Northern Leopard Frog (*Rana pipiens*)
Husbandry Manual

A Report Produced by

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For the
Leopard Frog Recovery Team

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1.0 Executive Summary

The Northern Leopard frog (*Rana pipiens*) is a medium-sized, semi-terrestrial amphibian. Historically, *R. pipiens* were widely distributed within North America, and locally abundant where they occurred. In the late 1970's the species experienced a drastic decline in western portions of the range in both Canada and the United States, as a result of habitat loss, disease, and/or introduced fish. Currently, there is only one known population remaining in British Columbia (BC), in the Creston Valley Wildlife Management Area, a 7,000 ha area composed of dykes, ponds and marshes. This small population exhibits low reproductive output, it has two breeding sites, and one overwintering area. Genetic analyses indicate that this population is related to other Pacific Northwest populations, but genetically distinct from eastern North America *R. pipiens*.

Due to the small population size and its limited distribution in BC, *R. pipiens* are listed as endangered. The Leopard Frog Recovery Team was established in 2001 to protect the species from local extirpation. A major goal of the Recovery Team is to increase the number of individuals in the existing population, and head-start new populations within the historic range of the species, through captive rearing and translocation. The long-term goal is to down list the species within 10 years.

In 2001, the Columbia Basin Fish and Wildlife Compensation Program (CBWCP) conducted a trial study to captive rear *R. pipiens*. Hatchling *R. pipiens* were collected in spring and reared in outdoor cattle tanks and fed a mixed diet of vegetables and protein. Daily and weekly maintenance of the tanks included regular water changes, water quality testing, waste removal, and health monitoring. The results from this trial demonstrated that *R. pipiens* could be successfully reared in captivity, with relatively low mortality rates; close to 500 metamorphs were released back into the wild at a previously occupied site (> 75% survival rate). Based on this success, the rearing effort will be expanded to: 1) increase the number of metamorphs released, and 2) to conduct research to determine optimal rearing conditions.

This purpose of this manual is to ensure that standard husbandry protocols are administered throughout the captive-rearing program, including data collection and documentation. The manual is divided into four sections: 1) Background; including a general description of the species, and the abiotic and biotic factors that should be considered during captive rearing, 2) Daily and Weekly Care Husbandry; outlining the protocols that animal care keepers must follow in terms of handling, feeding, tank maintenance, and growth measurements, 3) Special Considerations; with release protocols, disease issues, disinfection and decontamination, quarantine, euthanasia, and research and experimental design, and 4) Appendices (Data Forms and Postable Protocols). This manual will be updated regularly, as new information becomes available.

The intended audience for this manual includes the daily-care staff at the captive-rearing facility in the Creston Valley, as well as Recovery Team members. Other groups that may be interested in this manual include members of other amphibian Recovery Teams, the Alberta Conservation Association, and zoo and aquarium staff involved with amphibian husbandry.

2.0 Preface

2.1 Need for this Document

Rana pipiens west of the Rocky Mountains are listed as critically imperilled (S1) by the Conservation Data Centre (Cannings *et al*. 1999), and endangered by the Committee on the Status of Endangered Species in Canada (COSEWIC 2002); currently, only one known population remains within BC, with an estimated total population of approximately 1,100 individuals in 1999 (Waye and Cooper 2001).

In response to these low numbers, a Recovery Team for the Southern Mountain population of the Northern Leopard Frog was established in February 2001 to protect the species from local extirpation. A major goal of the Recovery Team is to increase the number of individuals in the existing population, and head-start new populations within the historic range of the species, through captive rearing and translocation. The long-term goal is to down list the species within 10 years by addressing habitat and disease issues.

In 2001, the Columbia Basin Fish and Wildlife Compensation Program (CBFWCP) captive-reared *R. pipiens* under the direction of the Recovery Team on a trial basis. The CBFWCP experienced fairly high survival rates of captive-reared *R. pipiens*, releasing close to 500 metamorphs (> 75% of the original hatchlings caught) back into the wild at a previously occupied site. Due to the success of this initial rearing program, future rearing efforts in 2002 will be based on the general design and techniques followed in 2001. This manual was developed to ensure that standard husbandry protocols are recorded and followed throughout this program, including data collection and documentation.

The intended audience for this manual is the husbandry staff involved with the daily care of *R. pipiens* in the Creston Valley, as well as Recovery Team members. Groups involved with other amphibian recovery efforts may also benefit from this manual (e.g., Recovery Teams, Alberta Conservation Association, zoo and aquarium staff, etc.).

2.2 Organization of this Document

This manual outlines the husbandry protocols used for captive-rearing *R. pipiens* in the Creston Valley, BC. It is organized into four main sections:

- Section I – Background
- Section II – Daily and Weekly Care Husbandry
- Section III – Special Considerations
- Section IV – Appendices (Data Forms and Postable Protocols)

Section I provides a general description of the species, and an overview of the abiotic and biotic factors to that must be considered in the care of captive *R. pipiens*. Section II was written as a reference for animal care keepers in their daily maintenance of captive *R. pipiens*. Section III is also an important reference for animal care keepers, but it covers issues that should not be a factor on a daily basis, such as release protocols and disease monitoring. Section IV includes all of the data forms required for documenting all aspects of the husbandry program, including egg monitoring, collection, daily and weekly care, growth and development monitoring, and marking and release.

2.3 Organization of Responsibilities/Tasks

The success of any captive rearing program for an endangered species is dependent upon the care provided by the animal keepers involved with the program. They are responsible for monitoring and maintaining the health of the animals for successful release. The success of the program is also dependent upon effective communication between the husbandry personnel and the Recovery Team.

The various tasks required for husbandry must be clearly assigned to the appropriate individuals/groups. Members of the Recovery Team are responsible for making decisions regarding collection sites, the number of animals to be collected per egg mass, the number collected from the population as a whole, when collection should occur, what research should be conducted, that staffing is adequate, and where animals should be released. Individuals involved with the daily care of captive-reared *R. pipiens* must ensure that equipment and nutritional needs are met, daily care is maintained, health assessments are conducted, diligent record keeping is maintained throughout, and that data are submitted to the Recovery Team at the end of each season.

A report should be produced each autumn, and submitted to the Leopard Frog Recovery Team, that summarizes the captive-rearing efforts for that year. In addition, the protocols in this manual should be updated regularly, as new information becomes available.

Within the captive-rearing staff, a head husbandry technician should be hired. Among the tasks that this person would complete, is the development of a daily to-do list for staff to follow. Before the onset of the rearing season, the head technician should be provided with comprehensive training regarding water quality chemistry and equipment use, appropriate clinical techniques for specimen preparation, and data management protocols. Each year, time must be allotted for a thorough training and orientation session for all captive-rearing staff regarding all aspects of the program, particularly decontamination and disinfection protocols, the use of water quality testing equipment and procedures, and food preparation and feeding. Lastly, all staff should have access to adequate references at the rearing facility, covering all aspects of amphibian biology, water quality testing, amphibian diseases, nutritional needs, and clinical techniques.

SECTION I – BACKGROUND

3.0 Introduction

3.1 General Description of **Rana pipiens**

The Northern Leopard frog (*Rana pipiens*) is a medium-sized (35-100 mm snout to urostyle length; Corkran and Thoms 1996), semi-terrestrial amphibian whose historic range included a vast geographic expanse across North America. In the western part of the range, *R. pipiens* inhabit a variety of wetlands and the backwaters of streams, and they appear to be more closely linked to water than populations in eastern North America (Seburn and Seburn 1998). In BC, the one known remaining population is found in the Creston Valley Wildlife Management Area, a 7,000 ha managed by the Creston Valley Wildlife Management Authority (Seburn and Seburn 1998).

Rana pipiens adults and juveniles have light bordered, large black or brown spots on the dorsal surface, a white ventral surface, a long, narrow snout, conspicuous dorsolateral folds extending the full length of the back, long hind limbs, and incomplete webbing on the hind toes (Seburn and Seburn 1998). Females lay only one clutch of eggs per year, up to 7,000 eggs in the shallow margins of water bodies in early spring, where waters are warmest. Eggs hatch in approximately 10 days, and hatchlings cling to the jelly mass for up to three days (Seburn and Seburn 1998; Waye and Cooper 2001). The greatest mortality of *R. pipiens* is during the tadpole stage; in the wild, survivorship ranges from 1.0 - 7.5% from hatching to metamorphosis (Seburn and Seburn 1998).

The size of *R. pipiens* froglets at metamorphosis is unknown for the Creston Valley population, but the smallest young of the year caught in the summer of 1999 was 30.7 mm snout-urostyle length[1] (SUL) and 39.9 mm SUL in 1997 (Waye and Cooper 2001). Average size of metamorphs in Alberta, Manitoba and Minnesota was 31-40 mm, 33-37mm, and 35-40mm SUL respectively; *R. pipiens* metamorphs from a shrinking pond environment in Minnesota averaged 25-30 mm SUL (Seburn and Seburn 1998).

Outside of the breeding season, *R. pipiens* continue to utilize wet areas as part of their summer range, escaping into water when disturbed. The majority of *R. pipiens* overwinter under well oxygenated water that does not freeze solid (Seburn and Seburn 1998). In the Creston Valley,

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[1] Close monitoring of tadpole growth and development in the wild should be conducted to compare these figures to captive-reared animals.

researchers believe that some radio-tracked frogs overwinter underground in small mammal burrows (Waye and Cooper 2001; Parris 1999).

3.1.1 Wild and Captive Populations in BC

Historically, *R. pipiens* were abundant in the East Kootenays and Okanagan Valley. Individuals were introduced to Vancouver Island, but the status of that population is unknown (Cannings *et al*. 1999). Currently, there is one known wild population of *R. pipiens* remaining in BC. This population is isolated, as the next closest potential source of wild *R. pipiens* is from Idaho (Seburn and Seburn 1998). The Creston Valley population has two breeding sites, approximately 1.75 km apart (Waye and Cooper 2001; D. Adama, pers. comm.).

In 2001, the CBFWCP released 496 captive-reared *R. pipiens* metamorphs at a previously occupied site within the Creston Valley Wildlife Management Area - Corn Creek Marsh, Compartment/Pond 1.

Rana pipiens are commonly retained and bred in public aquaria, zoos, and research laboratories, the majority of which are obtained from biological supply houses versus wild populations. Individuals at the Vancouver Aquarium were obtained from a biological supply house (T. Reynolds, pers. comm.).

3.2 Abiotic Considerations

Amphibians reared for reintroductions should be maintained under optimal conditions to reduce mortality and increase fitness (e.g., size at metamorphosis). Key abiotic factors should be monitored at breeding sites in the Creston Valley throughout the course of larval development (e.g., daily and weekly), and at the captive-rearing facility, to compare conditions and provide standards (i.e., temperature, pH, hardness, alkalinity, ammonia, nitrites, nitrates, and dissolved oxygen). Some of these variables vary over time so more readings are required to obtain standard levels. For example, hardness, alkalinity and pH are relatively stable, while dissolved oxygen, ammonia and nitrates are affected by temperature (e.g., from cool morning to hot mid day; C. Bishop, pers. comm.).

3.2.1 Temperature

Of all of the abiotic factors within an aquatic environment, temperature exerts the greatest force on the overall fitness of anuran larvae due to its influence on growth rates and time to metamorphosis (Ultsch *et al*. 1999). From laboratory studies, it is clear that increased temperatures usually result in faster developmental and growth rates, shorter time to metamorphosis and reduced body size at metamorphosis, until an inhibitory temperature is reached (see below). For example, in one study *R. pipiens* larvae reared at 13ºC were 3 times larger throughout all stages of development, including metamorphic climax, than those reared at 23ºC (Ultsch *et al.* 1999).

