: Sierra Blakeley). Image in the center is a portion of the northern extent of the East Channel taken on Aug. 5th, 2014. Image on the right is the East Channel on September 9th, 2014. Photo Credit: Chelsea Waddell & Cameron Smith.

Area Between East and West Channel

The area between the East and West Channel was surveyed two times (Table 2.2.4). Although, in order to get to and from the West Channel and the Area West of the West Channel, the area North of the small pond between the East and West Channels had to be crossed. For this reason, many more visits to this area occurred than were actually included in a formal survey. No aquatic animals were detected in this region, although some shallow water on the North end was present during the first survey session. Walking VES was used to survey this area.

West Channel

The West Channel was surveyed on 10 occasions, and a diversity of species was encountered. The channel typically had water present, and at times the water was too deep to survey using chest waders. Therefore, both Walking VES and Floating VES were used there. Figure 2.2.9 below shows 3 images of the West Channel: One at a southern location past a beaver dam where adults were captured, another at the central area where adults were also captured, and a third at the northern extension where an adult was captured. Water levels also decreased in the West Channel, but this was most noticeable in the northern extent of the channel.

Figure 2.2.9. West Channel. Top left is and image taken on Sep. 12th, 2014 of the West Channel at a southern location past a beaver dam where adults were observed. Top right is the West Channel at a central location taken on Aug. 8th, 2014. Bottom left image, taken on Aug. 7th, 2014, is of the northern extension of the West Channel. Bottom right image, taken on Sep. 11th, 2014 is of the northern extension of the West Channel. Photo Credit: Chelsea Waddell & Cameron Smith.

Clearing North of North Channel

A clearing North of the North Channel was visited 7 times during the surveys (Table 2.2.4), and numerous species were detected there including adult OSF. This area typically had water present, and connected to the southern and northern extent of the East Channel (Figure 2.2.10). Minnow traps were set out at this location and Walking VES was used for surveys. This area also had an observable decrease in water levels during the second survey session (August to September).

Figure 2.2.10. Clearing North of North Channel: The photograph on the left is of the main section of the clearing and was taken on Aug. 7th, 2014. The photograph in the center is of the channel leading to where the corner of the East Channel and North Channel meet it. The image on the right was taken on Sep. 5th, 2014. Photo Credit: Chelsea Waddell & Cameron Smith.

North Channel

The North Channel, which connects the East and West Channels, was surveyed 6 times and a single adult OSF was captured there (Table 2.2.4). This area typically had water present, but no minnow traps were used. This area was, at times, difficult to traverse because of its depth, and the presence of a large, impassable willow in the center. However, Walking VES surveys were used to survey this area. Figure 2.2.11 is two images of the North Channel taken from its connection point with the West Channel,

showing what the channel looked like during the first session (July to August) and the second session (August to September).

Figure 2.2.11. North Channel. Image on the left was taken on Aug. 7th, 2014. Image on the right was taken on Sep. 11th, 2014. Photo Credit: Chelsea Waddell & Cameron Smith.

Area West of West Channel

The area west of the West Channel encompasses a large area dominated by dense, tall vegetation with occasional clearing and channels. This area was surveyed 4 times throughout the survey, and only juvenile and metamorphosed OSFs were detected in the clearings and channels (Table 2.2.4). These areas typically had water present with the heavy *scrub/shrub/willow* (discussed above) growing out of the water. Figure 2.2.12 shows what the dense vegetation and clearings looked like at the time of the surveys. The clearings during the first survey session had water in them. However, during the second survey session, the water was no longer present. These areas were typically very difficult to traverse, which may have influenced detectability of OSF there.

Figure 2.2.12. Area West of West Channel: Top right and left, taken on Aug. 1st, 2014 are of the *scrub/shrub/willow* dominant vegetation in this area. The image on the bottom left is of one of the clearings on Aug. 7th, 2014. The image on the bottom right is of one of the clearings on Sep. 11th, 2014. Photo Credit: Chelsea Waddell & Cameron Smith.

Tilley Pond

A primary focus of this project was to determine whether adult Oregon spotted frogs resided in Tilley Pond and it's connecting channels (Figure 2.2.13). However, after an exhaustive effort (8 surveys), only one adult Oregon spotted frog was observed. An effort was made to capture this adult, but failed due the amount of vegetation present.

Many species were detected in Tilley Pond (Table 2.2.4). Walking VES was used to survey the edges and connected channels of this pond, but Floating VES was used to survey the center of the pond, as it was too deep to traverse in chest waders.

Observationally, water levels decreased in Tilley pond during the second survey session (August to September, 2014), especially around the edges of the pond.

Figure 2.2.13. Tilley Pond on July 25th, 2014. Photo Credit: Sierra Blakeley

POND SOUTH OF TILLEY POND

(aka. Pond Adjacent to Tilley Road; Beaver Creek Pond)

The pond next to Tilley Road is directly south of Tilley pond and was approached as an exploratory measure to see if adult Oregon spotted frogs were present. The surveyed area was heavily dominated with deep open water (Figure 2.2.14), with channels leading to more open water on the east end of the pond area. The pond is connected to Beaver Creek, which runs east to west in the area directly under Tilley Road, in lowland western Washington.

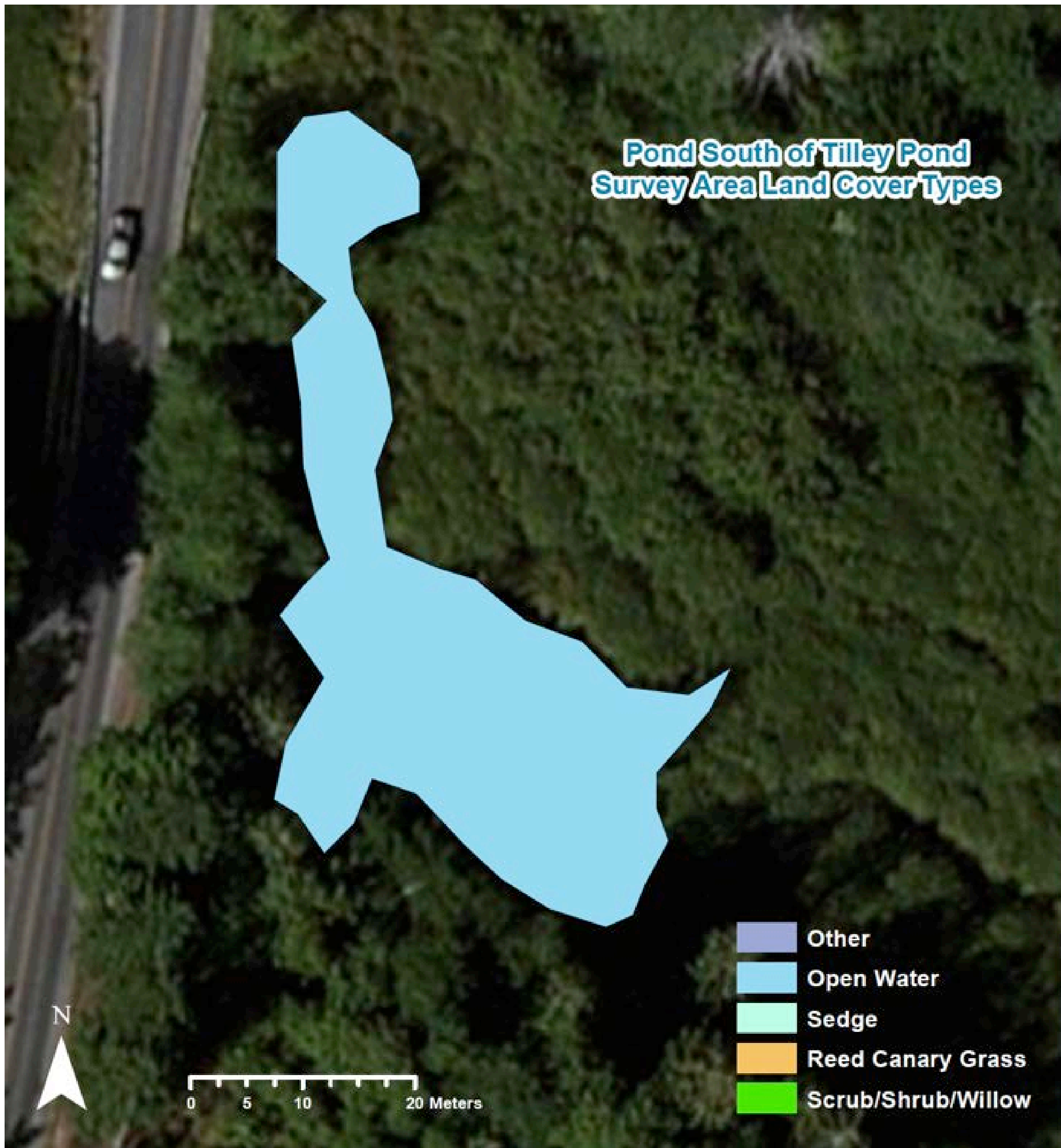

Figure 2.2.14. Pond South of Tilley Pond and adjacent to Tilley Road: Land Cover Type. Tilley road, which is represented in the map West of the pond.

**World Imagery Base map by ESRI 2015; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map developed by Chelsea Waddell (2015)*

The area (Figure 2.2.15) was surveyed two times (Table 2.2.4). This pond had water present during both surveys. Northern Red-legged frogs were observed in the pond, but no adult or juvenile OSF were observed or captured there. Floating VES were used to survey the area. No minnow traps were used in this area.

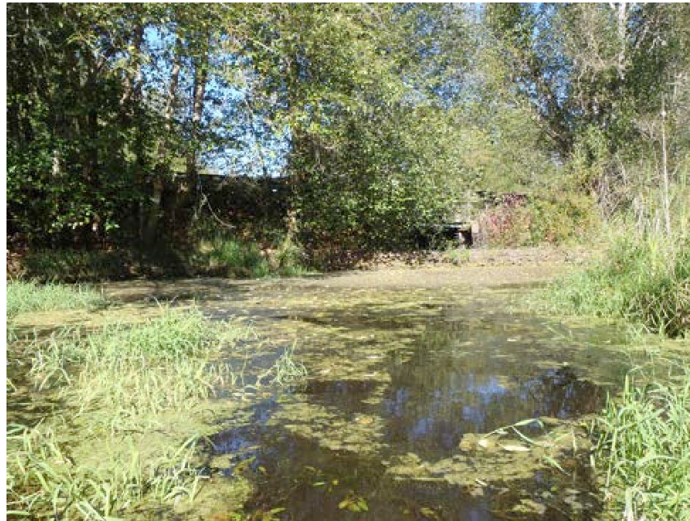

Figure 2.2.15. Pond South of Tilley Pond. Image taken on Sep. 12th, 2014. Photo Credit: Cameron Smith.

EAST SIDE SURVEY AREA

The East Side Survey Area (Figure 2.2.16) was visited twice (Table 2.2.4) during the adult survey time, as this area was particularly difficult and dangerous to traverse. The second survey was the most extensive survey of this area. The area included very deep mud, at times deeper than chest height. Land-cover type was assessed based on the methods described previously, and areas of open water often included large flowering lily pads, swarming with yellow jackets. Areas indicated as *other* were designated when survey points were not taken in those areas, and the vegetation was not distinguishable based on the ESRI World Imagery base map used (ESRI, 2015).

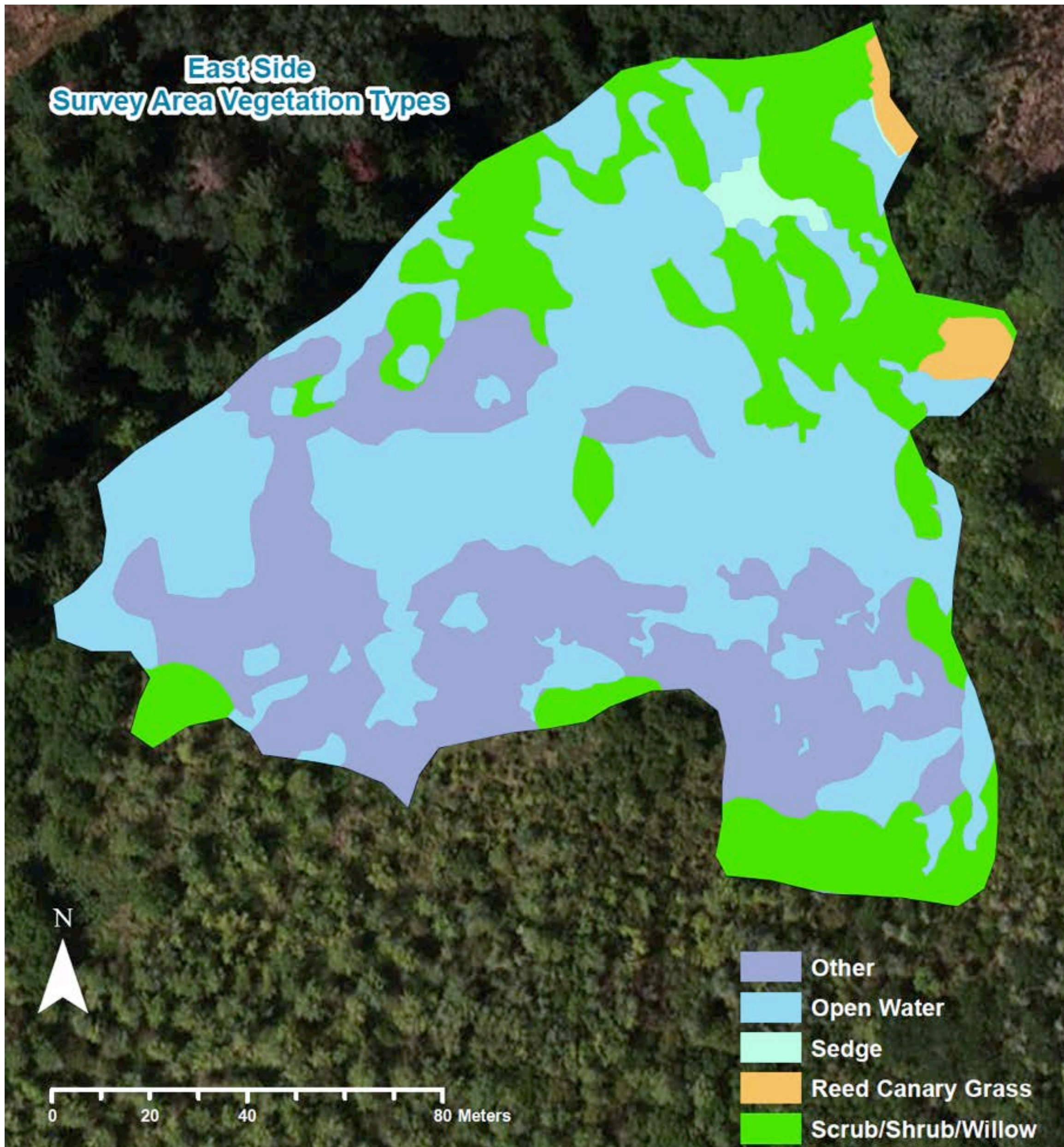

Figure 2.2.16. East Side Survey Area: Land Cover Type.

