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Improving Redclaw Crayfish (Cherax quadricarinatus) Aquaculture: Assessment of Invasive Impacts and Production of All-male Broods

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Improving Redclaw Crayfish (*Cherax quadricarinatus*) Aquaculture: Assessment of Invasive Impacts and Production of All-male Broods

by

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Abstract

The Australian redclaw crayfish (*Cherax quadricarinatus*) was first considered for aquaculture in the United States in 1990 and is quickly becoming an important species for aquaculture worldwide. Redclaw are valued as both a food source and as an ornamental aquarium species. Ecologically-sound aquacultural development requires the development of environmentally low-impact, yet cost-effective culture techniques. Because this species is exotic, its potential impacts on native fauna should be addressed before the development of an aquaculture industry in the Southeastern United States. Several aspects of aquaculture for the redclaw were assessed in a series of experiments which evaluated: 1) the impacts of adult redclaw on the native crayfish *Procambarus acutissimus*, 2) effective anesthetics and their ability to improve survival of juvenile redclaw exposed to physical stress, and 3) the effectiveness of androgenic gland ablation in sex-reversal of stage III juvenile redclaw crayfish with the ultimate goal of producing all-male broods.

A 24-week competition study (July – December) was conducted in outdoor tanks containing 9 native crayfish and a range of redclaw densities (0, 2, 6, 10, 16, and 20 per tank) with a constant number of shelters and identical feeding rates among all tanks. The experiment ended when all redclaw died in mid-December. Redclaw appeared to affect average size of *P. acutissimus*, but did not affect survival. Results supported previous studies that suggest redclaw cannot overwinter in most of the United States. Even though

redclaw invasion duration is likely limited by cold winter temperatures, they may have significant, negative effects on growth of male crayfish. However, reduced growth of female crayfish was not observed, decreasing the likelihood of indirect effects on egg production. The brooding season of redclaw and *P. acutissimus* overlapped, suggesting the potential for competition between YOY of both species, but these interactions were not addressed in this study. Food preference, assimilation efficiency, and relative ability to acquire food resources should be studied to further evaluate the effects of redclaw competition on native North American crayfish species.

Anesthesia has useful applications in handling, transport, and surgery of many aquaculture species. In this study, the effectiveness of different anesthetics was evaluated for their ability to produce heavy sedation and improve survival of surgery-stressed juvenile redclaw. Isoeugenol (Aqui-S) and ice bath anesthesia were both effective anesthetics for juvenile redclaw. Juvenile redclaw achieved heavy sedation in < 10 minutes with Aqui-S concentrations between 5 and 15 ml/L. Crayfish that were exposed beyond heavy sedation exhibited decreasing survival and increasing time to recovery with increasing exposure duration and increasing Aqui-S concentration. Ice bath anesthesia produced heavy sedation for juvenile redclaw at temperatures between 8 °C and 17 °C; lower temperatures were required for smaller crayfish to reach heavy sedation. Seventy-two hour, post-ablation survivorship was high for all juvenile crayfish regardless of age-class or use of anesthetic. Therefore, anesthesia was not used in the subsequent ablation evaluation study.

To evaluate the effectiveness of ablation in producing sex-reversed, neofemale broodstock crayfish, androgenic gland ablation was tested on pre-release juvenile

redclaw. Mating a normal male and a sex-reversed male (neofemale) together should result in the production of all-male progeny, which will greatly improve mono-sex male redclaw crayfish culture. Androgenic gland ablation was carried out on 70 stage III juvenile redclaw, ~1 week pre-release, from three broodstock females (N=210). Ablated juvenileswere grown out for three months along with non-ablated, control crayfish (N=90) in individual containers. Survival and growth rates were high and did not differ between ablated and control crayfish, showing that crayfish can be feasibly ablated at a very early age. The number of males (49% ± 6%) and females (50% ± 7%) did not differ in the control group, but the ablated group contained de-masculinized, unilateral males and eunuchs, that lacked external sexual characteristics. The ablated group was comprised of 24% ± 15% males, 64% ± 17% of which were unilateral males, 26% ± 12% eunuchs, and 48% ± 6% females. The proportion of female crayfish in the ablated group was not significantly greater than the control group, indicating that sex reversal was unsuccessful. However, the presence of de-masculinized males in the ablated group showed that ablation did have an effect on development of external sexual morphology of males. Future studies should attempt ablation at earlier age classes and at cooler culture temperatures to increase the probability of neo-female production.

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List of Abbreviations

AFW	Artificial Fresh Water
AG	Androgenic Gland
AGH	Androgenic Gland Hormone
SGR	Specific Growth Rate
GP	Genital Papillae
O	Ovopore
ROC	Rate of Oxygen Consumption
TAN	Total Ammonia Nitrogen
YOY	Young of Year

I. Introduction

Aquaculture is, in essence, farming of aquatic organisms primarily for food purposes. Aquaculture practices include breeding, rearing of eggs and juveniles, and growout of adults in a myriad of salt and/or freshwater culture facilities including: greenhouses, tanks, raceways, ponds, cages, and recirculation systems. Given the ever-increasing human population, the degraded ocean fishery, and the constant demand for quality protein sources, aquaculture has become an important source of food for many cultures. Traditionally, wild catch fisheries dominated the fish production with 67.2 million tons per year in the 1980's coming from wild catch and 5.7 million tons per year coming from aquaculture (FAO 2007). Over time, wild capture has leveled off at about 60 million tons per year, while aquaculture has expanded to nearly match the wild catch with 50.3 million tons in 2007 (FAO). Clearly, future generations will continue to increase their dependence on aquaculture in their food production strategy, and continual research is necessary for improving aquaculture production.

The Australian redclaw crayfish (*Cherax quadricarinatus*), hereafter referred to as redclaw, was first considered for aquaculture in the 1970's and is quickly becoming an important species for aquaculture worldwide. Redclaw are valued as both a food source and as an ornamental aquaria species. Aquaculture for the redclaw occurs in Australia, Southeast Asia, Africa, Latin America and to a small extent the United States. The redclaw is a subtropical species, native to Australia, with relatively high tolerance to poor water quality. In one growing

season (five to seven months), redclaw will grow to 60-120 grams, whereas red swamp (*Procambarus clarkii*), native to North America, will only reach 28 to 40 grams (Masser and Rouse 1997, Romaire et al. 2005). The edible tail portion, or dress-out percentage, of the redclaw is considerably higher than that of the red swamp - 30% and 15-20%, respectively (Masser and Rouse 1997). These traits, coupled with its gregarious and non-burrowing nature as well as its relatively large size and palatability make it a desirable species for aquaculture.