Anuran larvae have a preferred body temperature (PBT; when placed in an thermal gradient, larvae select a certain temperature or range), a critical thermal maximum (CT_{max}; temperature which results in impairment of locomotory function so that the animal loses its ability to escape harmful conditions that will lead to death), and an inhibitory temperature (where growth and differentiation fall below optimal levels). Temperatures that fall outside of the PBT for a species can alter active metabolic rate, maximum sustained speed, growth rate, food conversion efficiency, and learning and memory capabilities; the PBT for *R. pipiens* is from 20.0 - 30.0ºC depending on the acclimation temperature (Ultsch *et al*. 1999). The PBT of anuran larvae appears to increase with developmental stage, and the CT_{max} for anurans appears to be related to what the species has been exposed to in the wild; the CT_{max} of *R. pipiens* is 40.0ºC (Ultsch *et al*. 1999). The inhibitory temperature of most anuran larvae is well below the CT_{max}; in one study, differentiation and growth of *R. pipiens* tadpoles was greatest at 23ºC, and began to fall at 28ºC (Ultsch *et al*. 1999). During the final stages of metamorphosis, the temperature tolerance of anurans is reduced and should be monitored closely (Ultsch *et al*. 1999).

The majority of the research on the influence of temperature on amphibian larvae has been under controlled laboratory conditions, where larvae are usually exposed to constant temperatures throughout development. *Rana pipiens* larvae captive reared in the Creston Valley are exposed to ambient air temperatures, which fluctuate daily and seasonally. For this reason, the above review serves as a general guideline for developing captive rearing protocols, but may not reflect true patterns and conditions. The most suitable measure for monitoring water temperature may be comparing captive conditions to those in the wild. Water temperatures were recorded hourly at the two *R. pipiens* oviposition sites in the Creston Valley and in one captive-rearing tank from May to August in 2001 (Figure 1). Although the tank experienced greater daily fluctuations than the natural sites, the maximum and minimum temperatures were within tolerable limits and similar to natural conditions. The greatest daily differences between the natural sites and the rearing tank occurred in July, with temperatures in the rearing tank rising 6-10°C above natural sites during the day, and falling 5-7°C below at night. These temperatures remain below the lethal limit of 40ºC for *R. pipiens* (Ultsch *et al*. 1999).

A gradient of temperatures should be provided so that anuran larvae can seek out PBT, and water temperature measurements should be recorded daily to avoid thermal extremes. Larvae may be able to thermoregulate more efficiently if variable water depths are provided, as well as shaded areas, and/or by allowing slightly cooler water to gently flow through the system at one end of the tank. Extremes in temperature should be avoided; measures should be in place to ensure hatchlings do not freeze, or that tadpoles/metamorphs do not over heat. The threat of freezing is present for the first month after hatching (May through early June). As hatchlings (Gosner stage 20-25; Gosner 1960) can tolerate higher densities than tadpoles they should be retained in small plastic tubs during the first month after collection, so that they can be moved indoors if there is a threat of freezing. In contrast, air and water temperatures in July can reach 35+°C in the Creston Valley. Measures should be in place to shade, and lower temperatures, when air temperatures approach or exceed 35°C (e.g., suspending a tarp over the predator cover).

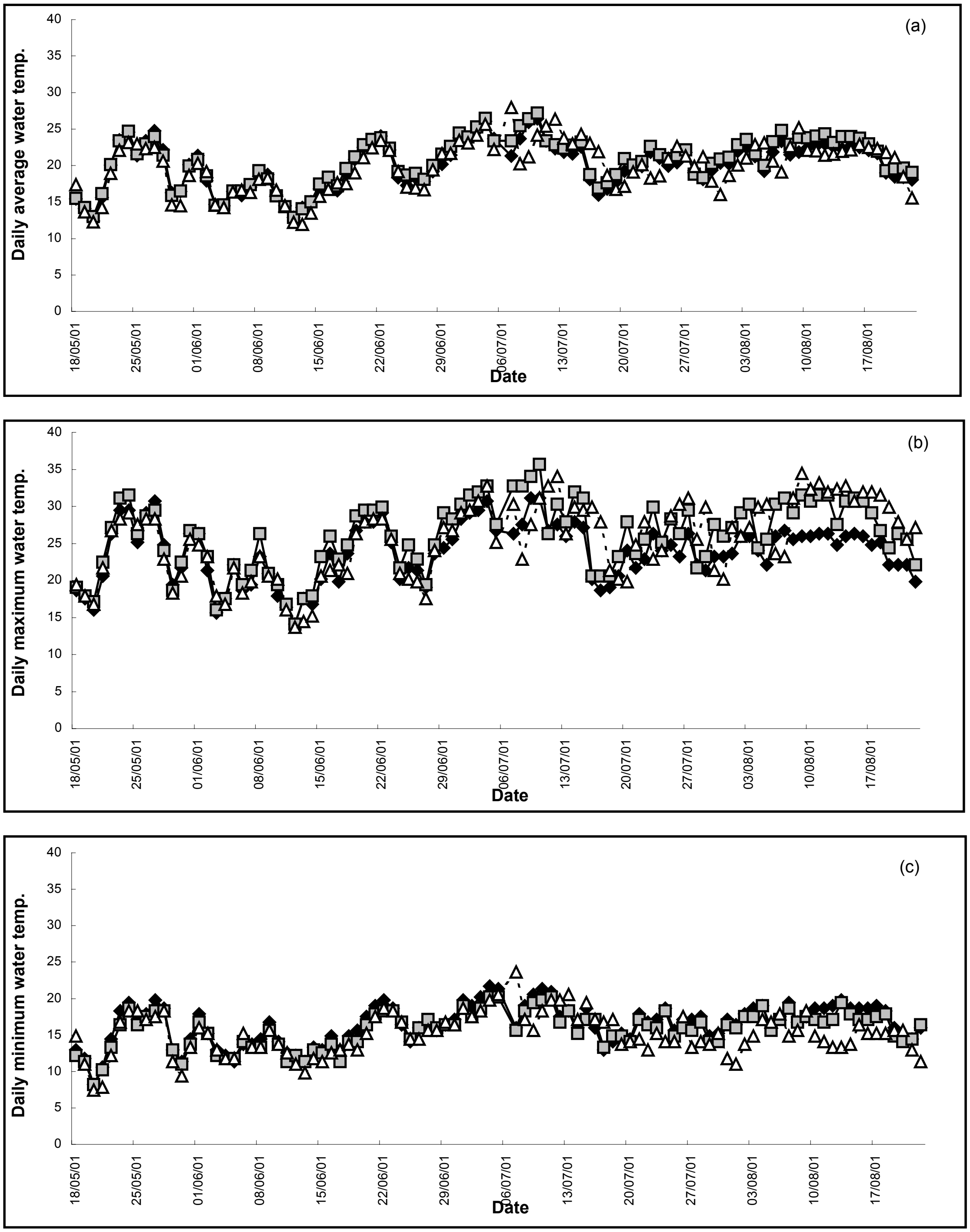

Figure 1. Daily average (a), maximum (b), and minimum (c) water temperatures at two *Rana pipiens* breeding sites in the Creston Valley, BC (grey square and black diamond), and one captive rearing tank (white triangle), recorded hourly from May 18 to August 22, 2001.

3.2.2 pH

pH is a measure of the concentration of hydrogen ions in water, indicating neutrality (7.0), acidity (< 7.0) or alkalinity (> 7.0). It is important to monitor pH because inappropriate levels degrade water quality by creating conditions suitable for harmful toxins (e.g., ammonia) and bacteria (Whitaker 2001). Wide swings in pH can be stressful to amphibians, and newly hatched tadpoles are particularly sensitive to inappropriate levels. pH is affected by the mineral ions present in the water (dissolved magnesium and calcium), as well as the buffering capacity of the water (see Section 3.2.3).

Ideal pH levels for captive-reared *R. pipiens* are unknown but should match those found under natural conditions. Whitaker (2001) recommends a pH of 6.5-8.5 for most larval amphibians, and a pH of 7.0 for species where the optimal levels are unknown. pH values observed in the wetland habitat used by the Creston Valley population were between 8.0 and 8.3 in 2001. These values fall within the pH range observed at other *R. pipiens* sites in Alberta and Ontario (Table 1). Although pH values are fairly stable over time, additional readings should be taken at the natural sites before any standards are set.

Table 1. pH values taken at natural *R. pipiens* sites.

BC	**Alberta**		**Ontario**	
Creston Valley oviposition site	Seburn and Seburn 1998[a]	Kendell 2002[b]	Bishop *et al*. 2000[c]	Hecnar and M'Closkey 1996[d]
8.0 - 8.3	8.5-9.5	7.6-8.2	7.5-8.1	8.3 (7.2-10.2)

[a] Range between four breeding sites.

[b] Range between three oviposition sites.

[c] Hatching success was > 80% at storm water ponds and a natural wetland where pH values were within this range.

[d] Average pH (and range) at 130 wetlands, 121 of which contained *R. pipiens*.

The pH of water in the seven captive-rearing tanks in 2001 averaged 8.8, which is slightly higher than the two natural oviposition sites, and other natural sites outlined in Table 1. These levels were likely elevated due to the use of concrete bricks placed inside the tanks to provide cover (see Section 3.2.3 below). In the future, concrete should not be used in the tanks as their lime content will elevate pH levels. In contrast, the buffering capacity of soft water is low, meaning that more frequent water changes and/or the addition of buffers are needed to maintain the desired pH (Whitaker 2001).

3.2.3 General Hardness and Total Alkalinity

General water hardness (GH) is a measure of the mineral ions present (dissolved magnesium and calcium), and carbonate hardness (KH; total alkalinity) refers to the buffering capacity of water, and reflects its ability to stabilize pH. GH and KH must be monitored because they interact with pH, which affects water quality, and continued exposure to unnatural levels of hard water can lead to skin lesions in amphibians (Whitaker 2001). The fresh water being added to tanks may be soft or hard depending on the source, and materials added to tanks or in contact with the water may influence GH or KH (e.g., concrete bricks).

KH and GH levels should be maintained at values similar to those observed at natural sites. The values for KH and GH at natural breeding sites in Alberta and Ontario, and a semi-natural site, vary considerably from recommended values (Table 2). The values from occupied sites in Table 3 suggest that *R. pipiens* fair well, and may prefer, relatively hard water. Whitaker (2001) acknowledges that optimal alkalinity values vary greatly among different species.

Table 2. Total alkalinity and general hardness (in mg/L) at *R. pipiens* natural sites in Alberta and Ontario, versus recommended levels.

	Levels at Occupied Sites			**Recommended Levels**	
	Alberta	Alberta	Ontario		
	Natural Breeding site (Seburn 1993)	Natural and Semi-natural Rearing sites (Kendell 2001, 2002)	Natural ponds[a] (Hecnar and M'Closkey 1996)	(Whitaker 2001)	(Nace *et al.* 1996)
Alkalinity (KH)	305-407	> 214.8 (nat. sites) 125.3 – 250.6 (2000) 125.3 - > 214.8 (2001)	195 (59-960)	15-50	150-250
Hardness (GH)	151-342	> 214.8 (nat. sites) 161.1 – 232.7 (2000) 125.3 - > 214.8 (2001)	226 (43-1108)	75-150	150-250

[a] Water samples taken from 130 ponds in Ont., 121 of which contained *R. pipiens* but were not necessarily breeding ponds; average value presented with range in brackets.