**World Imagery Base Map by ESRI 2015; coordinates collected by Chelsea Waddell, volunteers, and WDFW employees, 2014. Map developed by Chelsea Waddell (2015).*

To survey the East side survey area (Figure 2.2.17), walking and floating VES were used. Northern red-legged frogs were the most abundant species observed, and juvenile OSFs were observed.

Figure 2.2.17. East Side Survey Area. All three images were taken on Sep. 10th, 2014. Photo Credit: Chelsea Waddell.

DORSAL PATTERN RECOGNITION RESULTS

During field surveys, dorsal patterns were used to distinguish individuals who had been previously sampled, and those that had not yet been captured and sampled. During the first survey session, a PDA (Personal Digital Assistant) was used to view the pictures, however it is likely that the low resolution of the screen caused misidentification of individuals that had been previously sampled; thus causing repeat sampling of individuals. During the second survey session, images of previously captured individuals were viewed on an iPhone 4S, which had a substantially higher resolution and, observationally, made rapid identification simpler. Using this method in the field, 81 adult OSF were sampled, and deemed unique new individuals.

When adults were compared in the office on higher resolution screens in September 2014, the identification of repeat individuals was noticeably faster, and 58 of the 81 sampled adults were deemed unique, new individuals. Finally, when compared to the genetic results, 56 individuals were deemed unique individuals (See Chapter 3 Results for details on the results of this comparison).

CHAPTER 3

GENETICS

INTRODUCTION

OVERVIEW OF POPULATION GENETICS & AMPHIBIAN DECLINE

Population genetics is a growing and complex field in which genetic analysis informs researchers about the status of species populations. Areas of considerable concern in population genetics include gene flow, inbreeding depression, heterozygosity, allelic richness, and effective population size. These analyses can be performed at multiple spatial and temporal scales, and with varying objectives. Commonly, species' populations tend to decrease first at their extended range, causing them to decline inward to the center of their range (McKenzie et al., 2005). This pattern is evident in the Oregon spotted frog, as its current range is now much smaller than its historical one. OSF populations have declined in recent decades, and occupy only 10-30% of their original range (Blouin et al., 2010). The species historically persisted in southern British Columbia, western Washington, western Oregon, and northern California; it is now believed to be extinct in California, and parts of western Oregon (Blouin et al., 2010).

AMPHIBIAN POPULATION DECLINE

Amphibians are considered the most imperiled of the vertebrates, with 41% of them threatened with extinction (Monastersky, 2014). Many issues contribute to the decline of amphibians worldwide, but they can generally be broken down into two classes of factors, deterministic and stochastic. These factors affect amphibian population health, and can act additively or synergistically (Storfer et al., 2009). Class 1 factors, or deterministic factors, include habitat alteration and the introduction of invasive and non-native species. These factors can cause declines in food availability, and invasive species may prey on native amphibians or compete for resources (Storfer et al., 2009). Class 2 factors, or stochastic factors, include global climate change, infectious disease, and environmental contaminants (Storfer et al., 2009). As discussed in Chapter 1, deterministic and stochastic factors affecting OSF include the invasion of Reed Canary Grass, invasion of bullfrogs, and susceptibility to contaminants. Given the tremendous diversity of issues amphibian populations are facing, it is critical to understand how their populations are functioning in order to best manage them. Population genetics serves an integral role in endangered species management and has been increasingly used for many declining species, including the Oregon spotted frog (Blouin et al., 2010).

Habitat alteration and fragmentation can have major effects on amphibian populations. Landscape genetics has been used to address issues of gene flow (exchange of genes) among populations. Habitat loss and fragmentation can restrict the dispersal, and exchange of genes (i.e., gene flow), which is important for maintaining genetic diversity among populations (Storfer et al., 2009). Restrictions to gene flow can cause populations to have high susceptibility to inbreeding, which can cause further

demographic problems commonly associated with small population sizes (Storfer et al., 2009); this is also the case with the decline of the Oregon spotted frog (Blouin et al., 2010). Furthermore, when populations become small and isolated, they can lack genetic variability and thus, are less able to adapt to future environmental changes such as climate change and increased fragmentation (Storfer et al., 2009). With increasing habitat alteration and fragmentation, maintaining connectivity between habitats will enable gene flow and, ideally, functional populations. However, research needs to be conducted to determine how these populations function on large and small scales. The integration of population genetics with wildlife management undoubtedly increases the means with which we can manage threatened and endangered species.

The primary emphasis of OSF management has been to mitigate loss of habitat and fragmentation. These efforts have been supported with genetic information, which inform managers about their population structure, and how populations across their range differ genetically. However, more needs to be learned from looking at the small, isolated or distinct populations.

OREGON SPOTTED FROG GENETICS: CURRENT KNOWLEDGE

Genetic research on OSF has focused on understanding the divergence between populations and the population structure of the OSF across its range. This section directly addresses the current knowledge about genotypic variation across the OSFs geographic range. General knowledge of this information is critical to effectively managing their populations, and for interpreting the methods and results of this study.

Blouin et al. (2010) compared the genetic variability and health of the Oregon spotted frog to a related *ranid* species called the Cascade frog (*Rana cascadae).* Both species share much of the same general geographic range, but Cascade frogs are more abundant (Blouin et al., 2010) and generally occur at higher elevations. Based on 23 sampled populations, three major hierarchical groups, or clades, of Oregon spotted frogs exist across their range; a northern clade, a central cascades clade, and a southern Klamath basin clade (Blouin et al., 2010). Figure 3.1.1 is a map developed by Blouin et al. (2010) showing the OSF sample sites where genetic material was collected from OSF; there are circles around two of the three clades on the southern extent of the map. Locations north of the central cascades clade are considered part of the northern clade (Blouin et al., 2010).

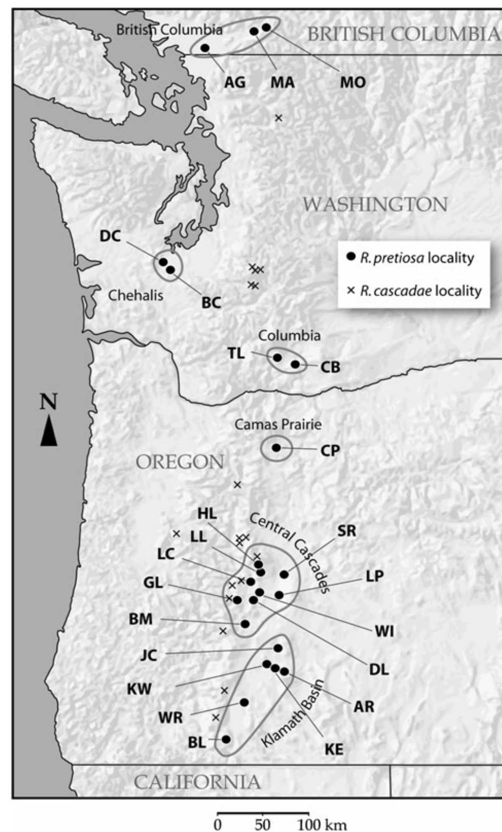

Figure 3.1.1. Sampled Range: Map of OSF sampling locations representing their geographic range, and clades. Figure adapted from Blouin et al. (2010).

Furthermore, hierarchical substructure was found within these three groups. Four subgroups exist within the northern clade, but weaker subgroup structure is evident in the central cascade and southern Klamath clades (Blouin et al., 2010). A Principle Coordinate analysis shows the genetic distances based on the allelic frequencies of all 685 individuals sampled in these three major groups and their substructures (Figure 3.1.2).

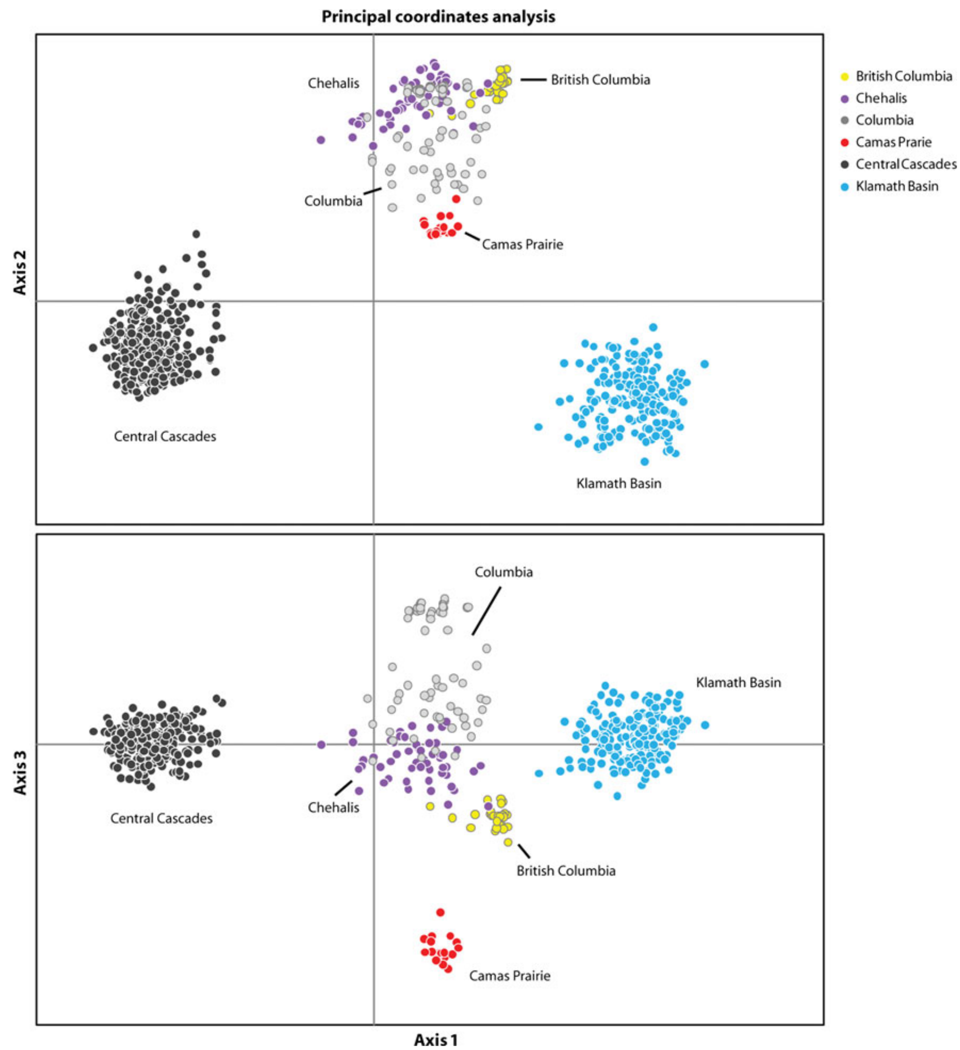

Figure 3.1.2. Principle Coordinates Analysis showing the Oregon spotted frog's three genetically distinct clades; genetic distances are based on the divergence of allelic frequency. Figure adapted from Blouin et al. (2010).

Given the amount of substructure in the northern clade, special attention should be paid to these populations (Blouin et al., 2010). The level of genetic distance between these groups indicates low connectivity and minimal gene flow between them. Gene flow is very small beyond 10km, and the distances between populations are typically larger than this (Blouin et al., 2010). It is therefore important to maintain healthy populations within the six subgroups (4 Northern, 1 Central, 1 South) because genetic rescue from nearby populations is not likely to occur. According to Blouin et al. (2010), these six subgroups should be considered Ecologically Significant Units (ESU), and therefore should be considered distinctly different for purposes of conservation (Blouin et al., 2010). The population of interest in my study is located within the Chehalis clade. While understanding this species divergence across its range provides critical information about the range-wide population structure, it does not paint the entire picture of individual population structures and functions.