The aquaculture industry for redclaw remains underdeveloped in the United States because of a lack of infrastructure, marketing, and general knowledge of the species among other problems. Because feed and labor are the major costs of production in aquaculture, it is important to reduce feed inputs and labor to a minimum. Mono-sex male culture of redclaw is the fastest and most efficient way to grow large crayfish that will yield a high market price (Curtis and Jones 1995, Naranjo 1999, Rodgers et al. 2006). Mono-sex male culture produces larger individuals than mixed-sex culture because males grow faster than females and, in the absence of females, channel their energy towards growth rather than reproduction (Curtis and Jones 1995, Rodgers et al. 2006). The Australian yabbie crayfish (*Cherax albidus*), a close relative to the redclaw, was grown in monosex male, female, and polysex cultures, and the monosex male culture yielded a 70% increase in the market value of the final product when compared to the other cultures (Lawrence et al. 1998). Similar results have been seen with the redclaw in monosex culture (Rodgers et al. 2006). In addition to the money wasted on feed for the slower growing female redclaw, separating the sexes by hand is a time-consuming, laborious process, which needs to be streamlined to improve profitability for farmers.

Because the redclaw is native to Australia, there is concern about their potential for invasiveness in North America. The redclaw has escaped captivity in Mexico and established breeding populations in lentic habitats, such as ditches and sloughs (Bortolini et al. 2007). Redclaw are not likely to establish populations in most of the United States because of their intolerance to cold winter temperatures and susceptibility to fungal diseases like *Aphanomyces astaci* or crawfish plague, to which the redclaw has no immunity. Thus, the impacts of escaped redclaw are likely to be seasonal in nature (Medley et al. 1993). Although they can reach a very large size, they are considered less aggressive than most North American species (Medley et al. 1993). However, the large size of the redclaw relative to the native crayfish species may give them a competitive advantage for food and shelter.

The recent global decline in species richness in crayfish has been tied to many anthropogenic factors including (1) habitat degradation; (2) chemical pollution; (3) introduction of non-indigenous organisms; and (4) overexploitation (Taylor et al. 2007). Crayfish aquaculture, or farming, may help ameliorate the issues associated with overexploitation of wild populations. Although the *Procambarus clarkii*, the most cultured and consumed species of North American crayfish, is not in peril (Eversole 2008), the use of aquaculture for redclaw production could alleviate pressures on wild finfish and marine shellfish fisheries. However, aquaculture activities often result in the translocation of a desirable aquaculture species outside of its native range (Eversole 2008). Translocations of exotic species can result in native species declines by providing a vector for range expansion and displacement of native species or hybridization of the exotic and native fauna (Taylor et al. 2007). With 363 identified taxa, North America has

the highest crayfish diversity in the world; however, only 52.1% of those species are considered stable, with invasive crayfishes identified as a major threat (Taylor et al. 2007). Many crayfish species that are considered invasive are native North American species that have undergone rapid range expansion through a myriad of anthropogenic pathways including aquaculture and bait-bucket translocations by anglers (Kulhmann and Hazelton 2007, Taylor et al. 2007, Eversole 2008). These occurrences have been documented for the *Orconectes rusticus* (Kuhlman and Hazelton 2007) and *P. clarkii* (Eversole 2008) as well as other North American crayfish species (Taylor et al. 2007). Non-indigenous species are typically successful at establishing populations beyond their native range when environmental factors are similar to the native range, and when the introduced species has competitive advantages over the native species that occupy a similar niche (Hayes et al. 2009). The effects of species invasion can expand to all trophic levels, in some cases altering the macrophyte and benthic macroinvertebrate communities (Rosenthal et al. 2006). The Southeastern United States has great potential for impacts from non-indigenous crayfish because it is the global 'hot-spot' for crayfish species diversity with 85 species occurring in Alabama alone (Schuster et al. 2008; Taylor et al. 2007). However, competition between native and introduced species does not always lead to extirpation of the native species, but rather an altered population dynamic (Gurevitch and Padilla 2004; Ricciardi 2004). Concerns about invasive potential must be addressed and researched when developing a new aquaculture species.

II. **Effects of a potential North American invader (*Cherax quadricarinatus*) on survivorship and growth of a native crayfish species (*Procambarus acutissimus*) in the Southeastern United States**

Introduction

The recent global decline in species richness in crayfish has been tied to many anthropogenic factors including (1) habitat degradation; (2) chemical pollution; (3) introduction of non-indigenous organisms; and (4) overexploitation (Taylor et al. 2007). Crayfish aquaculture, or farming, may help ameliorate the issues associated with overexploitation of wild populations. However, aquaculture activities often result in the translocation of a desirable aquaculture species outside of its native range (Eversole 2008). Translocations of exotic species can result in native species declines by providing a vector for range expansion and displacement of native species or hybridization of the exotic and native fauna (Taylor et al. 2007).

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Because the redclaw is native to Australia, there is concern about their potential for invasiveness in North America. The redclaw has escaped captivity in Mexico and established breeding populations in lentic habitats such as ditches and sloughs (Bortolini et al. 2007). Redclaw are not likely to establish populations in most of the United States because of intolerance of cool winter temperatures and fungal diseases like *Aphanomyces astaci* or crawfish plague, to which the redclaw is vulnerable at low temperatures (14 °C). Thus, the impacts of escaped redclaw are likely to be seasonal in nature (Roy 1993, Medley et al. 1993). Although they can reach a very large size, they are considered less

aggressive than most North American species (Medley et al. 1993). However, the large size of the redclaw relative to the native crayfish species may give them a competitive advantage for food and shelter.

Escapement from aquaculture facilities is a potential vector for redclaw dispersal in the Southeastern United States. To assess potential impacts of seasonal redclaw invasions, we examined competition between redclaw and another species that is native to the United States and has a similar life history strategy. *Procambarus acutissimus* is common in Alabama (Schuster et al. 2008). Although knowledge of this, and most other non-commercial species, is limited, it has been reported that *P. acutissimus* prefers lentic rather than lotic habitats, can occur in dense populations, and is not a strong burrower (Hobbs 1981). Because habitat and burrowing characteristics are similar to those of *C. quadricarinatus*, *P. acutissimus* is a good model species for examining effects of escaped redclaws on native crayfish species. Several redclaw invasion densities were simulated to test the following hypotheses: 1) *C. quadricarinatus* will not survive through the winter in an outdoor environment, and 2) redclaw "invasions" can affect native crayfish survivorship and growth within the course of a single growing season.

Materials and Methods

Collection and Pre-experiment Holding

This experiment was conducted at the E. W. Shell Fisheries Research Center, Auburn University in Auburn, Alabama, USA. *Cherax quadricarinatus* has been cultured in ponds and indoor recirculating systems at this facility for research purposes since the late 1980's. Overwinter survivorship in ponds was shown to be zero in previous studies (Medley et al 1993, Semple et al. 1995) and no escaped *C. quadricarinatus* have

been reported from nearby streams. Research stocks are occasionally enhanced by outside sources. Redclaw crayfish for this study were obtained from Megar, a redclaw production facility in Tamaulipas, Mexico. Native crayfish (*P. acutissimus*) were collected during June 2009 using baited traps and dip nets from roadside ditches near Old Town Creek, Shorter, AL (32°18'57.50" N, 85°55'01.05" W). Prior to the experiment, crayfish were held separately by sex and species in twelve outdoor (120 cm diameter X 45cm deep) polyethylene tanks with PVC shelters (5.3 cm inside diameter X 15 cm) and flow-through surface water from a reservoir.