It is difficult to adjust GH, KH or pH without affecting the others, so it is not advised to attempt this unless absolutely necessary (Nace *et al.* 1996). Section II provides some control measures that can be used to adjust the values of each of these parameters if necessary. Because these water quality measures are difficult to manipulate and maintain, recommended values for GH and KH are very broad at this time (i.e., 150-400 mg/L) until natural levels are known from the Creston Valley. General hardness (GH) and total alkalinity (KH) of the captive-rearing tanks should be measured regularly in 2002.

3.2.4 Ammonia, Nitrite, and Nitrate

Aquatic amphibians produce ammonia, a toxic form of nitrogen, as a waste product. In a balanced system, nitrifying bacteria break down the ammonia to nitrite, and then nitrate. The temperature and pH of the water affect the amount of ammonia present; the higher the pH the greater the proportion of toxic un-ionized ammonia in the water (Whitaker 2001).

The accepted levels for ammonia, nitrite and nitrate for amphibians are usually based on studies conducted on fish. Recently, it has been determined that the larvae of some amphibians, including some ranid species, appear to be very sensitive to even low levels of nitrites and nitrates in water (e.g. LC50's of *Rana pretiosa* after 15 days of exposure to nitrites and nitrates was 0.5 mg/L and 16 mg/L respectively; Marco *et al*. 1999). *Rana pipiens* tadpoles exposed to 20 mg/L of ammonium nitrate fertilizers (NH_3) for 96 hours experienced 50% mortality, and severe weight loss resulted in those that survived (Hecnar 1995). Exposure to 10 mg/L for 100 days resulted in significant mortality. Water samples taken from each *R. pipiens* oviposition site in the Creston Valley and southern Alberta in 2001 show only trace amounts of these chemicals present (Table 3).

Table 3. Levels of ammonia, nitrites and nitrates (in mg/L) at oviposition sites in the Creston Valley and southern Alberta in 2001, compared to LC50 values for *R. pretiosa* and recommended values for amphibians and fish.

	Natural Levels		**Lethal Levels**	**Recommended Levels**	
	Creston Valley, BC	Alberta (Kendell 2002)	*R. pretiosa* 15d LC50 (Marco *et al*. 1999)[b]	For Amphibians (Whitaker 2001)[c]	For Fish (EPA)[d]
Ammonia	0.03 [a]	0-0.5[a]		< 0.02	
Nitrite	< 0.005	0	0.57	< 1	5
Nitrate	< 0.002	0.5	16.45	< 50	90

[a] Average, or range, of ammonia, nitrites and nitrates for one water sample taken at oviposition sites in 2001.

[b] Results of a sensitivity study of 5 amphibian species to nitrates and nitrites. Based on the results of this study chronic exposure to $N0^2$ < 0.44 mg/L and $N0^3$ < 10 mg/L should result in < 25% mortality rates of *R. pretiosa* tadpoles.

[c] Recommended levels for captive care of temperate amphibians.

[d] U.S. Environmental Protection Agency's recommended levels for warm-water fishes (in Marco *et al.* 1999).

More readings should be taken from natural sites to base standards for captive rearing, as infrequent readings do not reflect average levels. Until natural levels are known, acceptable ammonia levels should be based on an average of the two natural site readings (i.e., < 0.03 mg/L), which is similar to that recommended by Whitaker (2001). Nitrites and nitrates should remain within safe levels for *R. pretiosa* – NO_2 < 0.44 mg/L and NO_3 < 10 mg/L.

3.2.5 Dissolved Oxygen (DO) and Biological Oxygen Demand (BOD)

Dissolved oxygen (DO) is a measure of the concentration of oxygen in water at a specified temperature and atmospheric pressure, while biological oxygen demand (BOD) is a measure of the amount of oxygen consumed by microorganisms (e.g., bacteria) during the decomposition of organic matter, including the conversion of ammonia to nitrite and nitrates. Both measures are used as indicators of the ability of water to support aquatic life. Although aquatic amphibian larvae can tolerate relatively low oxygen levels in water for short periods, important nitrifying bacteria also require oxygen. Water temperature and the input of waste and organic matter into water affect DO and BOD levels.

DO and BOD levels in tanks should reflect those observed at natural sites. DO levels have not been recorded on a regular basis at the *R. pipiens* breeding sites in the Creston Valley. At the time of collection in May, 2001 readings were approximately 9.0 mg/L. At four *R. pipiens* breeding sites in Alberta, DO ranged from 8.8 to 16.3 mg/L (> 80%; Seburn and Seburn 1998). DO levels in captive-rearing tanks in 2001 improved after water changes, in that levels were > 6 mg/L, values were similar among tanks, and the average in each tank was approximately 8 mg/L. DO levels must be recorded at natural sites regularly to make comparisons to these values, and to provide standard levels. BOD was not measured in tanks, but values at natural sites in the Creston Valley were 7 and 8 mg/L in 2001.

The amount of dissolved oxygen (DO) in water should remain above 80% saturation to maintain sanitary conditions (Whitaker 2001). DO is measured in mg/L (ppm), and must be converted to percent saturation based on water temperature and elevation (Figure 1). Note that this nomogram assumes that measurements are taken at sea level – the values decrease by 4% per 300 m increase in elevation due to decreased gas pressure. The saturation value can also vary slightly depending on barometric pressure with lower values expected when a storm front moves through as compared to bright and sunny, high pressure days.

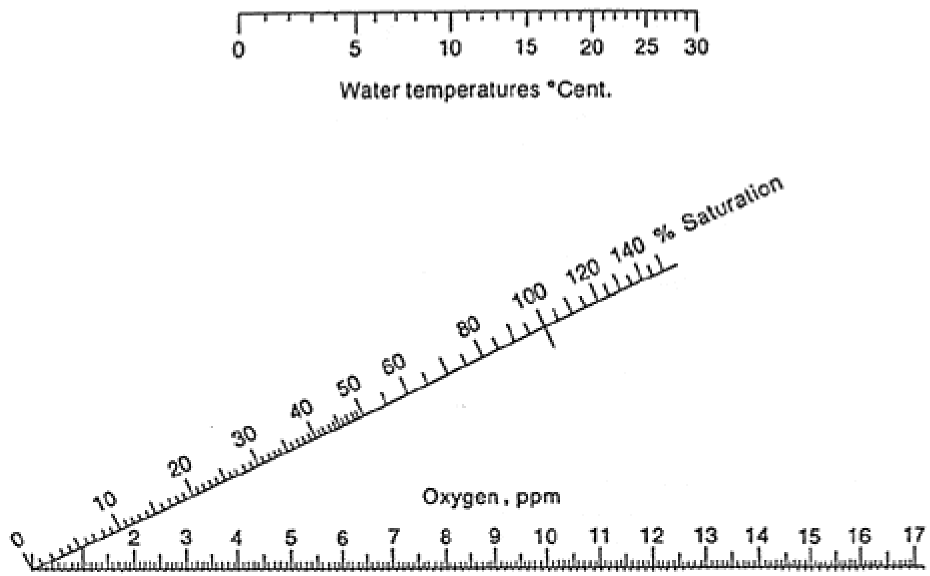

Figure 2. Percent dissolved oxygen conversion chart (nomogram). Draw a straight line between the water temperature and the ppm of dissolved oxygen - the percent saturation is the value where the line intercepts the saturation scale. (Taken from http://wow.nrri.umn.edu/wow/under/parameters/oxygen.html).

Oxygen can be added to water through aeration, circulation, and regular water changes. BOD can be more stable and reliable than DO but it is affected by temperature, so numerous readings are required to obtain standard levels (C. Bishop, pers. comm.).

3.2.6 Carbon Dioxide ($C0_2$)

Carbon dioxide (CO_2) in water is the by-product of respiration from amphibians and bacteria. CO_2 is an important water quality measure because high concentrations of carbonic acid may lower pH values (Whitaker 2001).

CO_2 was not measured at oviposition sites in the Creston Valley, so natural levels are not known. At four *R. pipiens* breeding sites in Alberta, CO_2 levels varied greatly (0 - 29.6 mg/L; Seburn and Seburn 1998). Whitaker (2001) recommends that dissolved CO_2 should remain below 5 mg/L.

The amount of CO_2 in water can be lowered through aeration, regular removal of organic debris, and agitation of the water. Regular water changes will likely keep this measure under control, so it not of great concern at this point in the program and does not need to be monitored regularly.

3.2.7 Salinity

Salinity is a measure of the concentration of salt in water. Due to osmosis, and the semi-permeable nature of amphibian skin, the majority of species do not survive in water containing salts higher than what is found within their body fluids (i.e., 200-300 mOsm; Diana *et al*. 2001). Salinity levels in tanks will be affected by water source.

Evidence suggests that salinity levels for *R. pipiens* larvae should remain below 2.0 ppt (Seburn and Seburn 1998), which is within the recommended level of 0.0 - 5.0 ppt for most amphibian species (Whitaker 2001). If well water it used, it should be tested for salinity levels, however, regular testing of tank water is not required as salt toxicosis is rare in captive amphibians (Diana *et al*. 2001).

3.3 Biotic Considerations

3.3.1 Diet

Anuran tadpoles feed on a variety of materials, including vascular plants, plant detritus, periphyton (a combination of cyanobacteria, diatoms, bacteria and desmids), ultraplankton, planktonic green algae and cyanobacteria, precipitates of dissolved organic matter, protozoans, pollen, mosquito larvae, fairy shrimp, other tadpoles, and amphibian eggs (see review in Kupferberg 1997). The main difference between these food items, is that the plant tissues contains more carbohydrates and lipids than protein compared to the animal materials (Kupferberg 1997). Although most anuran larvae feed predominantly on plant matter, they select for those with the greatest protein content. Kupferberg (1997) compared various algal diets and found that tadpoles selected for those with the greatest protein content (i.e., filamentous green algae with epiphytic diatoms), which resulted in faster tadpole development and larger size at metamorphosis. Numerous studies have demonstrated that anuran larvae develop faster and attain greater size at metamorphosis when animal tissue is included in their diet (Alford 1999). Animal tissue (e.g., eggs, meat) contains important nutrients, like vitamin B complex, which help combat various diseases such as spindly leg, scoliosis, and paralysis (see Section 12.0; Wright and Whitaker 2001).

Ideally, captive amphibians are provided with the same dietary options and nutrients as they have in the wild. However, relatively little information is available on the natural diets of tadpoles (Alford 1999). The mouth structure of tadpoles reflects their natural diet (Whitaker 2001), and *R. pipiens* are facultative filter feeders (Alford 1999); up to 97% of their gut content consists of free-floating food forms (Hendricks 1973 in Alford 1999). As with many anurans, *R. pipiens* larvae are largely herbivorous, however, protein should be included in the diet to improve overall health (Sredl and Healy 1999). Captive-reared amphibian larvae can be fed a variety of diets, ranging from purely natural (e.g., algae, detritus, etc.) to purely artificial sources (i.e., manufactured tadpole food). There are advantages and disadvantages to any approach, and the best solution is a balance between meeting the nutritional needs of the animals while falling within the logistical constraints of the rearing facility in terms of the time required for food preparation, maintaining water quality, and cost.

Captive amphibian larvae are often fed a vegetable-based diet (e.g., steamed/cooked lettuce) with a protein supplement (e.g., frozen bloodworms; T. Reynolds, pers comm.). A study compared the effects of four diets on the growth and development of *R. pipiens* tadpoles (Table 4; P. Jackman, unpub. data). The results varied between the four diets, but the Wards commercially prepared tadpole food/lettuce diet and the spinach diet appeared to be most successful in terms of relatively fast growth, low mortality, no deformities, and size of metamorphs.

Table 4. Growth and development rates of *R. pipiens* tadpoles fed four different diets. Each treatment was replicated three times, with 15 tadpoles per replicate/10L aquaria.