Blouin et al. (2010) tested for deviations from the Hardy-Weinberg and genotypic equilibrium, which is the assumption that there is a constant level of genetic variation from generation to generation, for each of the populations (n=23). They determined that all populations tested showed little genotypic disequilibrium between loci (locations of a gene) and were in Hardy-Weinberg equilibrium (Blouin et al., 2010). They also tested for allelic richness (number of alleles which are alternative forms of a gene, AR) and heterozygosity (H_e). They found a mean H_e=0.31, suggesting that 31% of loci characterized were heterozygous instead of homozygous, and an AR_{15}=2.46, indicating that in a population size of 15 individuals, the mean number of alleles per loci was 2.46

(Blouin et al., 2010). These results indicate lower allelic richness and heterozygosity in OSF than found in the comparison species, Cascade frogs (Blouin et al., 2010).

Effective population size (effectively breeding adults, N_e) is a foundational principle used by conservation genetics. Based on a single season analysis, the OSF is thought to have especially small effective (0.1-0.4) population sizes when compared to their census population (Total population, N) (Phillipsen et al., 2009). Fluctuation in population size and variance in family size are thought to impact effective population size, especially in pond breeding amphibians like the OSF (Phillipsen et al., 2009). The influence of habitat factors, especially in breeding habitat, is likely the cause of OSF boom and bust population changes from year to year (Phillipsen et al., 2009). Additionally, their family sizes may vary greatly, since females only lay 1 egg mass per year, and males only fertilize one egg mass per year (Phillipsen et al., 2009). These egg masses are often susceptible to freeze, desiccation, and disease based on the conditions that year. However, single population studies are not fully indicative of general effective population sizes across the OSF's range, and should be expanded to areas across their range. In part, this project adds to our current understanding of effective population sizes (N_e) across OSF's range. Furthermore, the addition of parentage analyses, looking at the linkage between parents and offspring within a single population, can be extremely useful for understanding family relationships between individual OSFs in a small population.

Knowledge of population structure across the OSF geographic range has established precedence for further studies. This is especially the case for small, potentially isolated populations, as these populations are the primary focus of management.

APPLICATIONS OF POPULATION GENETICS

As species continue to decline worldwide, we must integrate knowledge from multiple conservation fields to most adaptively conserve and manage biodiversity. Population genetics is a burgeoning field which has substantially advanced our knowledge of how declining and healthy populations function. Its applications are vast in the conservation community, and it is especially applicable to conserving declining amphibian populations such as the Oregon spotted frog. Furthermore, there are sub-fields within population genetics and genomics, such as parentage analyses, which can help answer specific hypotheses.

PARENTAGE ANALYSIS OVERVIEW

Parentage analyses have been widely used by ecologists in diverse fields to obtain knowledge about wildlife population structures and the behavior of these populations. Parentage analyses use genotypes of individuals to assign paternity and maternity (Frankham et al., 2003). It has become a prevalent practice in the field of molecular ecology and has advanced quickly (Jones & Ardren, 2003). “Patterns of parentage play a central role in the study of diverse ecological and evolutionary topics, such as sexual selection, patterns of dispersal and recruitment, estimation of quantitative genetic parameters, and conservation biology” (Jones et al., 2010). Additionally, parentage information helps managers understand the impacts of inbreeding, determine the effective population size, and verify pedigree so that the species can be managed based on their genetics (Frankham et al., 2003).

In the 1980s, DNA fingerprinting advanced the field, and parentage analyses were often conducted to determine the behavioral ecology of bird populations (Jones & Ardren, 2003). When microsatellites were discovered (See Methods: Microsatellites), they quickly became the chromosomal section of choice in parentage analyses. Until recently, parentage analyses were predominantly conducted with avian and fish populations, this was primarily due to a lack of identified microsatellites for other, less commonly studied species (Jones & Ardren, 2003). Currently, the field is still growing to incorporate more areas of the genome, and more advanced computational analyses.

Parentage analyses utilize DNA obtained from the focal organisms and, ideally, DNA from both parents and offspring should be obtained. "The basis of paternity comes down to the fact that in the absence of mutation, a child receives one allele matching each parent at every genetic locus examined" (Butler, 2005). Microsatellites are commonly used for parentage analyses because they follow the rules of Mendelian segregation, where a child receives one allele from each parent (Jones et al., 2010). However, it is possible for both parents to share alleles. For this, there are various statistical approaches to determining parents of specific offspring (Butler, 2005). The methods used for conducting these types of analyses are discussed in the Methods section of this chapter. Methods begin with obtaining the tissue samples from the organism, followed by laboratory methods for extracting (See Methods DNA Extraction Section) the DNA from those samples, and amplifying them via PCR (See Methods Polymerase Chain Reaction Section). Finally, the parentage-analysis methods are discussed, and the methods for determining the population's N_e, and allelic diversity.

GENETICS

METHODS

Field, laboratory, and analytical methods were used for the genetic components of this project. Genetic samples from adults and egg masses were collected in the field (as described in Habitat Chapter Methods). Once the samples were collected in the field and frozen at -20°C, a series of laboratory methods were performed.

Staff in the Molecular Genetics Laboratory (MGL) at Washington Department of Fish and Wildlife (WDFW) completed the genetic analysis of two individual eggs from 109 egg clusters. Laboratory analysis for all adults was done in the same laboratory as the eggs. I performed the bench work for the adults with the assistance of Cherril Bowman, a senior research technician in the MGL at WDFW.

A description of the methods used to collect and store buccal swabs, followed by the methods commonly used for population genetics studies is below. I then discuss the methods used to conduct the laboratory component of the analysis, and finally I describe the analyses used to assess parentage with the program CERVUS and population structure with the program COLONY.

FIELD METHODS

Buccal swabs (Epicentre©) were used to collect samples from each adult Oregon spotted frog by swabbing the inside and back of the mouth. Mouth swabs were performed in duplicate for each animal in order to ensure high yields of DNA in the extraction process. These swabs were dried immediately in the field, and stored at -20°C according

to the label. See Appendix B for field method protocol. A total of 162 samples were collected from adult Oregon spotted frogs during this study (n=81 x duplicate samples), and a total of 218 offspring (n=109 egg masses x 2 offspring).

SOURCES OF DNA

Obtaining DNA samples from organisms is a key component of conducting an analysis such as the one described here. Beyond collecting the samples, there is a lot of basic research that goes into determining which genes will give enough power to assess population structure or conduct parentage analyses, for example, and finally developing primers to isolate them. There are currently three ways of targeting regions or types of DNA that are commonly used in genetic analysis for population studies: microsatellites, mitochondrial DNA, and Single Nucleotide Polymorphisms (SNP). These common regions are typically chosen based on the objective of the study, and are used to represent genetic differentiation between individuals within a population. These approaches represent genetic variability differently, as they can represent chromosomal DNA (microsatellites, SNP), mitochondrial DNA, or areas across the genome (SNP). For this study, microsatellites were used because the primer sets to isolate them have already been established for the Oregon spotted frog. A discussion of mitochondrial DNA and Single Nucleotide Polymorphisms can be found in Appendix C. Additionally, microsatellites are commonly used for parentage analyses (Phillipsen et al., 2009; Blouin et al., 2010), and there were 12 markers (loci) available through the MGL. These characteristics sufficiently give this study a high degree of power.

MICROSATELLITES (MICROSATELLITE LOCI)

Microsatellite loci consist of tandem repeats of sequences which are between 1 and 6 nucleotides long and repeat between 5 and 100 times (Allendorf et al., 2013; Jehle & Arntzen, 2002). Microsatellites are present in every eukaryotic genome and typically occur in large numbers (Jehle & Arntzen, 2002). In population genetics, genome mapping, and parentage analyses, microsatellites are the most commonly used DNA markers (Allendorf et al., 2013). Microsatellites are very common among similar species and therefore primers can frequently be used more universally than other loci types (Allendorf et al., 2013). Furthermore, microsatellites tend to have high mutation rates due to slippage during DNA replication, and show high levels of genetic diversity, even in small populations (Allendorf et al., 2013; Frankham et al., 2003).). Microsatellite loci are commonly used in amphibian population genetics and have been used to understand the genetic variability of Oregon spotted frog populations (Blouin et al., 2010; Phillipsen et al., 2009). They are also used for looking at genetic diversity of other threatened species (Frankham et al., 2003).

LABORATORY METHODS: MICROSATTELITES

DNA EXTRACTION: SOLID PHASE

Once samples were collected from adult OSFs and stored, the DNA needed to be extracted because samples contained substances other than DNA (Butler, 2005). There are multiple methods for extracting DNA from samples, but Solid Phase extraction using silica bead columns, enables high-throughput DNA extractions, and is widely available for purchase from Qiagen (Butler, 2005). First, tissue was removed from the swabs, and lysed using a proprietary lysate solution (Qiagen).

For this study, Qiagen DNA extraction kits (DNeasy Blood and Tissue Kits) were used for extraction from mouth swabs and eggs. Extracted DNA was then stored in 96 well plates at 4°C in the short term, and -80°C for the long term (Butler, 2005). DNA was extracted independently from all of the mouth swabs collected in the field to minimize cross contamination between duplicate samples.

POLYMERASE CHAIN REACTION (PCR)

After the DNA was extracted from samples, PCR amplifications were run with 12 previously developed markers (loci), some of which were used by Blouin et al. (2010). These fluorescently labeled primers were used to isolate and amplify the microsatellites. “PCR is an enzymatic process in which a specific region of DNA is replicated over and over again to yield many copies of a particular sequence” or region (Butler, 2005). Primers were annealed to the 3’ and 5’ ends of each DNA strand, and billions of copies of the region of interest were produced.

Depending on the type of primer used, the annealing temperature differed (Table 3.2.1). For this reason, the primer sets were run in Multiplex, or combinations of different primers in the PCR reactions based on the annealing temperature requirements of the primer sets. Multiplexing streamlined this process by allowing me to use fewer resources and PCR blocks while working in the laboratory (Table 3.2.1). Total volume for each PCR reaction was 10µL, with the following final concentrations: 1µL template genomic DNA, 1.5mM $MgCl_2$, 20µM dNTPs, 1X Promega PCR buffer, 50µM Promega Gotaq® (taq polymerase), and diH2O (Deionized). Some of the PCR reactions followed a "touch-down" protocol, while others were amplified using a GO reaction (Table 3.2.1). Touch-down PCR began with an initial two minute denature at 94°C; then 3 cycles of 94°C for 30 seconds, followed by 30 seconds of annealing temperatures (Table 3.2.1), then 72°C for 1 minute; this process was then repeated 36 times; finally, the reaction was held at 72°C for 10 minutes, and held at 10°C in the PCR block until they were stored at 4°C for preparation for the 3730. GO PCR began with an initial two minute denature at 94°C; then 39 cycles of 94°C for 30 seconds, then varying annealing temperatures (Table 3.2.1) for 30 seconds, then 72°C for 1 minute; finally, the reaction was held at 72°C for 10 minutes, and held at 10°C in the PCR block until they were stored at 4°C in preparation for the 3730.

Table 3.2.1. Table represents: Column 1 – either the primer sets were run in multiplex or on their own, based on annealing temperature (Column 6). Column 2 signifies the marker type used, Column 3 is the fluorescent dye tag color (red, green, blue, or yellow), Column 4 is the primer number. Column 5 represents the type of PCR reaction performed.

Multiplex/ Single	Marker	Dye Label	Primer Number	Reaction	Annealing Temperature °C
M1	RP26	**VIC**	2748, 2749	Touch-Down	**58°- 55°**
	SFC120	**NED**	2760, 2761		
M2	SFC134	**VIC**	2762, 2763	Touch-Down	**57°- 50°**
	RP415	**NED**	2756, 2757		
	RP17	**PET**	2742, 2743		
M3	RP15	**6FAM**	2740, 2741	Touch-Down	**50°- 45°**
	RP461	**VIC**	2758, 2759		
	RP22	**NED**	2744, 2745		
S1	RP193	**6FAM**	2752, 2753	Basic GO	**50°**
S2	RP23	**VIC**	2746, 2747	Touch-Down	**50°- 45°**
S3	RP385	**6FAM**	2754, 2755	Basic GO	**50°**
S4	RP3	**VIC**	2750, 2751	Basic GO	**50°**

3730 GENETIC ANALYZER

The 3730, like many other genetic analyzers, uses fluorescent dye tags, which flag the primers, or other regions of the amplified DNA (Butler, 2005). PCR products were loaded with primers in sets (or multiplexes) in 96 well plates (Butler, 2005). The 3730 Analyzer at the MGL uses a series of capillaries to detect the fluorescence and size of the DNA fragments (C. Bowman, personal communication, 2014). The output file has

colored (blue, green, red, yellow) label peaks (DNA size and quantity) for each present allele for each animal sample (Butler, 2005). The actual size of these DNA fragments, and DNA locus genotypes (alleles), must then be calculated using an algorithm called the Local Southern Method in a genotyping software program called GeneMapper (Butler, 2005). The samples in this study were run on ABI 3730 DNA Analyzer either in multiplexes or as single reaction sets. Alleles were sized based on number of base pairs and calculated using the local southern method based on the GS500LIZ_3730 internal lane size standard.