Simulated Invasion Study

Twelve outdoor (120 cm diameter X 45cm deep) polyethylene tanks were used for this experiment. Each tank was stocked in mid-June with 9 native crayfish (5 males, 4 females) such that the initial stocking density of native crayfish was approximately115g/m^2 (average size 8.7 ± 2.5 g). Tanks were subsequently "invaded" with redclaw in July, after native crayfish were allowed to acclimate for two weeks. Two tanks were stocked at each of the following densities: 0, 2, 6, 10, 16, and 20 redclaws per tank (average redclaw size = 15.9 ± 3.5 g), each with a sex ratio of 1M:1F. Fifteen open-ended PVC shelters (5.3 cm inside diameter X 15 cm long) were placed in each tank. Although we could find no information on shelter-use by *P. actutissimus*, use of shelter by *Orconectes rusticus* (a native species undergoing range expansion) has been documented in the wild (Martin and Moore 2007). Agonistic behaviors between crayfish are typically low intensity interactions with the winner largely determined by size rather than shelter ownership (Martin and Moore 2007). The provision of shelter has been shown to improve the survival of redclaw crayfish in outdoor culture ponds (Jones et al.

2001). Therefore, the assumption was made that a ratio of 1 shelter to 1 crayfish would be an important threshold, above which crayfish would become shelter limited. In our study, the tanks initially stocked with 6 redclaw had the same number of total crayfish (native plus invader) as shelters. Tanks stocked with < 6 redclaws had more shelters than crayfish and tanks stocked with > 6 redclaws had fewer shelters than crayfish.

Critical standing crop is the biomass at which the growth of the population begins to slow from the maximum. In redclaw tank culture, crayfish are typically stocked at 200 g/m^2 (bottom area) and thinned at an estimated critical standing crop of 400 g/m^2 in order to maximize growth (Garza 2009). Therefore, crayfish in the mesocosms were estimated to become growth-limited at a biomass between 200 and 400 g/m^2 (Garza 2009). An initial combined crayfish biomass of ~ 200 g/m^2 was attained in the tanks with 6 redclaws and 9 native crayfish.

Crayfish in all tanks were fed daily with a 35% protein commercial, sinking shrimp feed (Rangen, Inc). Suggested feeding rate for this and similar feeds is 3% of crayfish biomass per day (Masser and Rouse 1997). All tanks were fed at a rate appropriate to 200 g crayfish $/m^2$ /day (200 * 0.03 = 6 g feed / m^2 / day). Thus, tanks stocked with < 6 redclaws were likely to have had excess food while tanks stocked with > 6 redclaws were more likely to have been food-limited at the outset of the experiment, assuming that 3% of biomass is an adequate feed rate for growth in both species. Feeding rates were decreased to 1.5% for the theorized 200 g/m^2 during November to adjust for decreased cold-weather feeding based on the presence of uneaten feed in the tanks.

Crayfish were fed daily at 6:00 PM and feeding data recorded. Water quality parameters were collected for the duration of the experiment. Temperature (HOBO logger), pH, and DO were taken in the morning (low diel values) (VWR International, LLC., Batavila, FL, USA, sympHony multi-probe meter). Total ammonia nitrogen, nitrite nitrogen, alkalinity, and calcium hardness were measured and recorded weekly (LaMotte water quality test kit). Every two weeks, all crayfish were removed from each tank and enumerated. Size (mm, rostral carapace length) and mass (g, wet weight) was recorded for each individual and gravidity and egg stage were recorded for each female. The experiment was continued until all redclaw had died in mid-December.

Data Analysis

Tanks that received the same number of initial redclaw were not considered to be true replicates since the number of redclaw (treatment) in each tank changed differentially during the course of the experiment. Therefore, to assess the effect of redclaw on native crayfish survivorship, native survivorship was regressed against current redclaw numbers during each sampling. Similarly, to assess the effect of redclaw on crayfish growth, average size of native crayfish was regressed against the current redclaw biomass and total biomass for each sampling event. Statistical analyses were performed using SYSTAT 12 (2007) (SYSTAT Software, Inc., San Jose, CA, USA) statistical software. Statistical significance was determined using $\alpha = 0.05$.

Results

Water quality parameters such as total ammonia nitrogen (TAN), nitrite nitrogen, calcium hardness, and total alkalinity were measured weekly. TAN and nitrite nitrogen remained below detectable limits for the duration of the experiment, < 0.2 mg/L and < 0.05 mg/L, respectively. Calcium hardness and alkalinity ranged between 20 and 40 mg/L, which are acceptable for redclaw culture (Masser and Rouse 1997). Temperatures ranged from 12 °C to 35 °C. Dissolved oxygen ranged from 4.2 to 9.3 mg/L and pH ranged from 6.2 to 8.4. All water quality parameters, aside from winter temperatures, were within acceptable ranges for redclaw aquaculture (Masser and Rouse 1997).

Increased redclaw abundance did not result in increased mortality of native crayfish. Native crayfish survival did not produce a significant trend when regressed with total redclaw abundance at week 4 (p-value = 0.2567), week 8 (p-value = 0.933) or week 16 (p-value = 0.513) (figure 1). In the tubs with highest redclaw numbers, the total number of crayfish exceeded the number of shelters until ~ week 16 (figure 1). Both species were observed in and out of the shelters during daylight hours throughout the study. Shelters sometimes had multiple inhabitants with both species occasionally observed within the same shelter. Approximately 40% of the native crayfish were still alive by the end of experiment, but 100% of the redclaws had died by mid-December when daily water temperatures were averaging 17.7 ± 1.9 °C (figure 2).

Growth rates (g /day) were estimated as change in average size during two periods of the study. Growth of *P. acutissimus* was calculated from the no-redclaw treatments only, whereas redclaw growth rates were calculated from all treatments where redclaw were present. In the first period (week 8, August 25, 2009 through week 14,

October 6, 2009), redclaw survivorship was relatively stable whereas survival of *P. acutissimus* was declining in a linear fashion (figure 2). Growth rates were 0.104 g/day and 0.220 g/day for the native and redclaw crayfish, respectively. In the second period (November 2, 2009 through December 16, 2009) native crayfish survivorship had stabilized and growth rate was estimated at 0.105 g/day. Redclaw growth rate was not estimated during this period because they had all died out prior to December 16.

Average size of native crayfish exhibited a significant decline with increasing redclaw biomass after week 12 of the experiment, but not before (figure 3 & 4). The slope of the regression of native crayfish average weight verses redclaw biomass became more negative after week 12 and remained statistically significant thereafter. From week 12 onward, redclaw biomass explained ~40 – 60 % of the variation in *P. acutissimus* average size (figure 4).