	Lettuce	Lettuce + Wards[a]	Wards[a]	Spinach
# days to rear buds (GS[b] 26-28)	46	22	26	33
# days to rear legs (GS 36-39)	60	33	33	33
# days to 4-legged frogs (GS 42)	66	48	46	50
% to 4-legged frogs	20	56	56	60
% found dead	0	2	36	4
% dead + missing	22	24	42	22
% remaining on test termination (day 97)	56	18	2	18
% abnormal	2[c]	0	13[d]	0
Avg. weight (g) for 4-legged frogs (std dev)	1.78 ± 0.43	2.67 ± 0.84	2.77 ± 0.72	3.02 ± 0.77
Avg. length (cm) for 4-legged frogs (std dev)	2.64 ± 0.49	2.68 ± 0.30	2.69 ± 0.27	2.78 ± 0.42

[a] Commercially prepared tadpole food

[b] Gosner Stage

[c] Crooked tail

[d] Crooked tail, bloated

As demonstrated in the above study, larvae fed spinach grow quickly and become large, which may be advantageous for young amphibians. However, the National Research Council notes that oxalate-containing plants (e.g., spinach and kale) should be avoided as they may result in renal disease (i.e., kidney stones; Barnet *et al*. 2001, McDiarmid and Altig 1999). However, the inclusion of small amounts of these vegetables in a mixed diet, versus providing spinach as a major food base, may contribute to the production of large, healthy metamorphs.

Larval diets have been developed that incorporate commercial foods with binders, to prevent disintegration and reduced water quality. For *R. pipiens*, 250 g of pulverized rabbit chow[2] can be combined with 20 g of granular agar (seaweed), and 14 g of unflavoured gelatine/litre of water (animal product; Nace *et al*. 1996). The mixture is brought to 100ºC (212ºF), after which it is allowed to solidify in flat pans where it can be sliced and stored indefinitely in the freezer or for up to 14 days in the refrigerator. The authors recommend that hatchling *R. pipiens* should be fed wilted lettuce for several days before starting on this agar-gelatin diet. This diet does not soil the water and requires minimum attention, as a full 1-2 day ration can be fed at one time (Nace *et al*. 1996).

In 2001, captive-reared *R. pipiens* tadpoles in the Creston Valley were fed a mixed diet of vegetables (i.e., 75%; spinach, zucchini and lettuce) and protein (i.e., 25%; frozen bloodworms; K. Lansley, pers. comm.), based on a dietary study conducted on captive-reared Oregon spotted frogs (*Rana pretiosa*, Csuti and Sellers 2000). Bloodworms were not fed to the larvae until they reached Gosner Stage 25 (approximately day 21 in the Creston valley; K. Lansley, pers. comm.). Although individuals reared on this diet in 2001were slightly smaller than their wild counterparts at metamorphosis, the abnormality rate of *R. pipiens* tadpoles was relatively low across the seven tanks (i.e., range = 0-6%; average = 2%). The smaller size may have been due to the amount of food they received versus the content of the diet (D. Adama, pers. comm.). The 2001 diet appeared to meet the nutritional demands of the tadpoles, and was easily managed by animal keepers.

In 2001, metamorphs were detained for up to six weeks post metamorphosis. Keepers found it difficult to keep up with the nutritional demands of the metamorphs. It is recommended that metamorphs be released as soon as metamorphosis is completed, so that they can begin foraging naturally before hibernation. If, however, some individuals must be retained, metamorphs should be fed a more standard captive diet of crickets (e.g., *Acheta domestica*), dusted with a mineral supplement (e.g., Herptivite or Reptical). These can be obtained from pet stores and biological supply houses. Metamorphs should not be fed maggots as this can lead to nutritional deficiencies because these invertebrates are not easily broken down in the digestive track (Nace *et al*. 1996).

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[2] Rabbit chow includes contents such as alfalfa, grass and/or hay meal, ground grains, soybean seed, safflower seed, rice bran, Bentonite, wheat bran, dried whey, and vitamins and minerals.

3.3.2 Density

There is a relationship between tadpole density and rate of growth and development. Adolph (1931) found that *R. pipiens* tadpole growth rates declined with increasing density under laboratory conditions, which he attributed to increased metabolic rate as a result of stress that results from constant contact between tadpoles. Increasing habitat complexity decreases contact between tadpoles, and reduces stress (John and Fenster 1975). The effect of density is more evident where tadpoles are locally dense (e.g., in small pools or under artificial conditions; Alford 1999). Stunted tadpole growth leads to delayed metamorphosis (Lynn and Edelman 1936), and smaller size of metamorphs (Wilbur 1976). Evidence suggests that larger wild *R. pipiens* metamorphs attain sexual maturity earlier (Seburn and Seburn 1998), which likely improves overall adult fitness (Alford 1999).

Research should be conducted to determine the optimal density for rearing *R. pipiens* in captivity, as these values are unknown. Measures that can be used to determine the optimal density level would be the size and weight of captive-reared metamorphs compared to their wild counterparts. Wild-caught metamorphs in the Creston Valley were reported to be approximately 38 mm snout to urostyle length (SUL), which falls within values observed elsewhere (i.e., 31 to 40.7 mm SUL; see references within Kendell 2002). Captive-reared metamorphs in the Creston Valley in 2001 were smaller than those observed at natural sites; approximately 28 mm (D. Adama, pers. comm.). In contrast, *R. pipiens* reared under semi-natural conditions in Alberta were 33.4 to 41.0 mm SUL (Kendell 2002). Research suggests that under ideal conditions (i.e., low density, etc.) metamorphs can reach 48-50 mm SUL, but will only attain an SUL of 25-30 mm under poor or stressful conditions (i.e., predator pressure, high tadpole density, or pond drying; see references within Kendell 2002).

3.3.3 Disease

Disease is an important consideration for animal husbandry and, in particular, in reintroduction programs (Cunningham 1996); diseases such as chytridiomycosis, iridovirus, *Aeromonas*, spindly leg syndrome, etc. Various stressors can increase the susceptibility of captive amphibians to disease and illness (Taylor *et al*. 2001). These include inadequate diet/nutrition, over crowding, high disturbance/agitation, and improper water quality. Captive-reared amphibians are particularly vulnerable to fungal outbreaks (e.g., *Aeromonas*; S. Raverty, pers. comm.). The risk of outbreaks can be reduced by minimizing stress, closely monitoring water quality, maintaining optimal densities, offering an adequate diet, and maintaining aseptic rearing conditions. This topic is covered in more detail in Section 12.0.

Captive-reared amphibians pose a threat to native amphibians once they are reintroduced back into the environment through their potential to introduce diseases. For this reason, a percentage of the captive-reared animals must be sacrificed for laboratory analyses before animals can be released into the wild (see Section 13.0). Serious consideration should be given to relocating the captive-rearing facility away from the wild population in the Creston Valley, to reduce the risk of contaminating the captive population through the spread of diseases (e.g., chytrid; D. Adama, pers. comm.).

3.3.4 Behaviour

It is important that captive-reared animals behave in similar ways to their wild counterparts, to ensure their survival after release, and to avoid selecting for certain genetic traits associated with unnatural rearing conditions. For example, by providing cover objects (e.g., rocks, plants, etc.) tadpoles are encouraged to seek refuge from predators and climatic extremes. Cover also provides greater habitat complexity, which reduces tadpole encounter rates and stress (Barnett *et al*. 2001; Kendell 2001). Metamorphs require some terrestrial habitat to begin foraging invertebrates on ‘land’.

3.3.5 Predation

Rearing tadpoles outdoors versus indoors increases the risk of predation by mammals, birds, reptiles, and aquatic invertebrates. A secure cover was placed over the rearing tanks in 2001, which was successful in keeping predators out of the tanks. However, some aquatic invertebrates were found in the tanks, likely through the pond water or aquatic vegetation used for cover. This can be reduced by altering the water source, filtering it, or ensuring that organic matter added to the tanks is free of larvae and eggs (e.g., cleaning plants, wood, etc.).

Invertebrate predators encountered incidentally during regular tank maintenance should be removed immediately. Predator screens should also be installed in water reservoir tanks. All covers should not be lifted off while tanks are unattended to prevent air borne beetles, such as predacious diving beetles (*Dytiscus* sp.), from entering the tanks. All invertebrates found in the tanks should be recorded on the daily care sheet (Section IV).

4.0 Physical Facilities

4.1 Artificial Versus Semi-Natural Rearing Conditions

There is a gradient in the design of captive-rearing programs, from completely artificial, indoor settings (i.e., aquaria, artificial lighting, climate control, commercially produced diets), to enclosures within natural rearing ponds, where the only input into the system by humans are the larvae themselves. In theory, the closer captive-rearing conditions are to the wild, the better adapted amphibians are for survival in the wild after release. However, there is a trade-off between the opposite rearing strategies, in that survival rates are often lower in semi-natural conditions due to predation, etc. For example, approximately 25% of *R. pipiens* larvae reared in large semi-natural ponds in Alberta survive to metamorphosis, versus 75% in the Creston Valley (Figure 2; Kendell 2000, 2001; D. Adama, pers. comm.).

Artificial conditions often lack climatic gradients, habitat complexity, and offer limited feeding/diet opportunities. Many of these variables can be relatively easily enhanced. For example, rearing animals outdoors provides more natural thermal and lighting regimes, and habitat complexity can be increased by providing wood, plants, and deep-water habitat so that animals adapt to utilizing cover to escape predators and climatic extremes.

Figure 3. Semi-natural ponds used to rear captive *Rana pipiens* larvae in Alberta (photo courtesy of Cal McLeod from the Alberta Conservation Association).

4.2 Considerations for Artificial Rearing

4.2.1 Rearing Enclosure

The purpose of utilizing artificial enclosures for a head-start program is to maintain greater control over captive-rearing conditions (e.g., reduce predation). The selection of adequate rearing enclosures should take into consider the depth of the tanks, the chemical make-up of the tank material (e.g., fish-safe plastics), the durability of the tanks, ease of cleaning and draining (presence of valves), heat absorption (i.e., colour), and storage (stackable).

In the Creston Valley, plastic cattle tanks were used in 2001 (Figure 3). Each had a 6' diameter, with a maximum capacity of 735 L (170 cm top width x 154 cm bottom width x 60 cm height). Water levels were 28 cm initially, so that water volume for the tadpoles was approximately 549 L. The cattle tanks proved to be a very effective means of captive-rearing *R. pipiens*. In 2002, the Recovery Team will be using larger tanks, with a 9' diameter and a maximum volume of approximately 5800 L (water volume will be based on 257.8 cm bottom width x 21 cm depth).

In 2002, during the first two weeks of captive rearing (until Gosner Stage 25), hatchlings were retained in small tubs (e.g., 109 L Rubbermaid Rough Tote), as a means of quarantine, to better account for mortality, and to allow the tubs to be brought indoors to prevent extreme temperature fluctuations (see Section 6.2). The same issues mentioned above must be taken into account when selecting small tubs for rearing hatchlings (e.g., depth, chemical make-up of the tubs, durability, etc.).

Figure 4. Plastic cattle tanks used to captive rear *Rana pipiens* in 2001.

4.2.2 Anti-predator Screen

Anti-predator screens are required over tanks to keep invertebrate and vertebrate predators from preying on, or injuring, *R. pipiens* larvae. The selection of appropriate screens should take into account their effectiveness at deterring predators, ease of handling, and durability.

The anti-predator screen installed over each tank in 2001 consisted of a fine wire window screening attached to a wooden frame, and held down securely over the tanks with rocks. No mammalian or avian predators entered the tanks. This method proved to be effective, inexpensive, and easily manipulated for daily maintenance of tanks. These same screens will be used again in 2002, with the potential modification to a more fitted/rounded frame with clamps.