LOCAL SOUTHERN METHOD & GENEMAPPER

With microsatellites, the number of tandem repeats that occur at each locus indicates the allele (Butler, 2005). These peaks are then compared to an allelic sizing ladder, which includes known sizes for each allele (Butler, 2005). The loci, which look like colored peaks in the file output, are sized using an "internal sizing standard", called the Local Southern Method (Butler, 2005). In this study, the internal sizing standard was the GS500LIZ_3730. For each allele, this method calculates the size of two peaks on both sides of the unknown peak being measured (Butler, 2005). The product was an allele genotype, which is the size/number of tandem repeats for an allele (Butler, 2005). For this study, this process was done in a program called GeneMapper, a commonly used program for scoring microsatellites (Butler, 2005).

Occasionally, scoring errors occur with this and other programs, typically from background fluorescence from other microsatellites run in the same matrix/multiplex (Butler, 2005). For this reason, I first allowed GeneMapper to score the alleles based on

its own programming, then systematically went through the generated scores to see if I agreed with them. Finally, I checked all scores with an experienced technician, Cherril Bowman.

The markers used for this study (n=12) and allele lengths were previously established and have been used in previous analyses conducted with the MGL. These allele lengths were in the form of bins, or shaded areas on the screen at a certain value. For example, the allele type 208 for the marker RP18 represents 208 base pairs, this allele was identified prior to my study, and it was therefore considered a bin. Each scored allele was put into these previously identified bins (alleles); no new alleles were discovered in the West Rocky Prairie adult population. Finally, these scored alleles were put together in a series of numerical values for each allele in a spreadsheet, giving me an output of the sample (or animal) genotype, based on the microsatellites used (Butler, 2005).

Within the same marker, 2 alleles would be scored. If there was a single peak at a number of base pairs, the individual was scored as homozygous. This means that both parents, according to Mendelian genetics, contributed the same allele. For example, for the loci marker RP15, a homozygous individual would have a score of 200, 200. However, if there were two peaks for an individual, that individual was deemed heterozygous. For the loci marker RP15, a heterozygous individual would have a score of 200, 208, which means that each of the individual's parents contributed different alleles. One element of scoring error may be the presence of null alleles, which are the absence of one microsatellite from one parent due to mutations at the primer's annealing site (Chapuis & Estoup, 2007). This can cause a misrepresentative higher ratio of homozygous alleles (Chapuis & Estoup, 2007), and is represented in the allelic richness

file outputs in parentage programs (see Parentage Below). These scores (number of base pairs) were then exported as a spreadsheet, which were then used to conduct multiple genetic analyses, including parentage assignments. Spreadsheets for both offspring and adult genotypes were developed, and used for parentage analyses. These spreadsheets included the sampled individuals, each of the 12 markers, and the allele scores.

MATCHING DUPLICATE SAMPLES

In order to determine whether the same adults were sampled more than once, an MS Excel plug-in, Microsatellite-Toolkit, which looks at repeat genotypes, was used. I used this method to determine the accuracy of the dorsal pattern recognition method used in the field component of the study. The output matches samples based on the scored alleles of all tested samples. Between two samples, it gives a score, which is the percent of alleles that match each other within the samples, the number of alleles that were compared, and the number of alleles that match.

PARENTAGE ANALYSIS: METHODS USED

Based on the literature, natural history, collection methods, and expert advice by Kenneth Warheit Ph.D and Maureen Small Ph.D, a number of methods were used to determine parental assignments for this project. As described below, there are five different types of methods to choose from for this type of analysis (Jones et al., 2010), and each researcher uses varying methods, depending on the objective of their study. The types of approaches used for this project include categorical allocation using a likelihood approach with the program CERVUS 3.0.7; Parental reconstruction, a commonly used

method for amphibians, was performed using the program FRANz, although the results of this analysis are not reported (Riester et al., 2009); and finally, the program COLONY (Jones & Wang, 2009) was used to determine parentage, the relationships between individual adults, and the effective population size. This section begins with a discussion of the two overarching methods used by researchers for parentage analyses. It then details the specific methods and programs used to run the parentage analysis in this study, and the methods and program used to determine the effective population and adult sibling relationships. See Appendix D for a discussion of the other types of methods and programs that can be used to meet different objectives for parentage analyses. Appendix D outlines the methods I also used for determining parentage using the programs FRANz and COLONY, as this information is only briefly discussed here, and not reported.

The two overarching approaches to conducting a parentage analysis are exclusion and likelihood. Parentage analysis by exclusion is preferable and relatively simple to understand, but is typically difficult to achieve. Therefore, multiple approaches to parentage analysis by likelihood have been designed for use when exclusion is not achievable.

EXCLUSION

Exclusion is considered the simplest technique in parentage analysis, and "is based on Mendelian rules of inheritance", where each parent contributes a unique allele to the offspring (Jones & Ardren, 2003). The genotypes of a candidate parent are compared with that of the focal offspring (Jones et al., 2010). "Any candidate parent who fails to share at least one allele with the offspring at any locus is eliminated from

consideration" (Jones et al., 2010). While perfect exclusion is the ideal, it is often difficult to achieve because parents may share, and therefore contribute the same alleles to their offspring. There are also multiple weaknesses to strict exclusion approaches, as genotyping errors, null alleles, and mutations often produce false exclusions (Jones & Ardren, 2003). These errors also become more common as datasets become larger because of scoring errors (discussed in GeneMapper section). When assigning parents to offspring, exclusion of individuals is attempted first, to exclude as many parents as possible. For the remaining non-excluded parents, parentage assignment/allocation is performed using statistical likelihood methods.

LIKELIHOOD

Likelihood methods either "assign progeny to nonexcluded parents based on likelihood scores derived from their genotypes" (Jones & Ardren, 2003), or they use a posterior probability to assign progeny (Jones et al., 2010). To assign parents to offspring based on likelihood, a likelihood-ratio is calculated for each adult using a frequentist statistical approach based on hypothesis testing. A likelihood ratio is calculated as the likelihood of paternity or maternity of a sampled adult/parent compared to the likelihood of paternity or maternity of an arbitrary adult/parent (Marshall et al., 1998). Alternatively, posterior probability uses a Bayesian approach, where parents are allocated with a probability using a known maternal or paternal genotype. Currently, there are five methods used for parental allocation, all of which have likelihood and posterior statistical approaches. Categorical Allocation and Sibship (Sibling Relationship) Reconstruction were specifically used for this project; other methods are discussed in Appendix D.

CATEGORICAL ALLOCATION & CERVUS 3.0.7 ANALYSIS

For parentage analysis, categorical allocation is the most commonly used and is a "method to choose the single most likely parent from a group of nonexcluded putative parents" (Jones et al., 2010). This is the primary method used for assigning parents to offspring in this project. Similar to exclusion, this method also requires a set of two candidate parents and a single offspring, and it serves as an excellent alternative for cases where perfect exclusion cannot be achieved (Jones et al., 2010). Both posterior probability and likelihood approaches can be used for categorical allocation (Jones et al., 2010); a likelihood approach was used for this study. Both approaches adhere to Mendelian transition probabilities, which is "the probability of the offspring's genotype given the genotypes of the mother and father" (Jones et al., 2010; Marshall et al., 1998). With categorical allocation, the entire offspring is assigned to a parent within the sample that has the highest likelihood or posterior probability of being the actual parent (Jones et al., 2010). Categorical allocation can be applied when a parent is already known for an offspring, or when no parents are known, as is the case with this study; it can also be used to assign one parent or parent pairs (Jones et al., 2010). This method can deal with scoring errors (See Laboratory Methods in this chapter) and mutations, and can calculate confidence in parentage assignment (Jones et al., 2010).

A program called CERVUS 3.0.7 is widely used to perform this type of analysis, and was used to determine parentage for this project. CERVUS 3.0.7 requires a number of assumptions, which were met by this study; it analyzes markers, such as microsatellites and SNPs (Described in Appendix C), assumes that the organism is diploid (two alleles per locus), and assumes that markers are inherited independently (Kalinowski et al., 2007). CERVUS 3.0.7 performs parentage analyses in three steps,

beginning with an Allele Frequency Analysis, followed by a Simulation of Parentage Analysis, and finally the Parentage Analysis.

Allele Frequency Analysis

Allele Frequency Analysis is required for parentage testing that uses likelihood (Kalinowski et al., 2007). A file with all genotypes (scored spreadsheet) from all sampled members of the population, including offspring and parent genotypes, were imported into CERVUS 3.0.7. From this genotype file, the frequency (how many and how often) of each allele present in the population is calculated for each locus tested (Kalinowski et al., 2007). It also calculates a suite of summary statistics for Hardy Weinberg Equilibrium, and chi-squared statistics for observed and expected heterozygosity (Kalinowski et al., 2007). This allele frequency file helps the program and researcher determine the suitability of the loci used for the continued analysis.

Simulation of Parentage Analysis

Simulation of Parentage Analysis is "used to calculate critical values of likelihood ratios (test statistic), so that when parentage analysis is carried out using real data, the confidence of parentage assignments can be determined" (Kalinowski et al., 2007). The allele frequencies file is used to run the simulation, along with the number of likely parents in the population and the percentage of the population that was sampled. For the population at WRP, 336 egg masses were laid in 2014; therefore, based on a 1:1 female to male sex ratio (Phillipsen et al., 2009), about 336 males and 336 females are expected to be in the population. The number of simulations run for this analysis was 100,000, as

suggested by the program to maximize power. Additionally, given that there are about 336 males and 336 females, the number of possible offspring genotypes is approximately 112,896, making 100,000 simulations an appropriate number.

Parentage Analysis

Parentage analysis uses both the simulated file and the allele frequency file to assign offspring to parents (Kalinowski et al., 2007). For each offspring sampled, the most likely parent was assigned based on the pre-determined level of confidence from the simulation or the offspring is left unassigned (Kalinowski et al., 2007). The parentage analysis that was run for this study included the parents gender based on field observations. The minimum number of loci typed was 6, meaning that if a candidate parent or offspring had less than 6 loci scored, they were not included in the analysis. The confidence results were partitioned into three different categories: strict assignment (95% confidence), relaxed assignment (80% confidence), and unassigned, which was partitioned into two sub-categories a) the most likely candidate that was not assigned parentage and b) unassigned (blank). In the analysis for this study, only the most likely candidate parent was assigned to each offspring.

Parentage-analysis output files included the identification of each offspring and its most likely mother, father, and family set. They also included a non-exclusion probability, which is the probability that the individual is included. The number of loci that were typed for each assigned parent and offspring, the number of loci that were compared between the offspring and parent, and the number of loci that do not match between the offspring and the parent were also in the output files. They also gave a pair

(single parent and offspring) and family (both parents and offspring) an LOD score, which is the log of the overall likelihood score. A positive LOD score means that the assigned parent is more likely to be the actual parent than not the actual parent (>50% likelihood) (Kalinowski et al., 2007). An LOD score of zero means that the assigned parent is equally likely to be the parent as it is not to be the parent (50% likelihood) (Kalinowski et al., 2007). A negative LOD score means that the assigned parent is not likely to be the actual parent (<50% likelihood) (Kalinowski et al., 2007). This LOD score is directly related to a Delta score, which is given for each pair and family (Kalinowski et al., 2007). The Delta is the difference in the LOD scores between the most likely parent and the second most likely parent. Finally, a measure of confidence is in the output file, where * is 95% confident assignment, + is 80% confident assignment, - is the most likely parent that was not assigned parentage, and blank is unassigned (Kalinowski et al., 2007).

Assignments were determined by a number of criteria that first relied on the assignment of the same parent to both offspring within an egg mass, based on the assumption that a single female lays an egg mass, and a single male fertilizes it (Phillipsen et al., 2009). For example, for offspring A and B from egg mass "1", if different mothers were assigned to A and B, then the offspring were not included in the final results. If offspring A and B from egg mass "1" were assigned to the same mother, the confidence was then assessed. Parent:offspring pairs with confidence as the most likely unassigned parent, 80% confidence, and 95% confidence were included; those left unassigned (blank) were not included for further analysis. LOD scores were then looked at, and if both offspring were assigned to the same parent with positive LOD scores they

were included. Negative LOD scores were infrequently included, unless it was the most likely candidate and the other offspring was assigned with confidence (>80%) to the same most likely candidate parent. Delta scores were then assessed. If Delta scores were the same or higher than the LOD score for that parent:offspring pair, the pair was included. If Delta was substantially lower than the LOD score, zero, or negative, the parent:offspring pair was not included. If the same parent was assigned to multiple egg masses, the parent pair was not included unless one of the assignments had >80% confidence. These methods allowed me to determine the parent:offspring assignments using categorical allocation and the program CERVUS.

FRANz ANALYSIS & PARENTAL RECONSTRUCTION

Besides using the program CERVUS, the program FRANz was also used to initially assess parentage for this study. However, FRANz uses a parental reconstruction likelihood approach, which requires >10 offspring from a single egg mass for adequate reconstruction. For this reason, the parentage assignments from FRANz are not reported, although a detailed description of the methodological approach FRANz uses in parental reconstruction is outlined in the Appendix D.

SIBLING RELATIONSHIP (SIBSHIP) RECONSTRUCTION & COLONY ANALYSIS

This approach is meant for studies where parents are not available for parental assignment, or it can be used to look at the relationships between parents. For the purposes of this study, it was used to assess the relationships (relatedness) between parents, and the effective population size (N_e) at WRP. This program can also be used for parentage analysis. It uses siblings, both full and half-siblings, to reconstruct the parental

genotypes, much like parental reconstruction except that the offspring are not assigned parents when they are not available (Jones et al., 2010). The siblings are assigned to different classes of relationships, typically full and half sibling relationships (Jones et al., 2010). Once the groups are identified, Sibship's can be used to reconstruct parental genotypes, and be used for parental analysis (Jones et al., 2010). Both likelihood, and Bayesian posterior probabilities can be used for Sibship reconstructions (Jones et al., 2010).