Average crayfish weight could also have been affected by the presence/absence of eggs, loss of chelipeds, and molting frequency, with these factors differentially affecting males and females. Therefore, average weight of males and females were regressed separately against redclaw biomass. Additionally, carapace length of male and female *P. acutissimus* was regressed against redclaw biomass (figure 5) since carapace length would not be as strongly affected by cheliped loss and egg production as would average size. Average weight and carapace length of male *P. acutissimus* decreased significantly with increasing redclaw biomass (*p*-values = 0.0215 and 0.0007, respectively) and yielded the following linear relationship: $Y = -0.02730\ x + 24.42$, where y is average weight (g) and x is redclaw biomass (g/m^2) ($R^2 = 0.4615$). The regression of male average carapace length against redclaw biomass yielded the following linear

relationship: Y = -0.02119 x + 45.28, where y is average carapace length (mm) and x is redclaw biomass (g/m^2) (R^2 = 0.7350). Average weight and carapace size of female *P. acutissimus* did not decline significantly with increasing redclaw biomass (*p*-values = 0.2016 and 0.1228, respectively).

At week 16, redclaw average weight did not decline significantly with increasing total crayfish biomass (*p*-value = 0.361); however, native crayfish average weight decreased with increasing total crayfish biomass (Y = 0.0189 X + 22.0327, y = native crayfish average weight, x = total crayfish biomass, R^2 = 0.3021, *p* = 0.037) (figure 6).

Molting frequency was reported as the proportion of individuals of each sex and species that exhibited either a soft exoskeleton or an obvious gap between the tail section and carapace. *Procambarus acutissimus* of both sexes molted throughout the study period (figure 7 a,b). Redclaw of both sexes molted until mid November (figure 7 c,d) when survivorship declined precipitously (figure 2). Native crayfish were observed with eggs during September and October, with the highest proportion of berried females (32% of native females) occurring in the first week of October (figure 8). Redclaw crayfish were berried from July into December (figure 8). However, increased fungal growth on the eggs indicated that egg health was diminished beyond mid-November, and no redclaw young-of-year survived through December. Direct egg counts were not conducted.

The sex ratio of both the native and redclaw populations shifted over the course of the culture period (figure 9). The initial sex ratio (M:F) for *P. acutissimus* was 1.25

(5M:4F), subsequently declining to a minimum of 0.77 at week 12 and subsequently increasing again to 1.4 by the final week (figure 9). The redclaw population began with an even sex ratio of 1 (1M:1F), but declined over time, maintaining a ratio of ~0.8 for most of the culture period but declining sharply after week 18 (figure 9).

Discussion

When discussing the effects of competition between two species, it is important to consider the possible effects that are realized by both species. Agonistic behavior between individuals is common in crayfish, particularly in males, which is believed to aid a dominant individual in attaining resources such as food, shelter, and mates (Gherardi and Daniels 2004). Shelter is of particular importance for increasing crayfish survival with regard to predator avoidance and cannibalism during ecdysis and may influence population dynamics (Gherardi and Daniels 2004; Jones and Ruscoe 2001). Molting frequency is directly related to growth, since the majority of weight gain occurs during and immediately after molting. If shelter provides protection during the molting process, crayfish that can aquire and defend shelters would be more likely to survive cannibalism. This would be particularly important for fast growing species with a higher molting frequency. However, in aquaculture settings, crayfish are frequently observed to molt away from shelters (pers. communication, D. Rouse, Auburn University). Thus, it is not clear that crayfish use shelter during the process of molting, when they are most vulnerable. In this study, crayfish of both species and genders molted throughout the experimental period (figure 7). If shelter limitation affected survival due to cannibalism during molting, then mortality should have been highest at higher crayfish densities (>15

total crayfish) when shelters were limited. The number of total crayfish (redclaw plus native) in shelter limited tanks decreased over time to match the number of shelters by week 16 (figure 1). However, results showed no significant decrease in native survivorship with increasing redclaw abundance before week 16 (figure 1), indicating that shelter limitation did not affect native survival. Shelter use was not directly measured in this experiment. However, crayfish of both species were observed inside and outside of shelters during each sampling event, with both species occasionally observed within the same shelter.

The threat of the redclaw as an invasive species in Alabama is further diminished because of its inability to overwinter (figure 2). The redclaw anesthesia data in chapter 3 suggest that temperatures of 14 °C and below render juvenile redclaw immobile, increasing the probability of predation and/or starvation. Semple (1995) found that redclaw can survive short-term exposure to temperatures as low as 7° C; however, for adult crayfish, prolonged exposure to 12 °C causes decreased mobility and feeding and eventually starvation. Additionally, winter water temperatures in Alabama are sub-optimal for redclaw, compromising their immunity to disease and increasing their probability of mortality (Medley et al. 1991, Roy 1993). Roy (1993) reported that redclaw that were exposed to *Aphanomyces astaci* were susceptible at 14 °C, exhibiting heavy mortality, but were not susceptible at 20 °C. In this study, we did not examine redclaws for infection by *A. astaci* and do not know whether the redclaw die-off was due to cool temperatures alone, or to decreased immunity to disease once temperatures declined below 20 °C. However, the December mortalities support the conclusions of

previous studies that redclaw cannot survive prolonged exposure to cool temperatures and would not be able to overwinter in most regions of the continental United States.

Native crayfish average weight did not decrease with increasing redclaw biomass (figure 3d) until week 12 of the study in early September (figure 4). Low density mesocosms maintained higher growth rates, while high density treatments exhibited restricted growth; this trend became stronger over time (figure 3 & 4). The lower average weight of the native crayfish in high redclaw biomass mesocosms falls at the peak of the brooding season for *P. acutissimus*, and could have been related to energy expenditures related to reproduction (figure 8). The combined sex ratio (M:F) of the native crayfish from all mesocosms fell steadily from the beginning ratio of 1.25 (5M:4F) at stocking to < 1 at peak gravidity (figure 9). This indicates that gender-specific mortalities caused the population to become more female by week 16, which could disproportionately skewed the average crayfish weight because female crayfish are typically smaller than males in these species. However, the sex ratios at week 12 did not yield a significant trend (*p*-value = 0.715) when regressed against redclaw abundance, implying that gender-specific mortality did not cause the observed decline in average size of *P. acutissimus* with increasing redclaw biomass. Also, at week 16, male native crayfish exhibited a decreased average size for weight and carapace length (figure 5) when regressed against redclaw biomass; whereas, native females exhibited no relationship. Increasing redclaw biomass negatively affected the growth rates of male *P. acutissimus* more than females. The mechanisms behind this relationship are unclear. Chelipeds of male *P. acutissimus* were quite large and may have comprised a substantial proportion of total body weight. Males may have been engaging in more aggressive intraspecific and/or interactions prior to the

breeding season, which can be interpreted through the shifting sex ratios toward more female populations (figure 9). Cheliped loss could potentially explain the negative relationship between average weight and increasing redclaw biomass. However, carapace length would not have been directly affected by chelipeds loss, but carapace length also showed a negative relationship with increasing redclaw biomass. Thus, chelipeds loss is not a likely explanation for the negative effects of increasing redclaw biomass on average size of *P. acutissimus* (figure 5). Further experiments are needed to determine why effects of redclaw biomass on *P. acutissimus* size were gender dependent.