4.2.3 Cover Objects

Amphibians require security and thermal cover to avoid predation and thermal extremes. Cover also provides some protection from potentially harmful UV-B exposure (Ankley *et al.* 2000), and it provides increased habitat complexity, which reduces stress and encourages natural behaviour patterns.

When selecting potential cover objects, the chemical make-up of the material (e.g., fish safe), and the safety of the design (e.g., stability, risk of injury/mortality) should be taken into account. The design should also take into account natural cover objects and tadpole behaviour. *Rana pipiens* oviposition locations are characterized by a loose layer of decaying and live vegetation on the bottom, which tadpoles utilize for cover (Waye and Cooper 2001).

In 2001, concrete bricks, foam lily pads made from place mats, rocks, and plastic plants were provided as cover objects in each tank. These materials proved effective, inexpensive, and easily cleaned. However, the concrete bricks may have affected water quality (see Section 3.2.3), and the foam lily pads must be designed in such a way that they do not float directly at the surface (i.e., the top of the pad should be above or below the water surface), as small hatchlings can become trapped in small water pockets on the surface.

Metamorphs should be provided with adequate haul out areas, as they are prone to drowning (Nace *et al*. 1996, Crawshaw 2000). Haul outs should be flush with the water surface to allow ease of access to and from the water. It is important that there are sufficient numbers of haul outs and surface area, so that metamorphs do not exhaust themselves swimming around the tank (K. Lansley, pers. comm.). Cover objects for tadpoles can serve as metamorph haul outs (e.g., rocks).

4.2.4 Filtration Systems

Biological filters, utilizing the actions of nitrifying bacteria, can significantly reduce the amount of ammonia present in water; these critical nitrifying bacteria must be preserved during filter cleaning to maintain a healthy system. However, even if a filtration system is being used, regular water changes must be conducted to reduce the level of ammonia present (Whitaker 2001). It may be too expensive and logistically challenging to use filtration in the large outdoor tanks being used in 2002. If water quality results suggest that water changes are not sufficient to maintain proper conditions, filtration will be considered as an additional cleaning tool.

4.2.5 Water Reservoir

Water reservoir tanks are required to store fresh water used during water changes. By storing water in tanks before water changes are conducted, the fresh water is the same temperature as that within the rearing tanks. The selection of appropriate reservoir tanks should take into account the chemical make-up of the tanks (e.g., fish safe), the capacity required to refresh the rearing tanks on a daily basis, heat absorption (e.g., colour), and ease of drainage (e.g., presence of valves).

Water reservoirs should be easily filled and drained (e.g., situated near water source, elevated off the ground to make drainage possible), and covered with an anti-predator screen (Section 4.2.2).

SECTION II – DAILY AND WEEKLY CARE HUSBANDRY

Section II of this manual serves as a reference guide for animal care keepers in the daily and weekly maintenance of *R. pipiens* during captive rearing. It is composed of five parts: 1) Record Keeping, 2) Amphibian Husbandry, 3) Disinfecting, 4) Feeding, and 4) Water Quality.

5.0 Record Keeping

All aspects of the captive-rearing program must be documented, including collection, daily and weekly care of tadpoles (feeding, measurements, etc.), tank maintenance (water quality tests, water changes, etc.), health assessments, pathology results, and release. Section IV includes data sheets to record all aspects of captive rearing, including a daily feeding schedule, water quality measures, daily minimum and maximum water and air temperatures, tank cleaning schedule, and growth and development measures.

All information must be entered into a computer database regularly (e.g., daily care and maintenance should be entered daily or weekly depending on available time), and backed up weekly. The head husbandry technician, in consultation with the Recovery Team representative working with captive rearing crew, should develop data-entry and back-up protocols.

6.0 Amphibian Husbandry

Every effort should be made to avoid stressing tadpoles and metamorphs. During daily and weekly maintenance, coordinate activities in such a way as to reduce the total number of hours keepers must attend to each tank. For example, water quality measures can be taken just before excess food is siphoned off each morning, and the general health of tadpoles can be inspected during weekly growth and development measures. Keepers should move around tanks in such a way as to reduce the amount of detectable movement by the tadpoles (e.g., keep > 2 m away when walking by, and avoid throwing a shadow across the tank).

Tadpoles and metamorphs should not be handled unless absolutely necessary. Animals can be temporarily chilled (4-6°C) to reduce activity level when they must be handled at length (e.g., during marking; Kendell 2001). Avoid contact with human hands as much as possible to reduce the potential of changing body temperature, to reduce stress, and to avoiding inflicting any pain or bodily harm; use nets to catch animals and place them into holding tubs during inspections or body measurements, etc. Wear vinyl or nitrile gloves when handling *R. pipiens*; do not use latex as these have proven to be toxic to amphibians (Gutleb *et al*. 2001). Ensure that gloved hands are moist when handling animals.

Care should be taken to prevent the loss or injury of *R. pipiens* during handling. For example, cover nets with hands when transferring tadpoles into inspection containers, and place secure lids over small tubs to reduce any chance of spilling containers onto the ground. Use small amounts of water in temporary holding containers, to reduce the chance of spillage. For metamorphs, all measurements, inspections, marking, etc. should be conducted over/in contained areas (e.g., a large tub) in case animals fall or escape. Wide-mouthed tubs with water should always be close at hand so that metamorphs can be released safely if handling problems arise (e.g., during marking).

6.2 Housing

After collection, each sibling group of hatchlings should be placed into an individual small plastic container (e.g., 109 L Rubbermaid Rough Tote) for the first two weeks. This facilitates transfer indoors if temperatures are low, it is easier to monitor and care for the small larvae(e.g., find dead individuals), and it facilitates quarantine (Section 14.0). The tadpoles can then be transfered into the large tanks after they reach Gosner Stage 25, following the same acclimation/water change rate suggested for acclimation after collection (Section 10.1).

The plastic totes and the cattle tanks should be situated on flat ground, in locations where the tadpoles are exposed to natural light levels, and where there is low human disturbance or risk of vandalism or damage by pests (e.g., bears, racoons). Each tote/tank must have a secure cover that is kept in place at all times outside of tadpole and tank maintenance. Various cover objects should be provided in each tote/tank for the tadpoles to seek refuge.

Tanks must be cleaned and disinfected before each captive-rearing season, and immediately after, following decontamination procedures.

7.0 Disinfecting

To reduce the risk of introducing or spreading infectious diseases, anything entering a *R. pipiens* tank, including equipment, water, and human hands, must be sterile. Separate equipment should be used for each tank (Nace *et al*. 1996). Disposable sterile, non-powdered nitrile or vinyl surgeon's gloves should always be worn when caring for *R. pipiens*. Gloves must be changed and disposed of between tanks.

Disinfection of tanks should not be a regular activity otherwise critical nitrifying bacteria will continually be lost (Whitaker 2001). Tanks should only be disinfected when they become abnormally soiled or infected (e.g., when animals are sick and/or die unexpectedly). Iodine should not be used as a disinfectant where amphibians are housed because iodine toxicity has been observed in animals where iodine became reversibly bound to plastic and wood in enclosures (Lentini 2000; Whitaker and Wright 2001). Soaps and detergents should also be avoided because they are difficult to rinse off (Whitaker and Wright 2001).

Chlorine bleach and ammonia are the least problematic disinfectants when used properly and thoroughly rinsed (Whitaker and Wright 2001). To be affective, the surface must be soaked in a 10% bleach solution (0.6% sodium hypochlorite) for a substantial period (e.g., 45 minutes) to inactivate some viruses (Brunner and Sesterhenn 2002). After disinfection, tanks should be rinsed with hot tap water and allowed to air-dry (Lentini 2000). Porous materials (e.g., bark or driftwood) should not be used in tanks.

Animal keepers should follow a decontamination protocol regarding the proper care of equipment, hands, and clothing at the rearing facility.

8.0 Feeding

The tadpoles should be fed a mixed vegetable and protein diet daily; mixed vegetables in the morning, and frozen bloodworms in the afternoon based on a dietary study conducted at the Oregon Zoo (Csuti and Sellers 2000). Feeding should take place after the previous days excess food and waste have been removed. It is the responsibility of the animal keepers to monitor the tadpole's feeding behaviour, to ensure that appropriate amounts are being fed, based on the general health of the animals and the amount of excess food that has to be removed each morning.

During studies investigating the effect of density on growth and development of captive-reared *R. pipiens*, the amount of food fed to each tadpole must be approximately equal. Based on the general feeding regime of 50 tadpoles in 2001, each tadpole received approximately 100 g of food throughout rearing. Early results from 2002 suggest that the diet should be reviewed and studied in more detail in future years.

8.1 Documentation

All aspects of feeding must be recorded for each tank at each feeding period, using the Daily and Weekly Care Sheet in Appendix A.

9.0 Water Quality

Maintaining appropriate water quality standards for captive-reared amphibians is essential to maintain a healthy population. Captive amphibians should not be exposed to poor water quality, especially where flow-through and filtration systems are not being used. It is challenging to provide filtration to outdoor rearing tanks, so water changes are required on a regular basis to reduce toxic levels of nitrogenous waste.

9.1 Monitoring

A field-friendly water-quality testing kit (e.g., Hach Company P.O. Box 389, Loveland, Colorado 80539-0389; 1-800-227-HACH; http://www.hach.com/contact.htm) should be used to monitor water quality on a regular basis; the use of this equipment requires adequate training at the beginning of the rearing season. The water quality of the captive-rearing tanks, and fresh water being added to the system, should also be tested regularly to ensure that they fall within acceptable levels. Municipal water sources must be dechlorinated before use, and well water and natural water sources (e.g., streams and ponds) should be tested for ammonia levels (especially where the surrounding area is utilized for agriculture), hardness (buffering may be required), and salinity.

Refer to Section I for a description of each water quality measure, and details regarding manipulation of each. Control measures should only be taken if values remain continuously outside of the recommended values.

9.1.1 Daily

- Parameters: Air and water temperature, and pH of tank water, should be monitored daily. Deviations from the values in Table 5 should be noted and the appropriate measures taken.

Table 5. Suggested levels for daily water quality measures.

Water Quality Measure	**Suggested Level**	**Control Measure**
pH	8.0 - 8.5	Review techniques, and identify cause of abnormal levels Lower = increase acidity (e.g., add peat moss to fresh water, or bubble CO_2 into tanks[b]) Raise = add buffer/increase alkalinity (e.g., sodium bicarbonate - baking soda[b])
Air temperature	5 > 35 ºC[a]	
Water temperature	5 > 35 ºC	Lower = shade tanks Raise = bring hatchling tubs indoors (but avoid extreme temperature change)

[a] Animal keepers should be prepared to take action if air temperatures are anticipated to go outside of this range. In spring (May), measures should be in place to move hatchlings indoors if temperatures are expected to exceed lower limits over night, and in July/August, shading should be provided to reduce water temperatures if critical levels are reached.

[b] pH, hardness and alkalinity are very closely linked, so adjusting one will affect the others. These measures can be very difficult to adjust and maintain. If values are continually outside of the natural conditions/desired levels the cause of the problem should be identified and addressed, versus adjusting the water chemistry manually - see Section 3.2.2 and 3.2.3.

- Documentation: Record the minimum and maximum air and water temperatures from the previous 24 hours, as well as the current pH level, on the Daily and Weekly Care Sheet in Appendix A. Care must be taken to ensure that thermometers are properly located (i.e., out of direct sunlight).

9.1.2 Weekly

- Parameters: The amount of ammonia, nitrites, nitrates, DO, hardness and alkalinity should be monitored in the rearing tanks weekly: before weekly water changes. Fresh water sources should also be tested throughout the year. Values should remain within those recommended in Table 6; the frequency of water changes should be adjusted to meet water quality demands.