COLONY was primarily used to identify the sibling relationships between the adults sampled by constructing all of the potential genotypes of sibling parents within the population and identifying those sampled parents that were siblings. These siblings were then compared to the parentage assignments based on CERVUS to identify whether any sibling parents were assigned to both eggs from an egg mass; if siblings were assigned to the same egg mass, they were not included in the parentage results. Also, these sibling relationships demonstrate the amount of relatedness within the population. Additionally, COLONY generated an output showing the N_e based on all sampled individuals. In order to assess the relationships between candidate parents, COLONY also ran a parentage analysis, which is not reported here, but is discussed in Appendix D.

SPATIAL ANALYSIS

In ArcMap 10.2 (ESRI, 2015), parents were selected from the total number of captured individuals, and a parent's feature-class was made which included all of the times each of the animals were captured. Then, for each parent, a new feature-class was made, which included all of their capture locations. Offspring within the parentage analysis were designated by geographical location (ex. East Side), not a specific latitude/longitude point for each individual offspring, as this information was not available. For this reason, the egg mass within each geographical region that had the most eggs within a cluster was chosen to represent the geographic area, as it can be assumed that it was the most likely to have the most eggs taken from it. For each geographical area, the number of individual egg masses present, the number of clusters present at each location, and the number of egg masses sampled per location was calculated. In ArcMap 10.2, the distance between parent points and offspring points (in meters) was generated using the Point Distance Analysis tool. This method was chosen because of the variability of the landscape and lack of knowledge about possible modes of travel between the points. The animals could have traveled down the channels or traveled across the wetland by any number of pathways.

GENETICS

RESULTS

SAMPLED ADULTS & OFFSPRING: A COMPARISON OF DORSAL PATTERN & MICROSATELLITE TOOL KIT

Of 81 duplicate adult samples collected in the field, all were genotyped in the laboratory to confirm whether repeat sampling of the same individual occurred and to confirm the efficacy of dorsal pattern recognition methods. The final number of adult individuals (n=56) was determined based on the spot pattern recognition discussed in Chapter 2, and the microsatellite plug-in for Excel, which was discussed in the Methods section of this chapter. In order to prepare the genetic data for an accurate parentage analysis, duplicate parents (n=25) were removed from the parent genotype files. In order to do this, I compared the “match individuals” based on genetics to the “matched individuals” based on the high-resolution comparison of the dorsal pattern images. The results of both comparisons are explained further in Table 3.3.1 below.

Three animal sets showed discrepancies between spot pattern recognition, and genetic analysis. Based on spot pattern recognition on September 23, 2014, adults 0003, 0022, 0033, and 0048 were matched. However, the genetic analysis did not match adult 0022 to the other three animals because marker Rp15 was likely mis-scored during the scoring process using GeneMapper. Animal number 0022 was deemed homozygous at Rp15, while the others were deemed heterozygous for Rp15. Based on the combination of spot pattern recognition and the likelihood that Rp15 was mis-scored, animal number

0022 was removed from the parentage analysis, and considered a duplicate individual. The representative sample for this group of samples from a single adult is 0003.

Animal codes 0007, 0028, and 0043 were matched based on high-resolution spot pattern recognition on September 23, 2014. However, due to the lack of the Rp15 and Rp23 markers in 0043's genotype, the microsatellite plug-in matching software did not recognize it as a match. Animal number 0043 was removed from the parentage analysis, as it is the same animal as the others based on the scores matching for all of the remaining 10 markers, and spot pattern recognition. The representative sample for this group of samples from a single adult is 0007.

The genetic analysis matched 3 sampled individuals, 0027, 0034, and 0041. However, 0027 was initially marked in the field as a female, and 0034 and 0041 were marked as male. Their spot patterns are nearly identical, as identified on September 23, 2014. Therefore, based on the genetics, and similarity of spot pattern, it is likely that this animal was misidentified in the field as a female, and is actually the same individual as 0034 and 0041. The representative sample for this group of samples from a single adult is 0034. The results of the microsatellite match program, which matches individuals based on genotype, are shown below in Table 3.3.1.

A total of 56 adult individuals and 218 offspring were genotyped at 12 polymorphic microsatellite loci for parentage analysis in this study. Of the attempted 218 genotyped offspring, 216 were successfully genotyped.

Table 3.3.1. A total of 81 samples were collected in the field, representing 56 unique individual adults. Based on dorsal pattern recognition and microsatellite Excel plug-in, this table shows the individuals that were sampled more than 1 time. This table does not represent all 56 individuals used in the parentage analysis.

Adult Sample	Match 1	Match 2	Match 3	Percent Alleles Typed/Matched	No. Alleles	No. Alleles Matched
0001	0055			100%	24	24
0003	0033	0048	0022	97.90%	48	47
0004	0054			100%	24	24
0005	0032			100%	24	24
0007	0028	**0043**		100%	32	32
0010	0036	0038		100%	36	36
0013	0051			100%	24	24
0014	0021	0040		100%	36	36
0015	0068			100%	24	24
0019	0030			100%	24	24
0023	0035	0039		100%	36	36
0025	0052			100%	24	24
0027	0034	0041		100%	36	36
0029	0042	0058		100%	36	36
0060	0070			100%	24	24
0074	0078			100%	24	24
0016	0026			100%	22	22

N_{total} = 56; Bold Type Font: individual was identified as a repeat based on dorsal-pattern recognition. See Sample Size results section for details. Data Generated by CERVUS 3.0.7 (Kalinowski et al., 2007), and adapted to include dorsal pattern matches.

EFFECTIVE POPULATION SIZE (N_e) ANALYSIS

The program COLONY (Jones & Wang, 2009), described in the Methods section of this chapter, was used to assess the number of individuals in the effective population based on the sampled population at WRP. COLONY calculated the N_e in three different ways. Based on our knowledge that OSF breed non-randomly, meaning that they select their mate, and generally only mate with a single individual in a given breeding year (Phillipsen et al., 2009), the N_e calculation by COLONY based on pair-likelihood score method, which assumes non-random mating was deemed most appropriate (Wang, 2009). N_e=25 (Table 3.3.2) is consistent with what Phillipsen et al. (2009) found, which was an N_e=36.7 (95% C.I. 19-71.9) based on calculating N_e from N_b. N_b is the effective population of a single breeding year based on Sibship reconstruction from egg masses taken in a single breeding season (Phillipsen et al., 2009).

Table 3.3.2. Effective population size of sampled population at WRP: Pair likelihood score method based on non-random mating.

Alpha:	0.02
N_e:	25
CI95 (Lower):	15
CI95 (Upper):	43

N_e (Effective Population Size); CI95 (95% Confidence Interval). Table is based on algorithms outlined in Wang (2009), and adapted from the generated N_e output by COLONY.

ALLELIC RICHNESS ASSESSMENT

The total number of individuals, which includes both adults and offspring, genotyped in this study is 273. For each of the 12 loci that were used for assessing genotypes of the WRP sampled population, the average number of alleles represented at each locus tested in the population is A_{mean}=3.833. The mean expected heterozygosity (H_e), which is the average of all expected heterozygosity calculated across all loci tested (Kalinowski et al., 2007), is H_e=0.5174. See Table 3.3.3.

Table 3.3.3. Number of individuals, loci tested, average number of alleles, and mean expected H_e

Number of Individuals:	273
Number of Loci:	12
Mean Number of Alleles per Locus:	3.833
Mean Expected H_e:	0.5174

H_e: Heterozygosity; Table adapted from the output generated by CERVUS 3.0.7 (Kalinowski et al., 2007)

Allelic Richness (A_{mean}) was measured to determine the variation of alleles present within the population at each locus observed. This information reflects whether the representative sample of the population, at specific loci, is at Hardy Weinberg Equilibrium. Based on chi-square goodness-of-fit analysis with Bonferroni correction, using observed versus expected heterozygosity for each locus, two loci are out of Hardy Weinberg Equilibrium ($p>0.05$ is in HW Equilibrium, $p<0.05$ is out of HW Equilibrium). This is likely due to the presence of null alleles (F null) at RP15 and RP461, which are generally caused by mutations at the annealing site of a primer, and can result in a higher

degree of homozygosity than actually occurs within the population (Chapuis & Estoup, 2007). Certain chi-square values were calculated with Yates Correction for continuity, while others were not. Table 3.3.3 below, shows the number of alleles represented within the sampled population at each locus, and the number of individuals typed for each locus. Additionally, Table 3.3.4 demonstrates the PIC (Polymorphic Information Content), which is the usefulness of each marker for linkage analysis (Elston, 2005) based on this sampled population.

Table 3.3.4. Allelic Richness: Summary table of CERVUS Allelic Richness Output

Locus	Num. of Alleles	Individuals Typed	H_eObs	H_eExp	PIC	Chi Squared	p value	HW Significance (Bonferroni Correction)	Null Allele (F)
RP15	2	268	0.873	0.499	0.374	**148.7596**	<0.001	***	-0.2734
RP17	2	268	0.101	0.096	0.091	ND	ND	ND	-0.0166
RP193	6	271	0.738	0.801	0.773	10.9599	0.0896	NS	0.0416
RP22	6	271	0.631	0.637	0.572	1.1804	0.7577	NS	0.0046
RP23	3	270	0.593	0.602	0.533	3.4311	0.3298	NS	0.0051
RP26	5	271	0.627	0.642	0.568	3.4136	0.3322	NS	0.0112
RP3	4	271	0.435	0.434	0.401	**0.1642**	0.6854	NS	0.0065
RP385	4	272	0.68	0.722	0.667	1.9667	0.5793	NS	0.0283
RP415	4	269	0.632	0.601	0.529	1.5446	0.672	NS	-0.025
RP461	2	252	0.21	0.321	0.269	**28.4627**	<0.001	***	0.2075
SFC120	6	272	0.349	0.364	0.341	**0.2233**	0.6365	NS	0.0188
SFC134	2	269	0.52	0.488	0.369	**0.9874**	0.3204	NS	-0.033

Bold Type Font – Indicates the use of Yates Correction for Continuity in calculating Chi-Squared values; *** Loci is not in Hardy Weinberg Equilibrium; ND: Not Done (No result to show); NS: Not Significant (Locus is in Hardy Weinberg Equilibrium); H_eObs: Heterozygosity Observed; H_eExp: Heterozygosity Expected. Data Generated by CERVUS 3.0.7 (Kalinowski et al., 2007).

ADULT RELATEDNESS

Based on the results of the Sibship analysis conducted in COLONY (Jones & Wang, 2009), it appears that there is a high degree of relatedness between the individuals in the adult sampled population. Table 3.3.5 shows the number of individuals within the sampled population (n=56) that have no sampled siblings, one sampled sibling, and two sampled siblings. These results indicate that 55.4% of the total sampled adult population has at least one of their full siblings within the sampled population. This level of relatedness can cause conflict in parental assignment, as siblings will typically have very similar genotypes, and if the actual parents are not sampled, there is a high likelihood that the parent's sibling (aunt/uncle) may be assigned to their niece or nephew (Wang, 2012). This information was taken into account for the parental assignments discussed in the Parentage Assignment Section in the methods, and in the results below.

Table 3.3.5. Sibling Relationships: Number of sampled adult individuals with siblings.

Number of Siblings	Number of Individuals
0	25
1	22
2	9

Data compiled from COLONY output (Jones & Wang, 2009)

PARENTAGE ASSIGNMENT RESULTS

Assignment of parents to offspring followed the methods outlined in the Methods section of this chapter with the program CERVUS 3.0.7 (Kalinowski et al., 2007). Based on log likelihood scores, delta scores, and confidence outputs from CERVUS 3.0.7 (Kalinowski et al., 2007) and the parameters outlined in the parentage Methods section, parental assignments were determined. A total of 12 individual parents were assigned offspring pairs in this study. However, no triplicate sets were assigned (mother, father, offspring). Table 3.3.6. shows the 12 assignments, the number of loci types for both offspring and the assigned parent, the number of loci that did not match in the assignment, the LOD and delta scores for each assignment, and the confidence based on parameters outlined by CERVUS 3.0.7 in (Kalinowski et al., 2007). If an assigned parent was matched to both offspring of another egg mass, then the number of times it occurred, the highest LOD and delta scores, and the number of loci mismatched are shown. Finally, the number of single offspring to which each parent was assigned is shown. Due to the high degree of relatability within this population, the best available methods were used to make these parentage assignments. Thus, 10 of the 12 assignments are based on the most likely, unassigned parent, with 2 of the 12 having an 80% confidence as the most likely assigned parent.

Table 3.3.6. Parentage Assignments based on log likelihood approach from CERVUS 3.0.7.