A central question is whether the decline in *P. acutissimus* size with increasing redclaw biomass was simply a result of the total biomass of crayfish in the mesocosms surpassing the critical standing crop or whether redclaw were outcompeting *P. acutissimus* as food resources became limiting. In the former case, we would have expected to see average size of both species decline similarly with increasing total biomass (native plus invasive biomass). However, if redclaw had a competitive edge over *P. acutissimus* we would expect to see average size of redclaw remain stable or decrease to a lesser extent with increases in total crayfish biomass. Results from this study suggest that redclaw were either outcompeting *P. acutissimus* for food or were better at assimilating commercial feed. At week 16, native crayfish average weight decreased with increasing total biomass, but redclaw average weight did not. During the first growth period (August through October), average individual growth rates for *P. acutissimus* and redclaw were 0.073 g/day and 0.154 g/day, respectively. The redclaw grew more than twice as fast as *P. acutissimus*. Although the effects of food capture were not directly evaluated, it is likely that resource competition between *P. acutissimus*

and redclaw favored redclaw as the winner (figure 6). This implies that redclaws out-competed *P. acutissimus* for food resources before they themselves became food limited; thus, at high densities, redclaw may out-compete natives over the course of a growing season. However, it may also be the case that redclaws were simply more efficient at assimilating the commercial feed pellets.

Efficiency of food resource capture was likely dependent on the resources available. Although feeding rates were maintained for a biomass of 200 g/m^2, other natural feed stuffs were available to the crayfish (i.e.- algae, snails, fish), because these experiments were conducted outdoors with flow-through surface water. The addition of natural food resources almost certainly supplemented the commercial feed resource provided at a rate estimated for a baseline of 200 g total crayfish biomass per meter square of bottom area (commercial feed input = 3% of crayfish biomass / day). Even with these additional, natural resources, average size of *P. acutissimus* declined with increasing redclaw biomass, suggesting that they were outcompeted by redclaw for food resources. Future studies comparing effects of increased native biomass to similar increases in redclaw biomass would help to further clarify whether the results of this study were due to interspecific competition. In addition, studies examining the relative ability of each species to assimilate commercial feed would be extremely useful.

The effects of redclaw invasions are likely to be species and/or context dependent. Medley et al. (1993) reported yields (kg/ha) from low density (1 crayfish/m^2) redclaw-*P. clarkii* growout ponds that were approximately three times lower in redclaw-*P. clarkii* interaction ponds than in monoculture ponds. In their study, average weight at harvest for *P. clarkii* did not differ significantly between treatments but slightly lower survival

rates for *P. clarkii* occurred in interaction ponds (13%) than in monoculture ponds (19%). Conversely, much lower survival of *C. quadricarinatus* occurred in interaction ponds (57%) compared to monoculture ponds (86%) (Medley et al. 1993). In our study, survival of *P. acutissimus* did not decline significantly with increasing redclaw density, but average weight of *P. acutissimus* did decline significantly with increasing redclaw biomass. Our mesocosms were stocked at a much higher densities (9 – 29 crayfish / m^2) than the ponds (1 crayfish / m^2) used by Medley et al. (1993) and it is possible that we would not have seen an effect on average size if we had limited our study to low density treatments. Because no redclaw monoculture treatments were tested in our study, the effects of *P. acutissimus* on redclaw survival could not be assessed. A direct comparison between these studies is not possible due to differences in species, stocking densities and experimental systems. However it is interesting to note that the difference in effects (i.e. survival vs biomass) found between the two studies.

Resource capturing, particularly food, directly affects overall health and gonad development for crayfish; which, in turn, affects the ability of a species to reproduce. Long-term effects of seasonal redclaw invasions may be manifested in reduced fecundity of the natives. Because some North American crayfish species reproduce in the spring and others in the fall, the effects of redclaw summertime invasions should have the greatest effect on fall-brooders due to direct competition during gonad development. The breeding season for *P. acutissimus* (a fall brooder) and redclaw crayfish did overlap, providing the possibility that resource competition may have affected the progeny of *P. acutissimus*. In this study, we documented overlap between the breeding seasons of redclaw and *P. acutissimus*. Competition between adults and offspring would be a strong

possibility for redclaws and fall brooders in invaded habitats. Because the redclaw began reproducing earlier than *P. acutissimus*, the redclaw young of year (YOY) may get a head start on food resources over the native YOY, but this remains to be tested and it is not known whether YOY native crayfish are typically food limited in natural environments. The effect of redclaw density on the proportion of gravid females was not analyzed because the number of females in each mesocosm was low (≤ 4) and the condition of any single female would have had a large impact on the proportion of gravid females in that mesocosm. Stochastic effects would have made it difficult to discern any significant trends. Future experiments utilizing larger numbers of females will be necessary to address this issue.

Our results show that even when redclaw invasion duration is limited by cold winter temperatures, they can have seasonal effects on a native species. These effects were reflected in reduced growth, rather than survival rates, and were gender specific. It is not clear whether reduced growth rates were simply due to increases in combined crayfish biomass or due to interspecific competition, but results suggest that redclaw were more efficient at assimilating food resources than *P. acutissimus*. Because *P. acutissimus* reproduces during this period of reduced growth, reproductive rates could have been negatively affected. However, the pattern of reduced growth with increasing redclaw biomass was driven by effects on male rather than female *P. acutissimus*. Thus the reduced growth rates were not likely to have strongly affected egg production.

The colonization rate of an organism is highly dependent on its reproductive rate. Because redclaw can produce multiple large broods per season, colonization of YOY redclaw could potentially be quite rapid. However, these effects should be diminished in

temperate climates (e.g. United States) compared to more tropical climates (e.g. Mexico) because YOY redclaw likely will not reach sexual maturity (~18g) within one growing season, are rendered immobile by cool temperatures (~ 14 °C), and are not likely to survive the winter. This study suggests that competition for food is the most likely mode by which escaped redclaw will affect native crayfish populations over the course of a season. Food preference, assimilation efficiency, and relative ability to acquire scarce food resources are dimensions of niche partitioning that should be studied to further evaluate the effects of redclaw competition on native North American crayfish species.