- Documentation: Record these parameters on the Water Quality Sheet in Appendix B. Enter this data, along with the data from the Daily and Weekly Care Sheet, into the computer database weekly.

Table 6. Suggested levels for weekly water quality measures.

Water Quality Measure	Suggested Level	Control Measure
Ammonia (NH_3)	< 0.05 mg/L	Review techniques, and identify cause of abnormal levels Lower = partial water change
Nitrite (NO_2)[a]	< 0.05 mg/L	
Nitrate (NO_3)	16 mg/L	
Dissolved oxygen (DO)	80% saturation[b]	Lower = stir water/agitate substrate[d] Raise = provide aeration
General hardness (GH)	150-400 mg/L[c]	Lower = add peat moss or distilled water[e] Raise = add calcium carbonate[e]
Total alkalinity (KH)	150-400 mg/L[c]	Raise = add buffer (e.g., baking soda[e])

[a] With limited time, this middle step can be skipped because the presence of acceptable levels of ammonia and nitrates in the water indicates that the nitrogen cycle is being successfully completed.

[b] Approximately 8 mg/L depending on water temperature (see Figure 2 nomogram in Section 3.2.5).

[c] Highly variable results from available data – very broad measures recommended until values from Creston Valley breeding sites are known.

[d] Too much oxygen in water can result in gas bubbles under the skin and eyes, and in the gastrointestinal tract of tadpoles. Tadpoles can tolerate relatively low oxygen levels, but critical nitrifying bacteria cannot – see Section 3.2.5 for details.

[e] These measures, in combination with pH, can be very difficult to adjust and maintain, so only consider tampering with them if values are continually out of the natural/desired levels - see Section 3.2.3.

9.2 Maintenance

Daily care of tanks is required to maintain water quality. This includes regular water changes and the vacuuming/siphoning off of organic waste material.

9.2.1 Water Changes

A minimum 50% water change is required for each tank per week, unless water quality results indicate otherwise. Fresh water should be collected > 24 hours prior to addition to the tanks to ensure temperatures match those within the tanks. Water will be changed using a siphon to help remove waste from the bottom of the tank. Care must be taken to ensure that tadpoles are not killed or overly stressed. Collected water must be retained in holding containers so that any tadpoles caught can be returned to the tank upon completion.

Waste water from tanks should be disposed of properly to avoid the introduction of pathogens into the environment. For example, waste water can be treated with a bleach solution (200 mg/L of sodium hypochlorite) for 15 minutes before being released (Lynch 2000). The disposal of waste water needs to be addressed in the Creston Valley (e.g., grey water pit).

9.2.2 Waste Removal

Organic waste and excess food should be vacuumed/siphoned off of each tank daily, before feeding time. A hose and pump will be used to siphon water from the tank into a holding container. Any tadpoles caught in the siphon can be returned to the tank upon completion. Care must be taken to avoid killing tadpoles.

9.2.3 Documentation

All aspects of tank maintenance must be recorded on the Daily and Weekly Care Sheet in Appendix A.

SECTION III – SPECIAL CONSIDERATIONS

Section III of this manual encompasses issues that are not part of the daily maintenance of captive-reared *R. pipiens*, but factors that will come into play during each captive-rearing season. This section is broken down into six parts: 1) Capture, 2) Growth and Metamorphosis, 3) Disease Monitoring - including a general overview of infectious diseases and parasites, collection and shipping of tissue and specimens, and health monitoring protocols, 4) Release - including marking and transportation, 5) Quarantine – including euthanasia, and 6) Monitoring, Research and Experimental Design.

10.0 Capture

Surveys are conducted in the Creston Valley Wildlife Management area from mid April to mid June to locate *R. pipiens* egg masses. Once located, the egg masses are monitored from deposition to hatching, to ensure that hatchlings are collected for captive rearing at the appropriate developmental stage. Dead eggs detected during monitoring should be collected and shipped to the laboratory for analysis following the procedures in Section 12.2.

Individual *Rana pipiens* egg masses are distinguishable (i.e., they are usually not laid communally) and the hatchlings remain close to the remaining jelly mass after emergence for up to three days (Seburn and Seburn 1998). This is advantageous to captive rearing for two reasons: 1) it facilitates collection of sibling groups for captive rearing, and 2) embryos can develop under natural conditions. The number of tadpoles collected from each female should be determined *a priori* by the Recovery Team based on the number of available masses, the objectives of the head start program, and the rearing capacity of the tanks.

Egg masses should be caged in mesh enclosures, and tadpoles counted to determine how many should be brought in from one egg mass. Hatchlings should be collected < 3 days after hatching (i.e., Gosner stage 21) usually early May at the east breeding site in the Creston Valley - where eggs are laid first). Tadpoles should be collected randomly from all sides of the mass using a hand net, sibling groups should be placed into individual plastic tubs or bags in water collected at the oviposition site, and filled to approximately 75% water volume (Sredl and Healy 1999). Barnett *et al*. (2001) suggest that only animals of the same size should be transported together. M. Demlong (in Sredl and Healy 1999) recommends stocking densities for Chiricahua Leopard frogs (*Rana chiricahuensis*) during transport as follows:

Embryos - one mass per tub/bag (28 x 27 cm)
Larvae < 1.5 cm (1/2”) - 50 per tub/bag (0.07/cm^2)
Larvae 2.5 cm – 4 cm (1”-1.5”) - 25 per tub/bag (0.03/cm^2)
Larvae > 4 cm (1.5”) - 10-15 per tub/bag (0.01-0.02/cm^2)
Recently metamorphosed frogs - 5 per tub/bag (0.001/cm^2)

The tubs or bags should be stacked in coolers to prevent damage and to avoid climatic extremes. Temperatures throughout collection and transport should be maintained within the range of water temperatures at the time of collection (e.g., 20-28ºC in 2001). Although temperatures in the Creston Valley are relatively cool in April and May, if they must be lowered during transport, chipped ice (in sealable bags and wrapped in towels) can be placed at the bottom of the cooler.

10.1 Acclimation

Upon arrival at the rearing facility, hatchlings should be subjected to a series of slow water changes (e.g., 20% every 20 minutes; Kendell 2001) to allow acclimation to the new water source, and fed a small amount of food immediately.

10.2 Documentation

The following information should be recorded for each mass before hatchling collection begins: date, collectors name, oviposition site, mass location, mass identification, approximate mass size/shape), water temperature, air temperature, mass depth in the water, water depth, general health of the mass, and tank destination. This information should be recorded onto the collection record data sheet in Section IV and entered into a computer database.

11.0 Growth and Metamorphosis

11.1 Growth Measurements and Health Assessments

One index for determining the general health of captive-reared *R. pipiens*, is to compare the growth and development rate of tadpoles at various stages of development to their wild counterparts.

Animal keepers should monitor the developmental stage of the hatchlings/tadpoles (i.e., Gosner stage) weekly, beginning immediately after collection. Once the tadpoles have been moved to the larger tanks (Gosner Stage 25), into their respective densities, 10% of individuals in all tanks will be measured weekly until release (weight, SUL and total length), including the smallest and largest tadpoles in each. Any abnormalities in terms of discolouration, size, lesions, gas bubbles, irregular movement, deformities, etc. will also be recorded.

Efforts should be made to capture and measure tadpoles and metamorphs for comparison.

11.2 Metamorphosis

Amphibians are particularly sensitive during metamorphosis, when the major transition from an aquatic, gill breathing, herbivorous form, to a terrestrial, lung breathing and carnivorous life stage takes place. It is important to minimize the amount of disturbance and stress the animals experience during this time.

A beach-type exit from the water must be made available to metamorphs to leave the water as required; metamorphs can drown if this is not provided (Nace *et al*. 1996; Crawshaw 2000). In 2001, several rocks and floating cover were provided for metamorphs to exit the water (Figure 4).

Figure 5. *Rana pipiens* metamorphs on rocks and artificial lily pads at a captive-rearing facility in the Creston Valley, BC.

In 2001, it took approximately one month from the onset of metamorphosis before all individuals were released into the wild. Metamorphs should be released upon metamorphosis to enable them to begin foraging in their new environement as soon as possible. Metamorphs will not be retained in captivity unless quarantine is required, (see Section 3.3.1). If individuals are retained, they should remain in their tank of origin and their growth should be monitored.

Held in captivity, metamorphs will be fed small crickets (e.g., *Acheta domestica*), dusted with mineral supplements (e.g., Herptivite and Reptical). Other dietary options include wingless fruit flies (*Drosophila melanogaster*) and/or sowbugs (e.g., *Oniscus aselus*), which are readily raised at 25-30°C in shallow pans with damp peat moss (covered with moist cardboard). Sowbugs are fed crumbled rabbit chow and crushed blackboard chalk, and under adequate conditions, a colony will double its number every 40 days (Nace *et al*. 1996).

11.3 Documentation

All aspects of growth and development of captive and wild tadpoles will be recorded. In addition to the weekly growth measurements, all aspects of metamorphosis (i.e., first and last date of transformation, number transformed/day, size at transformation, etc.) will also be recorded on the growth, development and metamorphosis data sheet in Section IV, and entered into the computer database regularly.

12.0 Disease Monitoring

All animal care keepers should follow the same routine during daily care and maintenance to allow tracking of disease outbreaks to its point of origin (i.e., care for tanks in the same order daily; Lynch 2000).

12.1 Overview of Infectious Diseases and Parasites

Common causes of death in captive amphibians are general bacterial infection and septicemia (e.g., *Aeromonas*, *Pseudomonas*, etc.; S. Raverty, pers. comm.). These bacteria are common in the environment, so infection usually represents a decrease in an animal's immune system in response to something in their environment such as a virus, nutritional deficiencies, or stress (Nace *et al*. 1996). For example, poor water quality can stress individuals and increase susceptibility to infection.

12.1.1 Aeromonas

It is difficult to identify animals infected with *Aeromonas* on the basis of appearance, but those that do show external signs often have a slumped posture, a disinclination to move when prodded, epidermal perforations and hemorrhages, and numerous neurological signs. Tetracycline has been identified as an effective treatment for amphibians infected with *Aeromonas* (Nace *et al*. 1996).

12.1.2 Iridoviruses

Iridoviruses have become a concern for wild and captive amphibians. There are four genera within the Iridoviridae family, two of which infect invertebrates (Iridovirus and Chloriridovirus) and two that infect vertebrates (Lymphocystivirus – fish, and Ranavirus - amphibians). Within the Ranavirus genus, there are two strains of concern for wild and captive amphibians - Frog Virus 3 (FV3) and tadpole edema virus (TEV; Green 2001). Under experimental conditions, *R. pipiens* embryos and tadpoles are extremely susceptible to FV3 (Green 2001). Iridovirus exists in the wild as a common infectious agent among fresh-water fishes, and *R. pipiens* populations may be infected before entering husbandry facilities (S. Raverty, pers. comm.). Infected embryos show "depigmentation, epidermal sloughing, and lordosis", while tadpoles show "ventral dermal hemorrhages, and abdominal edema" (Green 2001), while dead frogs exhibit liver, kidney and spleen necrosis (Lynch 2000). Infection by Ranaviruses can result in secondary infection by bacteria, which can make diagnosis challenging (Green 2001). Iridovirus is common among aquarium fish, so caution should be taken when purchasing products from pet stores. This virus is a very hardy, persistent disease that requires diligent cleaning to ward off (S. Raverty, pers. comm.).