Candidate Mothers

Offspring ID	Loci typed	Candidate mother ID	Loci typed	Pair loci compared	Pair loci mismatching	Pair LOD score	Pair Delta	Pair confidence	# of times matched to other offspring pair(s)	Highest Pair LOD	Delta	Loci Mismatched	# of times matched to single offspring
FQ0010a	12	0066	12	12	0	3.13	3.13	-	0	NA	NA	NA	10
FQ0010b	12		12	12	0	3.81	3.81	-					
FR0010a	12	0046	11	11	0	6.78	6.78	+	3	2.71	2.4	0	13
FR0010b	12		11	11	0	4.4	2.81	-		1.61	1.61		
FS0002a	12	0017	12	12	0	1.08	1.08	-	0	NA	NA	NA	5
FS0002b	12		12	12	0	2.38	1.36	-					
FU0010a	12	0075	12	12	0	2.27	2.27	-	0	NA	NA	NA	8
FU0010b	12		12	12	0	2.97	2.97	-					
FU0024a	12	0006	11	11	0	5.01	3.89	-	1	-1.78	0	1	12
FU0024b	12		11	11	0	0.97	0.97	-		1.77	1.77		
FV0007a	12	0008	12	12	0	4.92	1.38	-	1	1.65	1.38		10
FV0007b	12		12	12	0	2.37	0.93	-		-2.31	0	1	

Table Continued on Next Page

Candidate Fathers

Offspring ID	Loci typed	Candidate father ID	Loci typed	Pair loci compared	Pair loci mismatching	Pair LOD score	Pair Delta	Pair confidence	# of times matched to other offspring pair(s)	Highest Pair LOD	Delta	Loci Mismatched	# of times matched to single offspring
FU0007a	12	0062	12	12	0	5.87	5.87	+	1	0.02	0.02	1	7
FU0007b	12		12	12	0	4.66	0.9	-		-2.89	0		
FV0010a	12	0023	12	12	0	4.78	4.78	-	0	NA	NA	NA	5
FV0010b	12		12	12	0	3.78	1.52	-					
FV0016a	12	0005	12	12	0	3.3	3.3	-	0	NA	NA	NA	3
FV0016b	12		12	12	0	3.31	3.31	-					
FV0027a	12	0016	12	12	0	6.28	2.95	-	0	NA	NA	NA	6
FV0027b	11		12	11	0	4.01	0.08	-					
FU0025a	12	0004	12	12	0	2.42	0.13	-	0	NA	NA	NA	8
FU0025b	12		12	12	0	2.33	0.12	-					
FQ0006a	9	0061	12	9	0	2.8	0.68	-	0	NA	NA	NA	2
FQ0006b	12		12	12	0	3.02	0.68	-					

F*x* indicates the number and general location of the egg masses the eggs were taken from, *a* and *b* are the offspring; Candidate parents are assigned to offspring F*x* *a* and *b*; (-) Indicates that the candidate parent is the most likely parent; (+) indicates that the candidate parent is the most likely parent with 80% confidence. Data generated by CERVUS 3.0.7. (Kalinowski et al., 2007).

SPATIAL ANALYSIS OF PARENT AND OFFSPRING LOCATIONS

As described in the Methods section of this chapter, the distance between each parent and their offspring was calculated and mapped using ArcGIS 10.2 (ESRI, 2015). Nine of the 12 parents were captured in the South Pond (henceforth, Small Pond). Of those nine individuals, four (0062, 0004, 0075, 0006) laid their eggs in the West Central location, which is 101.91 meters away from the pond (Table 3.3.7, Figure 3.3.1). Another four (0023, 0005, 0016, 0008) laid their eggs in the West North location, which is 157.58 meters away from the South Pond (Table 3.3.7, Figure 3.3.1). The final animal from the South Pond (0061) fertilized an egg mass in the East side area, which is 2,147.83 meters away (2.15 km). Animal number 0017 was captured in 3 different locations, all within very close proximity to each other (2 in the ECH next to the pond, and one time in the PND). The distance for this animal was calculated for all 3 locations, and then an average distance was taken from the 3. This animal laid eggs in the Central West location, which is an average of 404.94 meters away. Animal number 0046 was captured in the northern extent of the West Channel, and laid her eggs in the Central East location, 1079.07 meters (1.08 km) away. Finally, animal number 0066 was captured in a portion of the East Channel, close to its junction with the North Channel. The animal’s eggs were laid in the East side oviposition area, 2230.15 meters away (2.23 km).

Table 3.3.7. Straight-Line Distance between Parents and Offspring

Parent	Mother/ Father	Offspring Location	Total Egg Clusters at site	Masses at site	Eggs sampled	Parent Location	Distance (m)
0062	Father	West Central	171	5	56	South Pond	101.91
0023	Father	West North	112	2	56	South Pond	157.58
0005	Father	West North	112	2	56	South Pond	157.58
0016	Father	West North	112	2	56	South Pond	157.58
0004	Father	West Central	171	5	56	South Pond	101.91
0061	Father	East	15	3	30	South Pond	2147.83
0066	Mother	East	15	3	30	East Channel	2230.15
0046	Mother	Central East	24	2	48	North section of West Channel	1079.07
0017	Mother	Central West	9	5	18	South Pond/East Channel	404.94*
0075	Mother	West Central	171	5	56	South Pond	101.91
0006	Mother	West Central	171	5	56	South Pond	101.91
0008	Mother	West North	112	2	56	South Pond	157.58
No Parent Assigned	NA	West South	5	2	10	NA	NA

Parent: The assigned parent & gender. Offspring location: Assigned offspring location. Total Egg masses at site (n): egg masses present at each oviposition site. Egg sampled (n): The number of eggs sampled at the site where the offspring came from. Distance (m): The straight-line distance the parent traveled from the assigned oviposition site to non-breeding summer habitat. *Indicates that the animal was captured in multiple locations.

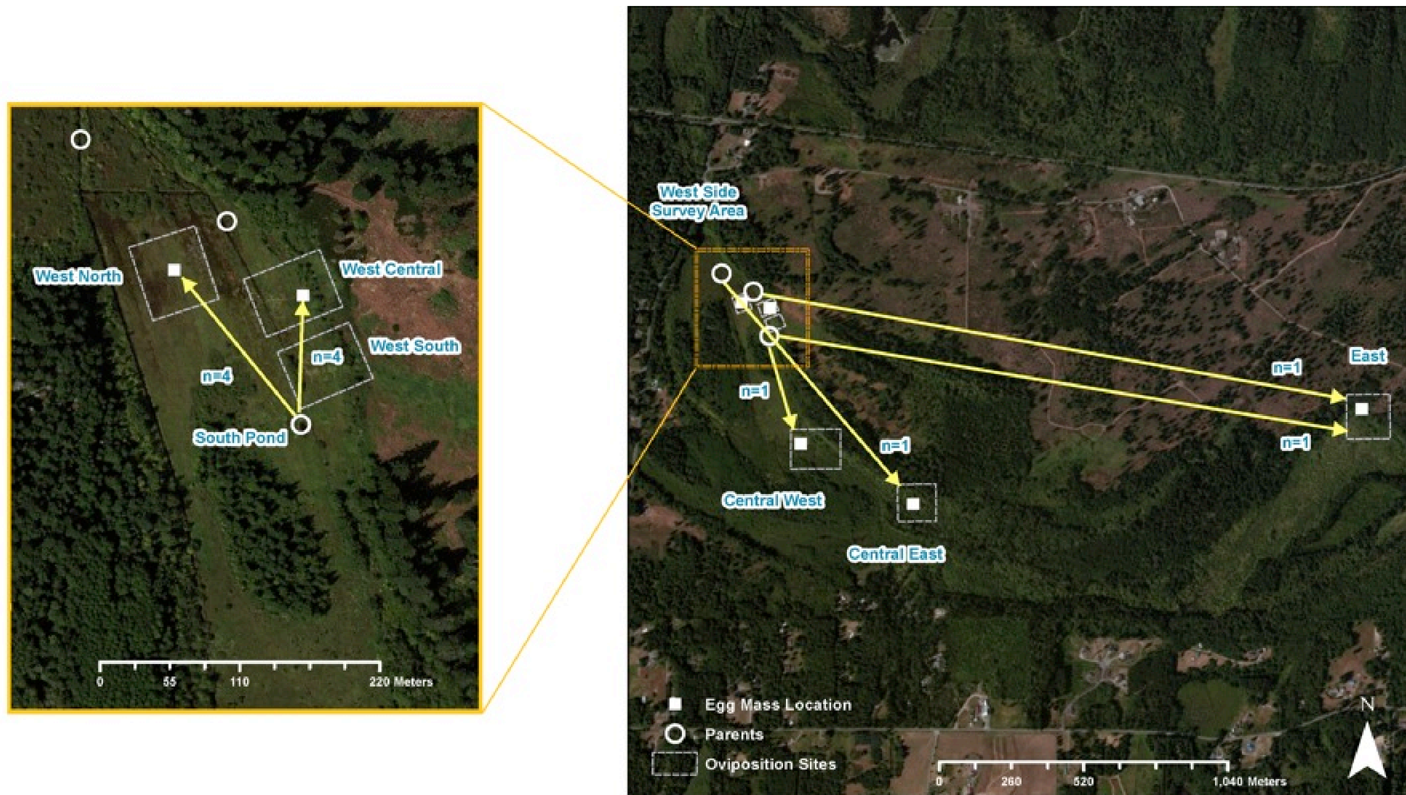

Figure 3.3.1. Locations of OSF parents (n=12), and their assigned egg mass locations (n=12). Right: West Rocky Prairie showing parents that traveled >400 meters to oviposit (n=4); From the South Pond, (n=1) parent went to the East Side, (n=1) parent went to Central East, and (n=1) parent went to Central West; From the East Channel, (n=1) parent went to the East Side. Left: Area in Orange box Zoomed in (n=8); From the South Pond, (n=4) parents went to the West Central oviposition site, and (n=4) parents went to the West North oviposition Site. **World Imagery Base Map by ESRI 2015; coordinates collected by WDFW (egg masses) & Chelsea Waddell, volunteers, and WDFW employees (adults), 2014. Map developed by Chelsea Waddell (2015)*

CHAPTER 4

DISCUSSION

PARENT OFFSPRING LOCATIONS

The locations of parents in relation to their offspring was measured and reported in the Genetics Chapter Results section. The distance parents (n=12) traveled between oviposition and active summer habitat were striking for a few of the animals, with the highest straight-line travel distance being 2.23 km. It is likely, however, that adult OSF in this study traveled farther than estimated using the straight-line distance, as they typically travel by water. However, Watson et al. (2003) showed that adult OSF in lowland western Washington typically travel several hundred meters to recolonize an area during the post breeding season, based on radio telemetry. Whereas McAllister & Walker (2003) showed that three adults, one male and two females, traveled an estimated 2.36 km by water between seasons at Dempsey Creek in lowland western Washington. These consistent findings suggest that adult OSF can travel substantial distances between late winter breeding and the summer non-breeding season. Additionally, this travel distance is likely closely linked to habitat availability, given the high abundance of individuals within the South Pond, and the fact that one parent traveled 2.15 km and resided in the South Pond. These consistent findings also suggest that the parentage assignment method coupled with the spatial analysis used in this study gave a robust and detailed view of OSF behavior as well as population structure. The straight-line distance measurement of distance traveled in this study is a conservative estimate of the distance these animals are

traveling throughout the landscape. Therefore, future spatial analysis of this data should investigate potential pathways these parents are traveling by water, as they may be traveling farther distances than currently realized.

Watson et al. (2003) showed that OSF movement slows during the dry summer months (Watson et al., 2003). Capture/recapture data from this study can be used to determine the active summer season movement range of each captured adult at West Rocky Prairie at a future time. It is likely that the adults captured during the summer season actually moved to those dry season habitat locations during the wet spring (post breeding) season, based on Chelgren et al. (2008).

Interestingly, in past studies of the populations at WRP, egg masses found at the East Side, West Side, and Central areas have been considered unique populations, and estimates of the population size at the WRP site have been established based on the number of egg clusters found at the East and West Sides (M. Hayes, personal communication). Parents of offspring found across all three general locations are utilizing habitat within the West Side survey area, so these populations should not be considered separate. Also, the calculation of egg masses for WRP should begin to include the Central locations. If this were done for the 2014 year, the number of offspring would be 336, and not 303. Therefore, the estimated breeding population size is actually 672, and not 606, as was estimated prior to this study. This indicates that the census population is likely much higher than may have originally been estimated. Most importantly, the population at WRP should be considered much larger than originally estimated by the West and East Side Oviposition areas (Tyson & Hayes, 2014), where the Central Oviposition area should also be included. The calculated effective population (N_e) represents the entire

population at WRP, including all unique adults and egg masses from the West, East, and Central Oviposition areas.

POPULATION STRUCTURE

EFFECTIVE POPULATION, ALLELIC RICHNESS, & RELATABILITY

The result that 55.4% of the sampled adult population had at least one full sibling within the sampled population indicates that some level of inbreeding may be occurring within the West Rocky Prairie population. This is further supported by the small effective population size (N_e=25), which is consistent with, but smaller than, the effective population size (N_e=36.7) of adults at the Sunriver, Oregon population studied by Phillipsen et al. (2009). However, the N_e in these two studies is calculated differently, which may explain this discrepancy. The Sun River population, studied by Phillipsen et al. (2009), also had an estimated breeding population of 90 adults at the time of the study. While small N_e is typical of pond breeding amphibians (Phillipsen et al., 2009) and ranid frogs (Hoffman et al., 2005), based on the results of the parentage analysis, the breeding population at WRP is projected to be around 672 individuals, based on the 1:1 male to female sex ratio used by Phillipsen et al. (2009). While these populations are in different locations, the Sun River population has an estimated breeding population that is 13.4% of the size of the estimated breeding population at WRP, with a comparable N_e. For the seven microsatellite markers used in Phillipsen et al. (2009), no more than 3 alleles were present at any locus, and the authors indicated that there was potentially a high degree of relatability in their studied population. This is consistent with our findings where we found that the WRP population had low allelic richness (mean *AR*=3.833) and high levels

of relatability. This may indicate that genetic drift is occurring within the WRP population. Genetic drift occurs when infrequently present alleles are lost from a population (Kliman et al., 2008). Blouin et al. (2010) found a consistent level of mean *AR*=2.46 across the OSF's range. Given the consistency of findings between these multiple studies, OSF populations tend to have low levels of allelic richness (*AR*) across their range, although this may be based on how robust the markers are for the OSF. Blouin et al. (2010) cautioned that isolated populations of *Rana pretiosa* may experience levels of inbreeding depression, and genetic drift, and that management should focus on mitigating these affects. Therefore, the risks of inbreeding depression and genetic drift within this WRP population may need to be further investigated in future analyses of this study's results and others results.