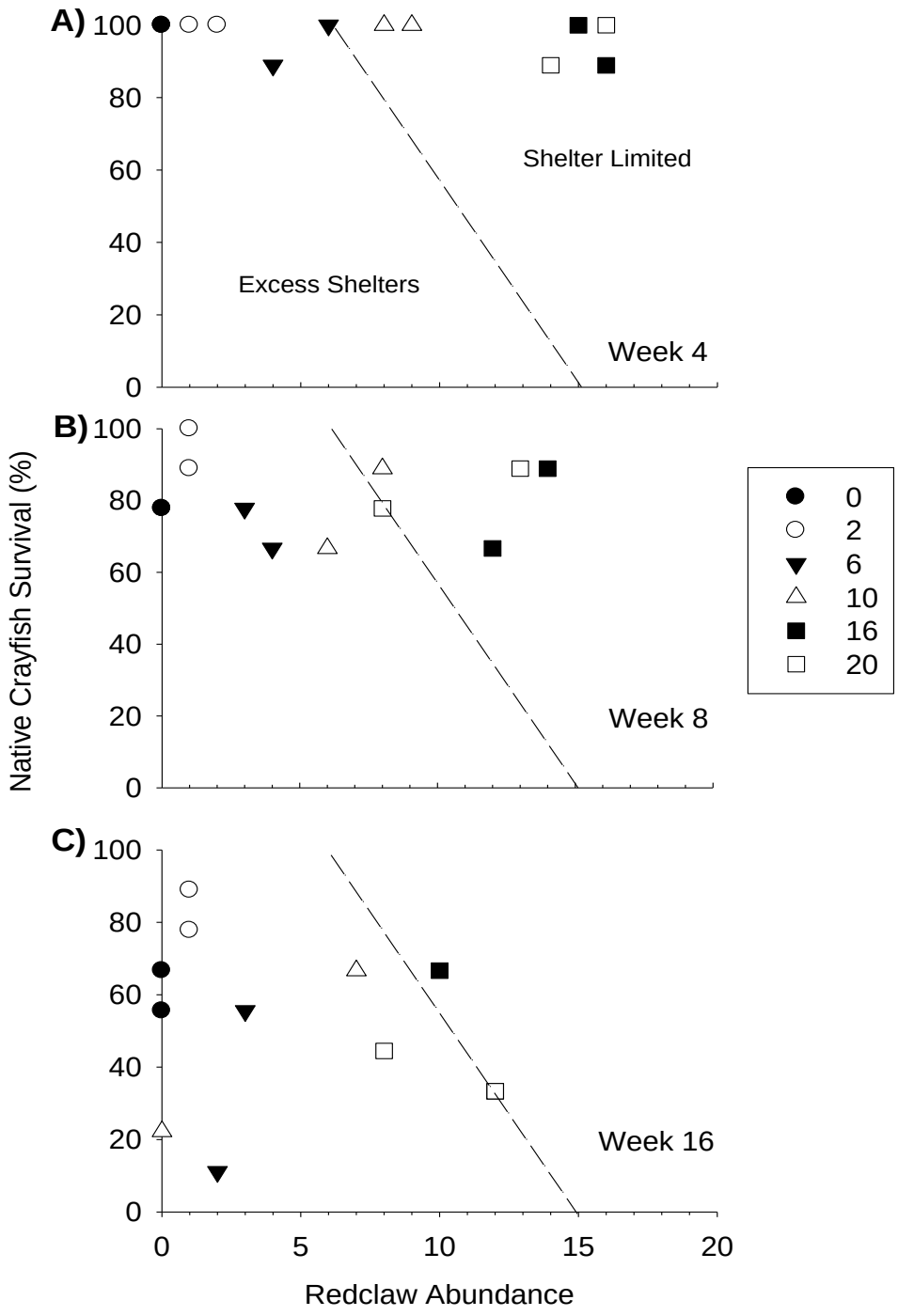

Figure 1. Native crayfish (*P. acutissimus*) survival vs. redclaw crayfish (*C. quadricarinatus*) abundance at weeks 4, 8, and 16. Trends were not significant in any week. Legend numbers represent the initial invasion density. Line represents the threshold of shelter limitation (15 shelters). Data points to the right of the line would represent mesocosms that were shelter limited, to the left would have excess shelters.

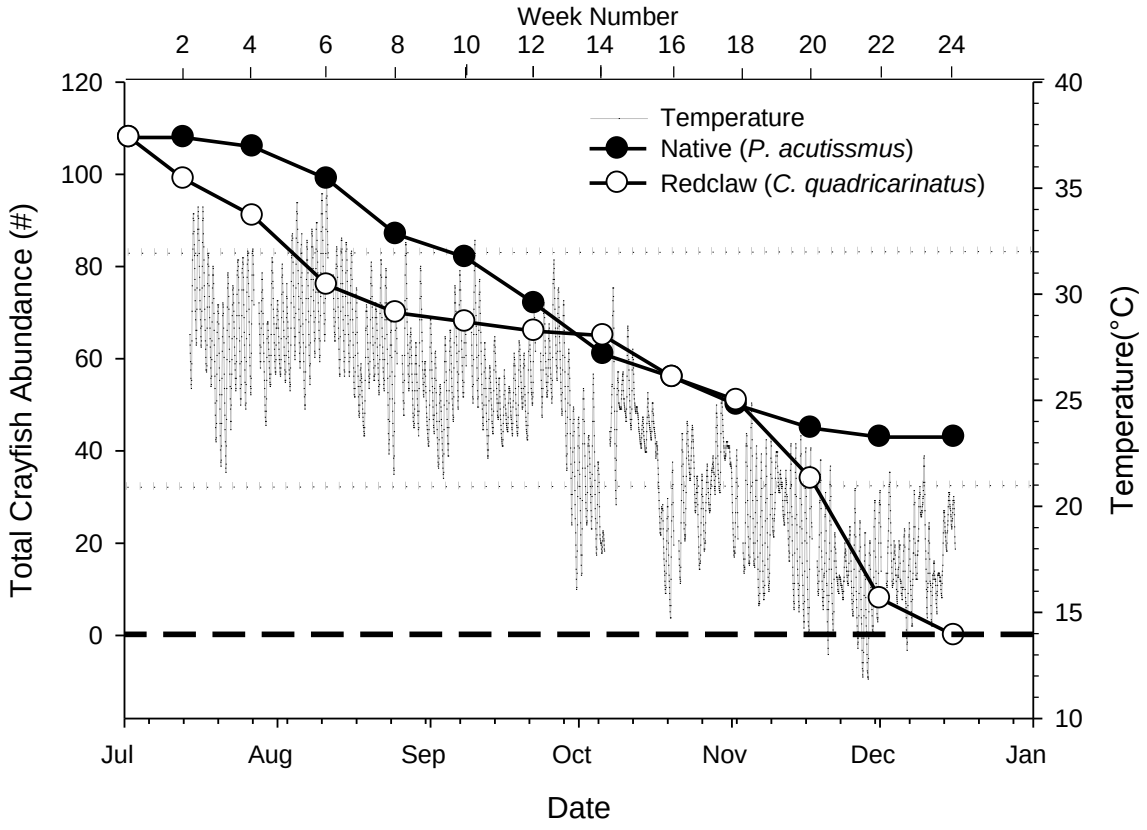

Figure 2. Total crayfish abundance for all mesocosms over time with respect to water temperature. Sampling occurred twice monthly beginning July 1 (week 0) and ended mid-December (week 24). The temperature range for redclaw growth (21-32° C) and threshold for crawfish plague susceptibility (14° C) redclaw growout temperatures are represented by the dotted and dashed lines, respectively.