12.1.3 Chytridiomycosis

Chytridiomycosis (*Batrachochytrium dendrobatidis*) has also become a problem in wild and captive amphibian populations since the late 1990's. This fungus is keratinophilic, meaning that it is only found in the mouth parts of infected tadpoles (the only keratinized area of the body), and that they will not be killed as a result. But, infected individuals will die once they metamorphosize into adults and the proportion of keratinized epidermis on the body increases. Clinical signs of this disease vary between species, and include lethargy, reddening of the ventral skin, convulsions with extension of the hind limbs, accumulation of sloughed skin over the body, and occasional ulcers (Berger and Speare 1998*a*). Skin of the ventral body, limbs and feet are most often infected. Death usually occurs within a few days of the onset of the disease. None of these signs are specific to this disease and diagnostic tests are required to confirm an outbreak. No airborne transmission has been observed, but all infected frogs must be kept separate to ensure that no water borne transmission of the disease can occur (Berger and Speare 1998*a*).

There is no effective prophylactic treatment for tadpoles, however, itraconazole appears to be an effective prophylactic treatment against chytridiomycosis for frogs (Lynch 2000). Some other antifungal agents have proven effective for a similar mycotic dermatitis as well (*Basidiobolus ranarum*; see references within Berger and Speare 1998*a*). If tadpoles are considered to be at risk for *Batrachochytrium*, a subset of individuals should be sacrificed during rearing for diagnostic testing. For highly endangered species, one alternative is to rear the animals through metamorphosis and then treat them with itraconazole bathing (Lynch 2000).

Currently, no immunizations are available for amphibians against ranavirus, Chytridiomycosis, or other significant infectious diseases (Cunningham *et al*. 2000).

12.1.4 Nutrional Disease

Nutritional disease must be monitored closely, as numerous deformities can result from poor diet. Larvae fed a diet high in spinach (i.e., oxalate-containing vegetables) can experience high growth rates, which may result in metabolic bone disease (Crawshaw 2000). Powdered supplements and high calcium invertebrate diets are required to combat this problem. In a *Rana pretiosa* tadpole diet experiment conducted at the Oregon Zoo, individuals reared on a spinach/bloodworm diet experienced the formation of gas bubbles in their digestive tract, which caused them to float to the surface after feeding; some also experienced tail deformities (Csuti and Sellers 2000). Gas bubbles may also result from improper water changes or source; e.g., supersaturated ground water (Somsiri 1994; Diana *et al*. 2001). Sustained exposure to supersaturated water (e.g., 6 d) can result in an increased infection rate by *Aeromonas* (Diana *et al*. 2001). There is no treatment for gas bubbles, only prevention (water should be held and aerated for at least 24 hours before use). Some tadpoles develop a curvature of the spine, and/or spindly leg disease, both of which may be related to a dietary deficiency in the vitamin B complex (e.g., vitamin B12). The cause of spindly leg is unknown, and tadpoles appear to develop normally until limb generation, at which time poorly muscled forelimbs appear. To combat these diseases, a vitamin B complex or living aquatic vegetation and algae can be added to the water (Wright and Whitaker 2001). Also, Crawshaw (2000) emphasizes the importance of a nutritional diet composed of fresh ingredients, including vitamins and supplements, as oxidation of some of the nutrients in the diet may be a factor in spindly leg disease.

12.1.5 Malformities

Some of the captive reared *R. pipiens* tadpoles in 2001 experienced malformations of the hind limbs that were likely the result of a trematode invasion in the joint during development (*Ribeiroia* sp.; Johnson *et al*. 1999). There is no treatment for these malformations, only prevention; the host organism for this parasite is a fresh water snail (*Planorbella tenuis*). These snails may be inadvertently introduced to *R pipiens* rearing tanks through fresh water sources, the addition of plant matter, rocks, wood, etc. from natural ponds, and through the movement of humans or animals between natural areas and the rearing facility. Rearing staff, and visitors to the facility, should follow decontamination protocols in terms of their clothing and gear, and domestic animals and wildlife should be discouraged from entering the rearing facility.

12.2 Collection and Shipping of Tissue and Specimens

All dead individuals found in the wild or at the captive-rearing facility must be fixed appropriately and immediately (see below), in order to ensure that the proper tests can be conducted (a postable protocol can be found in Section IV). These animals should be assigned to one of two treatments, so that both histology and pathology testing can be conducted on equal percentage of the animals; the 50% assigned to histology should be fixed in a buffered formalin solution (see below), and the 50% assigned to pathology should be frozen (unless they can be sent fresh). At the rearing facility, mortalities are expected and these animals will be shipped to the laboratory throughout the summer.

Where a suspicious illness arises, specimens are to be fixed and shipped, depending on the time of day, location, and how fresh they are, in the following ways (S. Raverty, pers. comm.):

1) If specimens can be sent to the lab immediately (e.g., during the day on week days), they should be placed into well-labeled, individual sterile bags, and shipped to the lab chilled (place a paper towel between the specimen bag and the ice pack to avoid direct contact with the cold surface).
2) On weekends, or in the evening, specimens should be kept frozen (-20ºC to -70ºC), and shipped right on the ice pack.
3) If dead animals are found and cannot be kept chilled or frozen, an incision should be made along the animal's abdominal wall (from the ribs to the pelvis), it should be fixed in a 10% buffered formalin solution (Gotte and Reynolds 1997) in approximately 20 times the volume of the specimen/tissue (DAPTF 2000), and sent to the lab as soon as possible.

4) If a bacterial infection is suspected, and fluid is present inside the abdominal cavity, a cotton swab should be taken by immersing the tip into the fluid and placing the swab into a transport media (i.e., vial); this should be shipped with the specimen (chilled).

The following formula produces a 10% formalin solution of neutral pH (Berger and Speare 1998*b*):

Chemicals required	
Di-sodium orthophosphate (Na_2HPO_4)	6.5gm
Potassium dihydrogen orthophosphate (KH_2PO_4)	4.0 gm
40% formaldehyde solution	100 ml
Distilled water	900 ml

Procedure
1. Dissolve salts in small part of water with heating.
2. Add remaining water, then formaldehyde.
3. Add 1-2 ml of methylene blue solution as a colour indicator.

12.3 Health Monitoring Protocol

Captive *R. pipiens* should be examined regularly for any signs of illness. This health assessment should correspond with a time when the animals are already being disturbed due to regular maintenance (e.g., during a feeding or cleaning period). Animal keepers should look for signs of lethargy, discolouration, loss of appetite, weight change, or behavioural abnormalities. An emergency procedure should be in place for the treatment of all ill or dying animals. All clinical care of *R. pipiens* should be referred to a veterinarian well versed in amphibian pathology (i.e., Stephen Raverty or Trent Bollinger – see below).

Regular sacrifices should be made of captive *R. pipiens* to gather baseline data on health issues within the species (S. Raverty, pers. comm.). The number to be sacrificed should be determined by the Recovery Team in consultation with the laboratories, based on the number being reared, the general health of the tadpoles, and the number that the Team is willing to lose. In 2001, approximately 5% of the animals in each tank were sacrificed for analysis. The Taronga Zoo in Australia sacrifices 15 tadpoles in each release group once per week for three weeks before any of their endangered frog species are released; five of the 15 are frozen, and 10 are submitted for histopathological analyses (Lynch 2000). Results of all pathology and histology analyses should be made available to members of the Leopard Frog Recovery Team as soon as possible.

All dead animals requiring pathology testing are to be sent to:

Stephen Raverty
The Animal Health Centre of the Ministry of Agriculture
Fisheries and Food Lab
1767 Angus Campbell Rd.
Abbotsford BC, V3G 2M3.
Phone: 1-800-661-9903.

Histology analyses (e.g., for chytrid) and PCR (e.g., for iridovirus) will be completed by:

Trent Bollinger
Canadian Cooperative Wildlife Health Centre,
Department of Veterinary Pathology,
Western College of Veterinary Medicine,
52 Campus Drive,
Saskatoon, SK, S7N 5B4
Phone: (306) 966-5153

The laboratories should be contacted prior to shipment, and confirmation of receipt of specimens requested.

12.4 Documentation

All packages containing dead specimens or tissue must be clearly labeled, and historical information should be included with the shipment (e.g., wild or research animal, age/developmental stage, location, date, contact person's information, and notes on any internal or external abnormalities; S. Raverty, pers. comm.). All information should be entered into a computer database immediately after shipping.

13.0 Release

13.1 Release Sites

It is the responsibility of the Recovery Team to determine when, where and how many *R. pipiens* will be released at the outset of the rearing season.

Metamorphs may be released at formerly occupied areas, or current ones. Releases at currently occupied sites pose greater risks to the wild population than historical sites, due to the potential for the introduction or spread of diseases and parasites, and negative impacts to the genetics of the wild population. These threats should be partially alleviated through sterile rearing protocols and disease monitoring. The health of captive-reared animals, and that of the wild population should be examined before releases take place (Woodford 2000). Sites where evidence of ranavirus or chytridiomycosis are found should not be used as release sites (Cunningham *et al*. 2000).

In 2001, captive-reared *R. pipiens* were released at an historically occupied site in the Creston Valley - Corn Creek Marsh, Compartment/Pond 1. In 2002, the Recovery Team plans to release 2/3 of individuals at a reintroduction site and 1/3 back at their site(s) of origin/collection, once it has been established that the captive-reared metamorph population is free from disease.

Release events should take place in the morning, when temperatures are lower. Metamorphs should be transported in containers with damp paper towels, and released in suitable habitat; with adequate cover and in close proximity to water.

13.2 Release Assessment

Wild metamorphs remain at breeding ponds until their tails are completely resorbed (Waye and Cooper 2001), suggesting that releases should not take place until the animals have reached this developmental state, and they naturally begin to disperse (i.e., Gosner stage 46).

The general health of all metamorphs should be assessed before they can be released. Animals with lesions, poor body condition, etc. are automatically rendered unfit for release on welfare grounds (Cunningham *et al*. 2000). All animals must be free from any therapeutic drugs that may have been administered by veterinarians for at least one week prior to release, to ensure that the drugs are not masking any signs of disease (Woodford 2000).

13.3 Marking

All released metamorphs should be marked, to determine the success of the captive rearing program. The most cost effective and reliable marking technique for amphibians is the use of Visible Implant Fluorescent Elastomer tagging system (VIE), developed by Northwest Marine Technology, Inc., Shaw Island, WA (Davis and Ovaska 2001). This technique involves fluorescent dye implants that are injected into the webbing of the hind toes (Figure 5), in colour and toe patterns that allow identification to at least the year and treatment level (P. Govindarajulu, pers. comm.). Each animal should receive at least two marks, in case one mark is lost. In 2001, the majority of metamorphs were marked on their right hind foot, in webbing #3 and #4 (M. Beaucher, pers. comm.). To facilitate marking, Kendell (2000) chilled metamorphs (4-6°C) so that movement was limited.

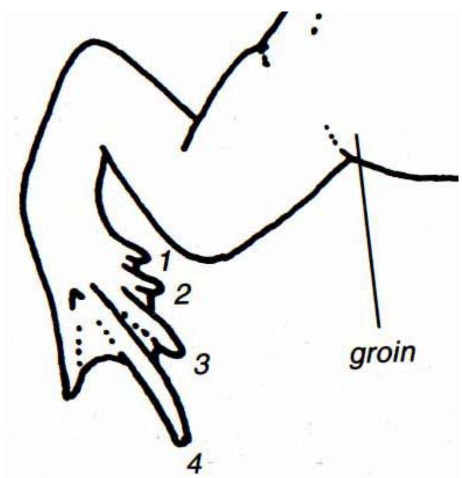

Figure 6. Webbing location and numbering system for injection of VIE dyes for marking *R. pipiens*.

The needle used for injecting the dyes should be cleaned and disinfected between animals. In order to protect against bacterial, viral and fungal agents, the tips of the needles will be immersed briefly in ethanol.