HABITAT AVAILABILITY & POPULATION SIZE

The effective population size observed in this analysis may indicate a recent rapid increase (bottleneck) of OSF at WRP due to increased habitat availability from management practices (Maureen Small, personal communication). Since the summer of 2000, sporadic mowing treatments of invasive reed canary grass (See Introduction) have been done at the WRP site to increase breeding habitat for the species. In 2000, Kapust et al. (2012), found 107 egg masses between the West Side survey area, and the central oviposition areas. Based on the same 1:1 male to female ratio, an estimated 214 breeding adults were within the WRP site, although this did not include the East Side oviposition area. Based on the observations of Tyson & Hayes (2014), and the findings from this study, a total of 672 individuals are part of the WRP site, with 15 egg masses on the East

Side. The population has therefore increased more than three times since 2000, which may be linked to the mowing treatments at WRP (Kapust et al., 2012).

The findings from our research bring to light some important issues about habitat availability and increasing population size. There was likely a recent bottlenecking because of a small starting population, which then likely rapidly increased since breeding habitat availability was increased. However, the amount of non-breeding habitat appears to be limited, according to the results of the parentage analysis component of this study. Individuals appear to be breeding in potentially far away locations and are primarily located within a small pond (South Pond). Therefore, if we wish to continue this increased population trajectory, we may need to consider increasing non-breeding habitat availability for these small populations in the form of small ponds similar to the size and hydrology of the South Pond.

Increases in OSF populations, such as the one at WRP, are beneficial for the recovery of this species, but the low level of allelic richness and relatedness in this population and other OSF populations may be cause for concern. Genetic rescue may be necessary for these rapidly increasing populations, as suggested by Blouin et al. (2010). However, the use of captive rearing should be implemented carefully, as the addition of locally poorly adapted individuals may actually harm established populations. This perspective is reinforced by statements made by Blouin et al. (2010).

GENETIC MARKERS

Twelve microsatellite loci were used to assess relatedness, N_e, and parentage in this study. While this number of markers is typical for studies assessing the OSF (Blouin et al., 2010; Phillipsen et al., 2009), the addition of new markers for the analysis of OSF genetics would be beneficial. The addition of SNP (Single Nucleotide Polymorphisms), and more microsatellite markers will likely increase the resolution and robustness of genetic analyses for this species. The addition of more robust markers would have likely increased our ability to define parents with higher confidence, as the level of relatedness within the population, and the low levels of allelic diversity at each marker, did not fully represent the distinction between candidate parents. Furthermore, the addition of new markers will aid the future management of this federally listed species. Additionally, the low level of diversity within the markers used, and the high level of relatedness within the population, may have caused assigned parents to be the sampled sibling or first cousin of the actual, un-sampled parent. However, the methods used to distinguish parents were done with a high level of stringency. Most importantly, even if the selected candidate parent is the sibling of the actual un-sampled parent, this still indicates that WRP inhabits a single OSF population, and that the members of the population are moving across the landscape to find non-breeding habitat. Investigating the site fidelity of individual breeding frogs across breeding years, and their subsequent offspring, would further determine if there is consistency in site selection within families to support this conclusion. Currently, OSF adults tend to utilize similar sites across years (Tyson & Hayes, 2014), but a more focused analysis of individual's site fidelity may provide new information about breeding habitat selection for this species.

LAND COVER CHARACTERISTICS

Based on this study, adult OSF at West Rocky Prairie preferred open water habitat over vegetated habitat, as 100% of the frogs captured in this study were found in open water. This is consistent with their fully aquatic nature (Green et al., 1997). However, Watson et al. (2003) found that adult OSF were found under hardhack (*Spiraea douglasii*), which was designated in this study under *scrub/shrub/willow*. Watson et al. (2003) used radio telemetry to identify the locations of adult OSF, whereas this study depended on VES (Visual Encounter Survey), which likely decreased our ability to detect adult OSF under heavy vegetation.

Between the first survey session (July to August) and the second survey session (August to September), there was a noticeable decrease in water availability across the landscape (see Habitat Survey Chapter Results). This is likely due to the nature of the dry season in lowland western Washington, and consistent with the findings of Watson et al. (2003). This decrease in water availability may be a factor in non-breeding habitat limitation for adult OSF. For example, animal number 017, a parent, was captured twice in the East Channel adjacent to the South Pond during the first survey session. During the second survey session, when that portion of the East Channel no longer had water, animal 017 was captured in the South Pond. This suggests that animals are moving to the most available water source, and, as demonstrated by the high number of individuals found in the small pond (South Pond), areas with year-round water sources are limited throughout the year. Further investigation of the movement across the WRP landscape of adults in this study may bring to light important information about the movement of adults during the non-breeding season.

An initial hypothesis of this project was that adult OSF may be using Tilley Pond during the summer season. However, only one adult OSF was observed there. This suggests that either adult OSF were actually present in the location and not detected, or that they are not actively utilizing that pond during the non-breeding season. Tilley pond was quite deep, and needed to be surveyed using Floating VES. This may indicate that adult OSF prefer more shallow water during the non-breeding summer season, as is present in the channels and South Pond. Watson et al. (2003) found that tracked OSF preferred remnant pools (23.6 ± 1.0 cm) in the non-breeding summer season (June-August). Although, the small South Pond, with a high concentration of individuals, was deeper (chest height) than what Watson et al. (2003) found. Photo documentation of the South Pond water level meter was collected for this study, and an average water depth will be computed in further analysis associated with this study.

LOCATIONS OF ADULTS

A majority (83%) of adults captured during this study were present in a small pond, called South Pond. This pond, as discussed previously, is 10m × 6 m, and was surveyed 13 times during the study. This predominance of adult OSF in the small pond could indicate that there is limited suitable habitat for adult OSF at West Rocky Prairie. This sentiment is further reinforced by the results of the spatial analysis of parents and offspring.

Interestingly, all six of the male parents resided in the South Pond, whereas two female parents were more scattered throughout the landscape, and one moved from the East Channel to the South Pond. It is possible that the high number of adults within the

South Pond may limit resources within it, and females seeking food resources for yolk production during summer months (M. Hayes, unpublished data) may be moving to less desirable habitats due to overcrowding. Future investigations with the data collected during this study can be used to determine if there are differences in habitat preferences between adult males and females at WRP by looking at the abundance and distribution of males and females across the landscape, and in the small pond.

HABITAT VARIABLES & THEIR EFFECTS ON OREGON SPOTTED FROG CAPTURE

Frequently, the capture of animals was difficult depending on the orientation of the animal, and the tools being used. Walking VES and floating VES were used to survey for adult OSF, and either hand captures or dip nets were used to capture them. Depending on the orientation of a prospective capture, a decision was made to use either the dip net or hand capture. If the animal was in the middle of a channel, or otherwise in open water, the dip net was used. Whereas when an animal was present on or near the edge of the water on a bank, hand capture was the primary method. Minnow traps yielded very few adult OSF, as discussed in the Habitat Survey Chapter Methods and Results section. Watson et al. (2003) found that 88% of the adult OSF they monitored were found at the water's surface, indicating that some individuals may reside underwater. Given that some adults may not have been present at the surface, new methods should be introduced in future studies in order to account for those undetected animals. New methods could include the use of scuba gear, or bottom trawling. Voris & Glodek (1980) used bottom trawling to determine the habitat of File Snakes (*A. granulatus*) in salt-water habitats.

Although, these methods would likely cause a high level of disturbance to OSF and other species present within the wetland, and consideration of these factors should be taken into account before they are used. Information obtained by these methods could determine the habitat utilization of adult OSF under water and the number of individuals present in those locations. Additionally, radio telemetry would be a useful addition to a study such as this to determine where OSF are going during the summer season, and to track parents across the landscape for longer periods of time. Radio telemetry is a commonly used method for OSF, and would also be useful for tracking the spatial and temporal movement patterns of parents across the landscape.

OREGON SPOTTED FROG BEHAVIOR & DETECTABILITY

While conducting field surveys for the capture of adult Oregon spotted frogs, many were seen basking in the sun, floating on the surface, or on the edge of a bank.

TEMPERATURE

Animals were primarily captured during the time interval between 10:00 and 12:00, which likely correlates with air and water temperature during that time of day. Based on observation, air and water temperature played an important role in the detectability of adult OSF, and should be investigated further through future analysis of the data collected during this study.

DIET

It has long been thought that adult OSFs do not eat small fishes (M. Hayes, personal communication). A single female at the South Pond regurgitated an Olympic

Mudminnow while having her mouth swabbed for buccal samples. This observation brings to light new details about the diets of adult OSFs at West Rocky Prairie.

PRESENCE OF NORTHERN RED-LEGGED FROGS (Rana aurora)

Multiple adult and juvenile Northern red-legged frogs (*Rana aurora*) were detected in the East Side Survey Area, and adult Northern red-legged frogs were detected in the pond next to Tilley Road (Beaver Creek Pond). Additionally, a single adult Northern red-legged Frog was captured in the East Channel, and juveniles were detected in the West Channel. However, no red-legged frogs were detected in South Pond, Tilley Pond, the North Channel, or the clearing North of the North Channel. The areas with the most red-legged frogs detected were the small pond next to Tilley Road, and the East Side Survey Area. It is possible that the presence of red-legged frogs may decrease the presence of adult OSF and may account for the high level of adult OSF in areas where red-legged frogs are not present, or in low abundance. This may also be due to differences in non-breeding habitat preference between Northern red-legged frogs and OSF. These phenomena should be investigated further, and may aid management in future endeavors for finding adult OSF during the non-breeding season.

JUVENILE OREGON SPOTTED FROGS

Juvenile OSF were typically found in open water habitats and were present in all aquatic survey areas except for the pond adjacent to Tilley Road (Beaver Creek Pond), as described in the Habitat Survey Results Chapter. This may indicate that juvenile OSFs have a more generalized habitat preference compared to adult OSF. Additionally, the

presence of juvenile OSF and lack of detection of adult OSF in the East Side Survey Area and Tilley Pond may indicate that these are habitat types that are not preferred by adult OSF. Future analysis of the data collected in this study may indicate that the distribution of juveniles and adults within the WRP site differ. These observations may warrant further research into the habitat preference differences between adult and juvenile OSF, as they may need to be managed differently.

DORSAL PATTERN RECOGNITION METHOD

Dorsal pattern recognition is a method that has been used for OSF and Cascade frogs by the Habitat Program at WDFW for 12 years (M. Hayes, unpublished data). Dorsal pattern recognition was used during field surveys, for in-office animal comparison analysis, and compared to the results of matched individuals based on genetics. This method is certainly useful, as once OSF reach adult stages, their spot patterns remain consistent across years (M. Hayes, personal communication). However, this method was very time consuming in the field, as each captured animal had to be compared to all images of previously sampled adults. The use of high-resolution devices in the field will likely increase the number of correctly identified individuals and should be used for future studies. If this method is coupled with genetic sampling in future studies, pictures and swabs could be taken for all captured individuals in the field, then marked as recapture or unique individual by analysis of the pictures in the office, rather than in the field. This will likely decrease the in-field processing time, and increase the amount of time devoted to capturing adult OSF by all surveyors. While the buccal swab method is

considered less invasive than the more commonly used toe-clipping method, it may cause stress to the animal, and should be used for this listed species with care and caution.

MANAGEMENT IMPLICATIONS & FUTURE RESEARCH

The high abundance of adult OSF in the small South Pond, and the distances parents are traveling through the landscape to reside there during the summer months, suggest that non-breeding summer habitat may be limiting for adult OSF at WRP. Also, given the noticeable presence of Northern red-legged frogs in the East Side survey area, and the low number of OSF egg masses detected there (n=15), the parents of these egg masses are likely traveling to the West Side to find more suitable habitat in the summer. The addition of a duplicate small pond, similar to the size and hydrology of the South Pond could be warranted in order to increase habitat availability for all adults within the WRP population. The most logical location, based on the information collected in this study, would be to build another small pond near the Central East Oviposition location. This would provide refuge to the parents of East Side egg masses, and provide more non-breeding habitat for parents of egg masses laid in the Central East and Central West Oviposition location.

Future studies of OSF adult habitat utilization at WRP should investigate the southern extent of the West Channel. Two adults were found in the West Channel near the Central West oviposition habitat location, but they were not assigned to any offspring. Given the variety of distances adult OSFs travel between breeding and non-breeding seasons, there may be adults in the areas of the West Channel that were not surveyed during this study.

This spatial analysis indicates a conservative estimate of the distance parent's travel between the breeding and non-breeding seasons at WRP, and future analysis of this data will include estimates of their potential travel paths across the landscape. For future studies, the addition of radio tracking devices coupled with parentage analysis could warrant important information about the true paths parents choose to take across the landscape in a single year.