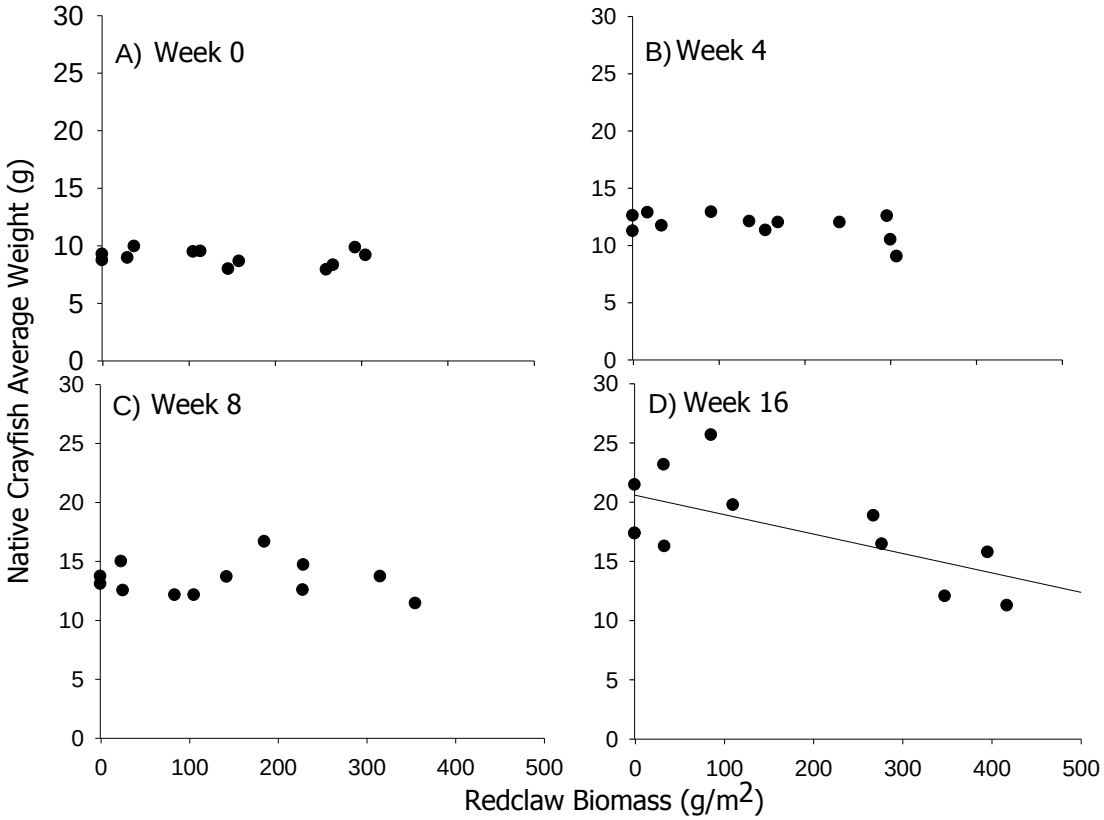

Figure 3. Native crayfish average weight vs. total redclaw crayfish biomass at weeks 0 (A), 4 (B), 8(C), and 16 (D). Trends were not significant for weeks 0, 4, and 8 (*p*-value >0.05), but was significant at week 16 (slope = -0.0163; *p*-value = 0.0234; R^2 = 0.4165).

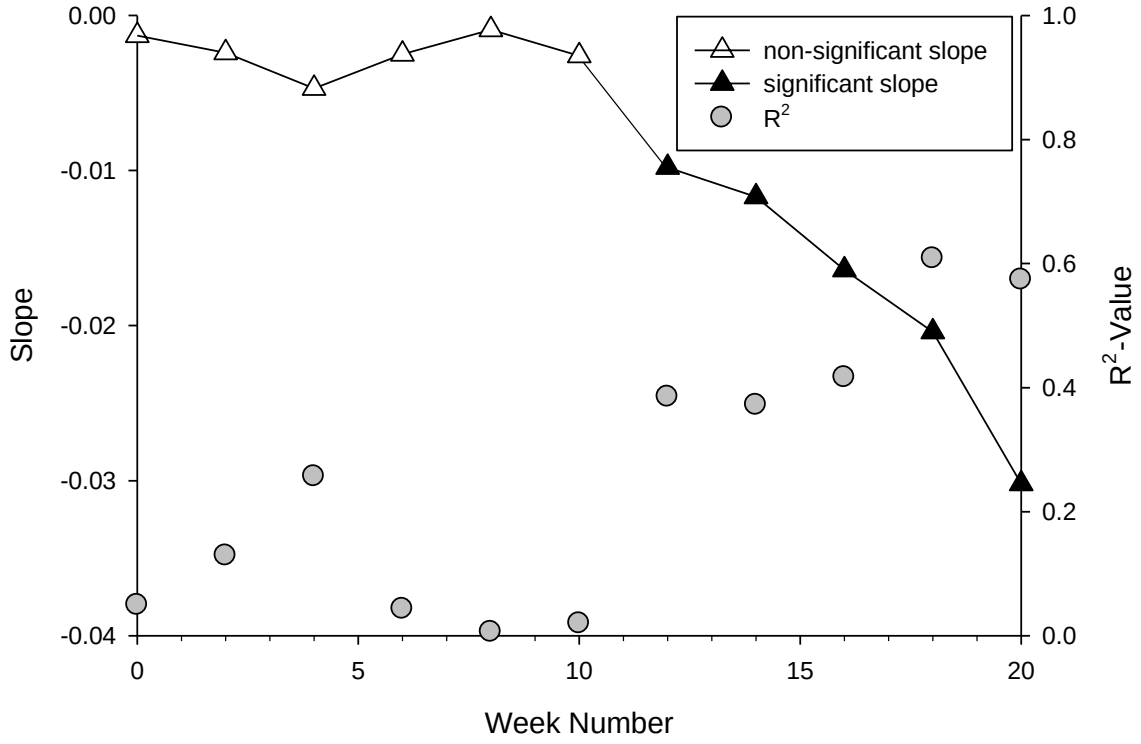

Figure 4. Slope plot from regression analysis for native crayfish average weight vs. redclaw crayfish biomass for weeks 0 thru 20 during the experiment. White triangles represent statistically non-significant slopes (*p*-value > 0.05), while black triangles represent statistically significant slopes (*p*–value < 0.05). Weeks 22 and 24 were not included because redclaw were virtually absent, and slopes were not significant. R^2 values are represented by gray circles.