Animals should be marked > 24 hours before release to allow the dyes to cure and to minimize stress. Metamorphs should be retained in tubs with damp paper towels during marking. Because the colours can appear quite similar and marks can be small, the ultra violet light and glasses provided with the VIE kit must always be used to confirm marks. The effectiveness of marks should be confirmed before metamorphs are released.

13.4 Transportation

As the rearing facility is in close proximity to the release site (i.e., 20 minute drive), the density of metamorphs during transport may be higher than that recommended for more distant sites (e.g., 0.001/cm^2; M. Demlong in Sredl and Healy 1999). Metamorphs should be placed into small plastic tubs with damp, unbleached paper towels. The tubs must have air holes and be stacked in coolers for transport. Temperatures should be maintained at ambient air and water temperatures recorded at the time of collection from the tanks.

13.5 Documentation

All aspects of the marking procedure and the release should be recorded in the release record data sheet in Section IV and entered into a computer database immediately.

14.0 Quarantine

Procedures must be in place to retain ill animals in quarantine, away from the remaining captive population. Sick animals should be removed from their tank immediately and held in quarantine. If a large number of animals in a tank become ill and/or die, all sick animals should be quarantined, and the remaining animals should be removed from the tank for cleaning and disinfection following the procedures above. A veterinarian familiar with amphibian pathology must be contacted immediately for a health assessment, and instructions regarding immediate care, potential shipping of tissue for analyses, and the duration of quarantine.

Animals that have to be removed from existing captive populations due to illness should not be returned to their original enclosures until the disease has been controlled for at least two weeks (Nace *et al*. 1996), and a veterinarian confirms the health of the animal. The total duration of the quarantine will depend on the incubation period of the disease concerned (Woodford 2000). Quarantine should be performed on an "all in, all out" basis - if an individual in a group being held in quarantine contracts an illness, that animal should be isolated from the group, and a new quarantine period should begin for the entire group (Lynch 2000; Woodford 2000).

Equipment should be devoted to the quarantine facility only. Animal care keepers should care for healthy animals before quarantined animals on a daily basis, and they must clean and disinfect their hands before and after caring for each group of animals following decontamination protocols.

14.1 Euthanasia

There are various acceptable methods for euthanizing amphibians, some of which do not lend them selves to histopathological analyses. As a result, the clinician should be the one to conduct the euthanasia when ever possible (Wright 2001). The following procedures the most easy and efficient for animal keepers to administer if required:

- *Tricaine Methane Sulfonate* (MS-222) *Overdose* – prolonged immersion does not appear to be stressful (AVMA 2001; Wright 2001). The AVMA (2001) recommends a concentration of ≥ 250 mg/L for fish (left in the solution for at least 10 minutes following cessation of gill movement), but a lower solution can likely be utilized for tadpoles (e.g., 150 mg/L in titration will likely result in death in 1-2 minutes; S. Raverty, pers. comm.). For low alkalinity waters (i.e., < 50 mg/L), the solution should be buffered with sodium bicarbonate to achieve a pH of 7.0-7.5 (AVMA 2001). A stock solution can be made and retained in a refrigerator or freezer in a brown bottle, replaced monthly, especially if a brown colour is observed (AVMA 2001).
- *Ethanol Overdose* – initial sedation in 5%, followed by exposure to a higher concentration (20%+; Wright 2001).

Animal care keepers should not attempt to brain pith animals as a form of euthanasia due to the accuracy required for this procedure to be properly administered (AVMA 2001; Wright 2001).

15.0 Monitoring, Research and Experimental Design

The purpose of captive rearing *R. pipiens* is to increase population numbers through a head start program. Research can play a significant role in optimizing the number of animals that are produced by determining the most effective conditions for rearing. The number that survive to release, their growth rates and size at metamorphosis all serve as indicators of a successful rearing program. However, to understand the overall success of the captive-rearing program, animals should be monitored after release throughout their lifetime, to record and compare growth rates, their ability to successfully breed, and overall longevity.

Research that poses a high mortality risk to embryos, larvae or adult frogs should not be conducted. Therefore, the upper and lower limit of research treatments should fall within known or suspected acceptable levels.

15.1 Research Priorities

Because size at metamorphosis is important in terms of the overall fitness and potential survival rate of amphibians (Wilbur 1976), research during captive rearing should focus on factors that influence the health, size and survival of metamorphs. Three of the main factors that affect the growth, development and survival of captive-reared tadpoles are density, temperature, and diet/nutrition.

The Recovery Team suspects that tadpoles reared in 2001 were slightly smaller than their wild counterparts at metamorphosis. The size discrepancy may be due to density and/or diet/feeding regime. Because these variables interact with each other they may be most effectively investigated under a factorial design.

15.1.1 Density

It is important to determine the optimal density for rearing *R. pipiens* as there is a cost-benefit relationship associated with this variable. More animals will be produced if they are reared at higher densities, but the animals reared under these conditions may be smaller than their lower-density counterparts. The ideal density for captive-reared *R. pipiens* tadpoles should maximize fitness and production, but remains unknown due to the fact that the density of tadpoles, and the feeding levels, varied within and among tanks in 2001. The literature does not provide adequate information on this variable, as it does not suit the scale of this captive-rearing program. As a result, this variable is a research priority for 2002.

Although the effect of density can be altered through various means (e.g., constant water levels and different numbers of animals/tank, a constant number of animals and varied water levels, and through increased habitat complexity), the best design will keep other variables as constant as possible. For example, in a design that has variable water levels and a constant numbers of tadpoles in each tank, it would be difficult to separate the effect of density from that of temperature in outdoor tanks.

15.1.2 Diet/Nutrition

Diet plays a major role in the growth and survival rate of captive-reared amphibians. The nutritional content of the food, as well as the amount fed, will both affect growth and fitness. Amphibians fed a varied diet will likely experience lower mortality rates and greater overall health (e.g., lower deformity rates) because there is a greater probability that all of their nutritional demands are being met. Amphibians fed adequate amounts may tolerate higher density conditions, as long as water quality can be maintained.

Investigating the quality and quantity of food fed to *R. pipiens* tadpoles is a research priority because there is greater effort/cost involved with providing hand-made food versus the practicality of utilizing commercially produced tadpole and fish flake foods. The diet currently being offered to the tadpoles is labour intensive to prepare versus commercially available/prepared foods, and may lack some essential nutrients. To investigate this, compare the effects of artificial/prepared foods (e.g., fish flake food, spirulina, and bloodworms) with the natural foods currently being used (mixed vegetables and blood worms) in terms of growth rate, development, survival and size at metamorphosis, as well as cost and labour involved. It may be useful to have the nutritional content of the foods analyzed.

15.1.3 Temperature and Other Factors

Amphibian larvae grow faster in warm versus cold water, and under natural conditions, larvae seek out the shallow, warm water areas of ponds. However, in a natural setting, a thermal gradient exists so that tadpoles can adjust behaviourally to meet their thermal demands. Tadpoles reared in outdoor tanks will be exposed to greater daily temperature fluctuations and extremes than their wild counterparts. The effects of this on overall health are unknown, but may affect growth and survival rates.

It is difficult to alter the water temperature of outdoor tanks, without influencing other factors as well. For example, shading would also influence the amount of UV-B tadpoles are exposed to, and providing a constant fresh water source into the tanks to maintain a more constant temperature would likely alter water quality. This would be best studied under laboratory conditions, where ambient air and water temperature can be controlled.

Other factors that may influence captive-reared tadpole health and fitness include: developmental stage at collection, extent of bottom surface area of the tanks, and exposure to ultra-violet radiation (e.g., UV-B).

The collection stage may be a critical factor in the survival rate and fitness of captive-reared *R. pipiens*. Determine whether the survival rate to metamorphosis, and size at metamorphosis, is greater for *R. pipiens* when they are collected for rearing as eggs, versus hatchlings or tadpoles.

In addition to density of tadpoles within the water column itself, the amount of bottom surface area may play an important role in growth and development, especially where tadpoles of a species spend much of their time lying on the bottom. Rear some animals in small tanks and some in larger tanks, at the same density.

Studies have shown that amphibian embryos and larvae may be sensitive to UV-B. Tadpoles in outdoor cattle tanks may be exposed to harmful levels of UV-B, because they are limited in where they can seek shelter. Provide 100% UV-B protection over a proportion of the tanks (continuously and/or partially during the day), to see if this affects overall health, growth, and survivorship. Alternately, provide more cover objects in some tanks (although the results of this may be influenced by density issues as well).

15.1.4 Monitoring the Survival of Metamorphs

Efforts should be made to monitor released R. pipiens as a measure of the success of the program. Spring calling surveys should be conducted at release sites two years after the first animals were released (i.e., starting in 2003), when released animals should reach sexual maturity. Surveys for previously released animals should be conducted in summer prior to the release of the current batch of captive-reared *R. pipiens*. Released animals should be caught and their movements monitored by radio telemetry to determine habitat use at release sites.

15.2 Study Design

Experiments must be designed carefully, and should take into account numerous factors. It is essential that the design of the study include an appropriate research question, appropriate variables to be measured for testing, a hypothesis, high internal validity to maintain adequate power, and defined boundaries to which the results apply (i.e., laboratory population only, wild populations, etc.; Hayek 1994). Also, appropriate sample sizes and the biological significance of results must be considered. This may prove challenging with the limited number of *R. pipiens* remaining in the Creston Valley, and the small geographic extent of the population. The design and results of controlled, captive studies must be placed into the context of natural conditions.

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SECTION IV – APPENDICES (DATA FORMS AND POSTABLE PROTOCOLS)

Section IV of the manual provides Appendices of data record sheets and postable protocols for all aspects of captive rearing, including egg mass monitoring, hatchling collection, water quality testing, daily and weekly maintenance of *R. pipiens* tadpoles, growth and development monitoring, and marking and releasing animals. ***Please note***: all data sheets are available in a Windows Excel file to allow for ease of manipulation to columns and rows where adjustments might be needed, and for immediate data entry and analyses.

Postable Protocol - MORTALITY: FIXING AND SHIPPING

If you find a dead tadpole/metamorph/frog:

1. Record mortality on Appendix C-1 data sheet, including body measurements, tank number and date collected.
2. Assign 50% of animals to fixation in formalin, and 50% to freezing.
3. If a known illness (e.g., chytrid) is suspected, or an abnormal mortality has occurred, contact the appropriate Recovery Team member (e.g., Doug Adama) to discuss the situation with Stephen Raverty (contact information below) – fix accordingly and send to the lab as soon as possible.
4. Specimens should be shipped to the laboratory in Abbotsford, where Stephen Raverty will determine the appropriate course of action (i.e., whether specimens should be sent for histology testing, etc.). Ship fresh or frozen animals to the lab chilled - place a paper towel between the specimen bag and the ice pack to avoid direct contact with the cold surface for fresh animals; frozen animals can be shipped right on the ice pack.
5. Label the bag/container with the specimen number, collection date, organization/location of origin, and recorder's name.
6. ***If dead animals are found in the field and cannot be kept chilled or frozen***, an incision should be made along the animal's abdominal wall (from the ribs to the pelvis), it should be placed into a 10% buffered formalin solution in approximately 20 times the volume of the specimen/tissue, and sent to the lab as soon as possible. If a bacterial infection is suspected, and fluid is present inside the abdominal cavity, a cotton swab should be taken by immersing the tip into the fluid and placing the swab into a transport media (i.e., vial); this should be shipped with the specimen (chilled).

Shipping Address

Stephen Raverty The Animal Health Centre of the Ministry of Agriculture Fisheries and Food Lab 1767 Angus Campbell Rd. Abbotsford BC, V3G 2M3