This study provides an important snapshot of the population and spatial distribution of the OSF population at WRP. However, N_e estimates from single season data do not take into account fluctuations in populations over time (Schmeller & Merila, 2007). Additionally, ranid frogs tend to experience boom and bust population changes over time (Phillipsen et al., 2009). Future studies of N_e in the WRP and other populations should include multiple years of genetic information to gain a robust understanding of the changes in effective size these populations experience across years. Also, the low allelic richness and relatability seen in this study and others (Phillipsen et al., 2009; Blouin et al., 2010) suggests that more robust markers are needed for this species. Genetic analysis of threatened and endangered species can only be as good as the resources the researchers have available. Future efforts for the development of more robust genetic markers should be considered by any management efforts aiming to use molecular techniques in their research. These efforts could consist of the identification and development of polymorphic markers such as SNPs or microsatellites.

Finally, future investigation into the metabolic cost of traveling long distances for the OSF should be conducted to determine and quantify energy expenditure, and to

ascertain the cost and benefit of traveling. Also, future research should address their dietary preferences to see whether food availability is driving movement.

SPECIFIC APPLICATIONS TO THE OREGON SPOTTED FROG RANGE-WIDE

Buccal swabs, and the corresponding methods used to obtain these samples, are a minimally invasive and effective method for obtaining tissue samples from this federally listed species. Of the 81 duplicate samples obtained from adults in this study, DNA was successfully extracted from all of them. This high success rate suggests that buccal swabs are an excellent alternative to the invasive toe-clipping method, which has traditionally been used for this species (M. Hayes, personal communication).

Dorsal pattern recognition with high-resolution devices can be a useful method for determining whether individual OSFs are recaptures or new captures, and could replace other, more invasive methods for determining recaptured individuals.

The use of parentage analysis coupled with spatial analysis can be useful for tracking where individuals have moved throughout the landscape, and temporally. Additionally, genotyping can help researchers understand the effective population (N_e) at a site and the relationships between individuals in the sampled population. Management of OSF is often focused on relatively isolated and site-specific populations. Genetic information, such as what was obtained in this study, can be invaluable to management of those small, relatively isolated populations. Information about the movement of parents and adults can help management allocate effort toward managing the adult demographic within these small populations.

The successful breeding of larvae and juvenile OSF can only be fully successful if these individuals, as adults, have available habitat. Incorporating parent/offspring analysis for managed small populations can glean information about the actual size, and spatial distribution of these populations. For example, based on the results of this study, and M. Hayes (personal communication), the East, West, and Central oviposition sites are less likely to be viewed as distinct populations.

The emphasis on oviposition and tracking of egg mass locations does not adequately describe this species, and specifically does not address where adult OSF are going and what habitat they are utilizing. Monitoring oviposition sites only describes a small, yet significant, component of the OSF lifecycle and habitat. In order to manage this species most adequately, knowledge of where these adults are going is essential. Additionally, since some adults at WRP are traveling >1 km to find summer habitat, this may be an important factor in the decline of this species. Therefore, the incorporation of summer surveys of adult non-breeding habitat within all known OSF sites, and the collection of buccal swabs from those adults and egg masses during oviposition, is an essential addition to inform the management of this species.

In order to best manage, and recover the Oregon spotted frog, it is essential that we understand, monitor, and manage all habitats and aspects of its life cycle.

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Production of Maps

All maps created by Chelsea D. Waddell in this document were created using ArcGIS® software, ArcMAP™ 10.2 with World Imagery Base Maps by Esri®.

APPENDICES

APPENDIX A: Standard English and Scientific Names for flora and fauna found in the WRP Survey Area.

Fauna	
Common Name	**Scientific Name**
Oregon spotted frog	*Rana pretiosa*
Olympic Mudminnow	*Novumbra hubbsi*
Three-Spined Stickleback	*Gasterosteus aculeatus*
Northwestern Salamander	*Ambystoma gracile*
Common Garter Snake	*Thamnophis sirtalis*
Red-Legged Frog	*Rana aurora*
Leech	*Hirudinea spp.*

Flora		
Common Name	**Scientific Name**	**Grouped Classification**
Sedge	*Carex spp*.	Sedge
Reed Canary Grass	*Phalaris arundinacea*	Reed Canary Grass
Willow	*Salix spp.*	Scrub/Shrub/Willow
Hard Hack	*Spiraea douglasii*	Scrub/Shrub/Willow

APPENDIX B: Field Survey Protocol for Buccal Swab Sampling. Protocol developed by Chelsea D. Waddell (2014) for the purposes of this study.

Protocol for Oregon Spotted Frog Buccal Swab Sampling
July 2014 – Courtesy of Chelsea Waddell

Supplies:
Epicentre Catch-All Sample Collection Swabs (Hard Pack)
70-90% EtOH (or EtOH cleansing wipes)
Paper towels
1 Pliable ruler
Small Cooler
Cooler Packs (frozen at -20°C)
Tube racks
Plastic container with holes in the top and sides

Protocol:
1. Sterilize the ruler with EtOH and paper towels (or EtOH cleansing wipes)
2. Be sure the ruler is completely dry
3. Prepare 2-3 sterile swabs with labels for each animal sampled
4. Hold the animal gently with the stomach of the animal facing away from you
5. Place the corner of the ruler at the side of the mouth and gently push to open the mouth.
6. Gently push up on the belly to encourage them to open their mouth.
7. Remove swab from sterile tube and place it in the animal's mouth. Swirl and rotate the swab around the roof of their mouth and to the sides of their tongue to completely coat the swab with buccal cells. Be sure to not get any debris from the animal's prior meal.
8. Repeat for each swab collection.
9. Dry swabs as soon as possible in plastic container, as oral enzymes will begin breaking down the cells and DNA quickly. They can be dried in a rack in a plastic container in the sun if humidity is low, or dried in the car on the dash board. Keep this drying process as sterile as possible (do not allow the tip of the swabs to touch anything). Swabs should be dry within 10-15 minutes.
10. Place dried, capped swabs into a cooler with multiple ice packs until they can be stored at -20°C.
11. Store swabs at -20°C, ASAP. The samples can be kept at room temperature for up to 3 days. However, this time includes the laboratory process. Minimize the time these samples stay at room temperature as much as possible.
12. Samples can be stored at -20°C for up to 1 year. After 1 year, extraction yields will likely decline.

APPENDIX C: A discussion about other DNA types (mitochondrial DNA and Single Nucleotide Polymorphisms) that can be used to assess genetic diversity in amphibian populations.

Mitochondrial DNA (mtDNA)

Mitochondrial DNA (or mtDNA) barcoding is an even more standardized approach than microsatellites for determining genetic differentiation between individuals (Storfer et al., 2009). This type of DNA barcoding uses universal primers to generate short DNA sequences, which vary among species. These sequences can be applied to large taxonomic groups (Storfer et al., 2009). However, mtDNA is only inherited maternally, and therefore does not give adequate representation of genetic diversity (Storfer et al., 2009). Since male chromosomal influence is not represented in mitochondrial DNA, this method is more appropriate for rapidly identifying specimens as a particular species and should not be used to establish genetic diversity (Storfer et al., 2009), or parentage. Another added challenge to using mtDNA is that these fragments typically represent one loci; to best represent genetic diversity within an individual and between members of the same species, multiple loci should be used (Storfer et al., 2009). The use of multiple loci is important because genetic variability within a species cannot only be represented by one loci, and not all members of a population across their range will have all the same loci (Storfer et al., 2009). Therefore, the law of large numbers is warranted, and multiple loci should be used to adequately represent a population's genetic variability. Many researchers still use mtDNA to look at amphibian population genetics, but this is typically supplemental to microsatellites, as was done by Blouin et al. (2010). Researchers can also use Single Nucleotide Polymorphisms (SNPs) to understand genetic variability.

Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphisms (SNPs) are single base-pair (point mutation) variations at particular sites along an organism's genome (Storfer et al., 2009). "SNPs are bi-parentally inherited and expressed, commonly have two alleles, can occur in protein coding and non-coding regions of the genome" and are easily sequenced using PCR methods (Storfer et al., 2009). This method is more powerful than microsatellites and is excellent for assessing genetic distinctiveness among populations (Storfer et al., 2009). Typically, SNP data is obtained from Next Generation Sequencing methods because of its high yield of sequencing outputs compared to traditional sequencing methods. Next Generation Sequencing is a relatively new method, which has been gaining popularity in the past few years. However, the process can be very expensive, is not yet widely available (Storfer et al., 2009), and SNPs for the Oregon spotted frog have not yet fully been developed (K. Warheit, personal communication). Given that Next Generation sequencing technology is not yet widely available, SNPs are less widely used (Storfer et al., 2009), these two methods were not an option for this study.

APPENDIX D: This appendix outlines the types of parentage analyses that can be performed that were not done for this study. Additionally, it details other methods that were used to inform the results of the parentage analysis, but were not reported in the text.

UNREPORTED METHODS USED

FRANz & Parental Reconstruction

The parental reconstruction approach "uses the genotypes of offspring in full- or half-sib (sibling) families to reconstruct parental genotypes" (Jones et al., 2010). In the case of amphibians, who typically lay their eggs in masses, each sibling within the mass shares one parent, the mother. The unknown parental genotype can be estimated by subtracting the known parent's alleles, thus reconstructing the shared parent's genotype (Jones et al., 2010). When the parental reconstructions are developed, they can be compared to the actual parents to determine which individuals have the highest likelihood of being a parent (Jones et al., 2010). There are multiple statistical approaches to this technique, which include complex algorithms, likelihood, and Bayesian posterior probabilities (Jones et al., 2010). Disadvantages to this approach include the need for hyper variable (highly polymorphic) loci, and 8-10 full and half-sibs to confidently reconstruct parents (Jones et al., 2010).

For parental reconstruction analysis in this project, the program FRANz was used (Riester et al., 2009). FRANz develops likelihood scores for each parent assignment, much like CERVUS 3.0.7. However, unlike CERVUS 3.0.7, it takes into account the sibling relationships between the offspring and assigns parents based on posterior probabilities. FRANz develops a simulation of parent:offspring and 50,000 iterations were run in the simulation for this study. It also generates a file of allele frequencies within the sampled population, which includes observed and expected heterozygosity.

The analysis method I used to assess parentage with the FRANz output is very similar to the method I used from CERVUS, where both offspring from a single egg mass had to be assigned to the same parent based on posterior probability. If a parent was assigned to both offspring from multiple egg masses, they were discarded from the assignment. These assignments were done using a pivot table in Microsoft Excel, where the parent with the highest likelihood's posterior probability was displayed and compared to the offspring.

Sibship (Sibling Relationship) Reconstruction & COLONY

Sibship reconstruction is an excellent method for reconstructing parental genotypes, as it does not have the same numerical restrictions (8-10 offspring) as parental reconstruction.

The program COLONY (Jones & Wang, 2009) was used in this project to determine parentage assignments in the form of posterior probabilities. It was run two times for parentage, in a short run and a medium run, the resulting assignments were compared to each other and a final assessment of the assigned parents was determined. To assess parentage using the COLONY outputs, I first compared the results of the short run and medium run to see if they were consistent. If both offspring from a single egg mass had to be assigned to the same parent based on the posterior probabilities in both the short and medium run, they were included. If a parent was assigned to both offspring from multiple egg masses, they were discarded from the assignment.

OTHER PARENTAGE ASSIGNMENT METHODS:

Fractional Allocation

Another parentage assignment method not used for this study is fractional allocation, which is very similar to categorical allocation except that instead of assigning the entire offspring to the most likely parent, it "assigns a given offspring partially to each nonexcluded candidate parent" (Jones et al., 2010). Each offspring and parent is assigned a percent likelihood of being matched. Fractional allocation assigns these unexcluded parents based on the same likelihood or posterior probability calculations as in categorical allocation (Jones et al., 2010). While there are some statistical advantages to using this method, it is rarely used in empirical studies because it does not fully assign parents to offspring (Jones et al., 2010). While categorical allocation will generate full assignment of offspring to the sampled parents, fractional allocation can be extremely useful.

Full Probability Parentage Analysis

Another method for parentage assignments is full probability parentage analysis, but it was not used in this project. Full probability parentage analysis estimates "population level variables of interest at the same time as the patterns of parentage" using a single modeling approach in a Bayesian framework (Jones et al., 2010). It allows for the incorporation of habitat and spatial information, such as physical barriers, which may be important to the relationships of the population of interest (Jones et al., 2010). In categorical and fractional allocation, uncertainty in parentage assignments is not incorporated when the researcher is looking at other variables of interest (i.e. Habitat

restrictions), causing an inflated level of confidence (Jones et al., 2010). In full probability analyses, the uncertainty in parentage assignments is included in "the estimation of the population-level variables of interest", which allows for increased confidence (Jones et al., 2010). This incorporation of uncertainty helps researchers answer questions about population level processes. Furthermore, in categorical and fractional allocation, all parents in the analysis are considered equally likely to be true parents of an offspring (Jones et al., 2010). With full probability, each parent is not considered equally likely because it takes "into account relevant ecological information, such as territoriality, spatial location, breeding status, etc." (Jones et al., 2010). A major disadvantage to this approach is that not all of this ecological and mating behavior information can be known (Jones et al., 2010). This can result in an inaccurate model and confidence in parentage assignment could be weakened. Full probability models should be run in conjunction with tests for the actual probabilities and likelihoods for parentage to be sure that the model is accurately assigning parents (Jones et al., 2010).