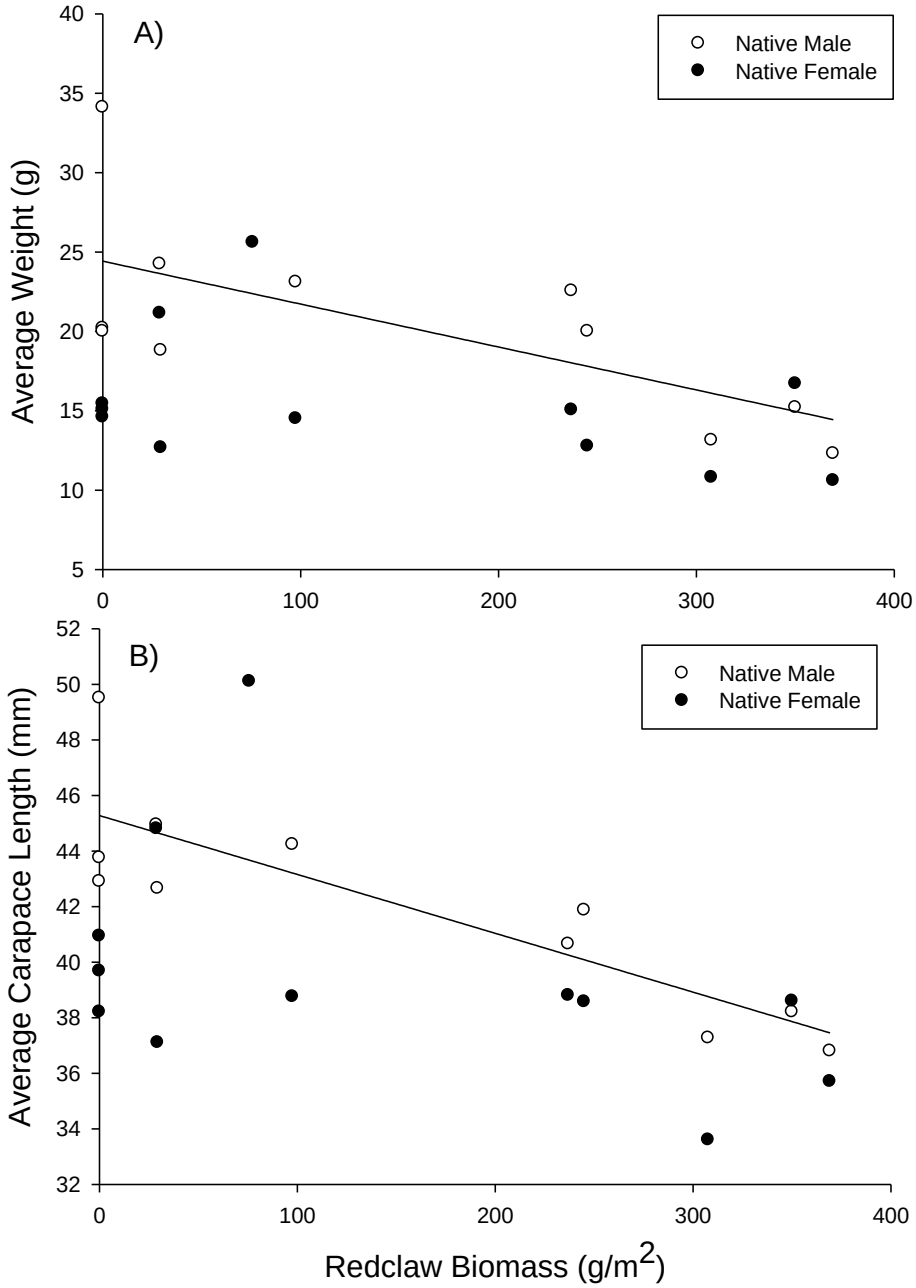

Figure 5. Average size of the *P. acutissimus* separated by sex and regressed against *C. quadricarinatus* biomass at week 16. A) Regression of native average weight against redclaw biomass. The male native average weight against redclaw biomass yielded the following linear relationship: Y = -0.02730 x + 24.42, (*p*-value = 0.0215, R^2 = 0.4615), not significant for females (*p*-values = 0.2016). B) Regression of native crayfish carapace length. The male native average carapace length against redclaw biomass yielded the following linear relationship: Y = -0.02119 x + 45.28 (*p*-value = 0.0007, R^2 = 0.7350); not significant for female natives (*p*-value = 0.1228).

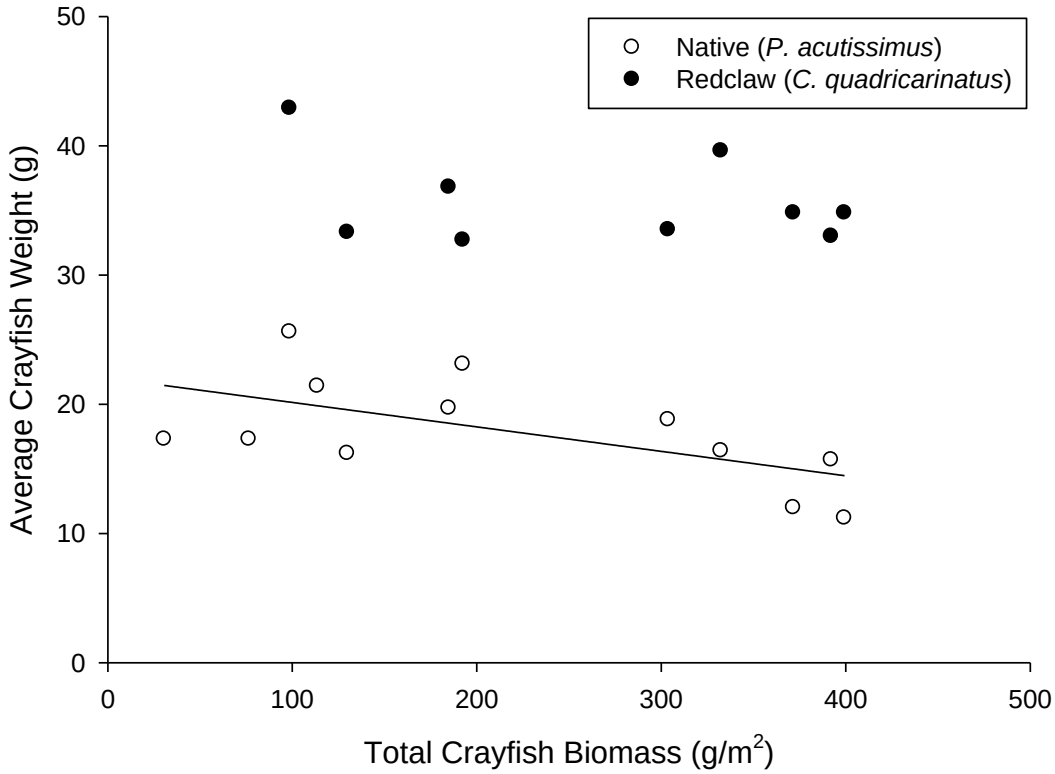

Figure 6. Average crayfish weight vs. total crayfish biomass (redclaw + native) at week 16. Native crayfish (*P. acutissimus*) average weight exhibited a significant decrease in average weight with increasing total crayfish biomass (*p*-value = 0.037) and followed the linear regression Y = -0.0189 X + 22.0327 (R^2 = 0.3021). Redclaw average weight did not significantly decrease with increasing total crayfish biomass (*p*-value = 0.361).

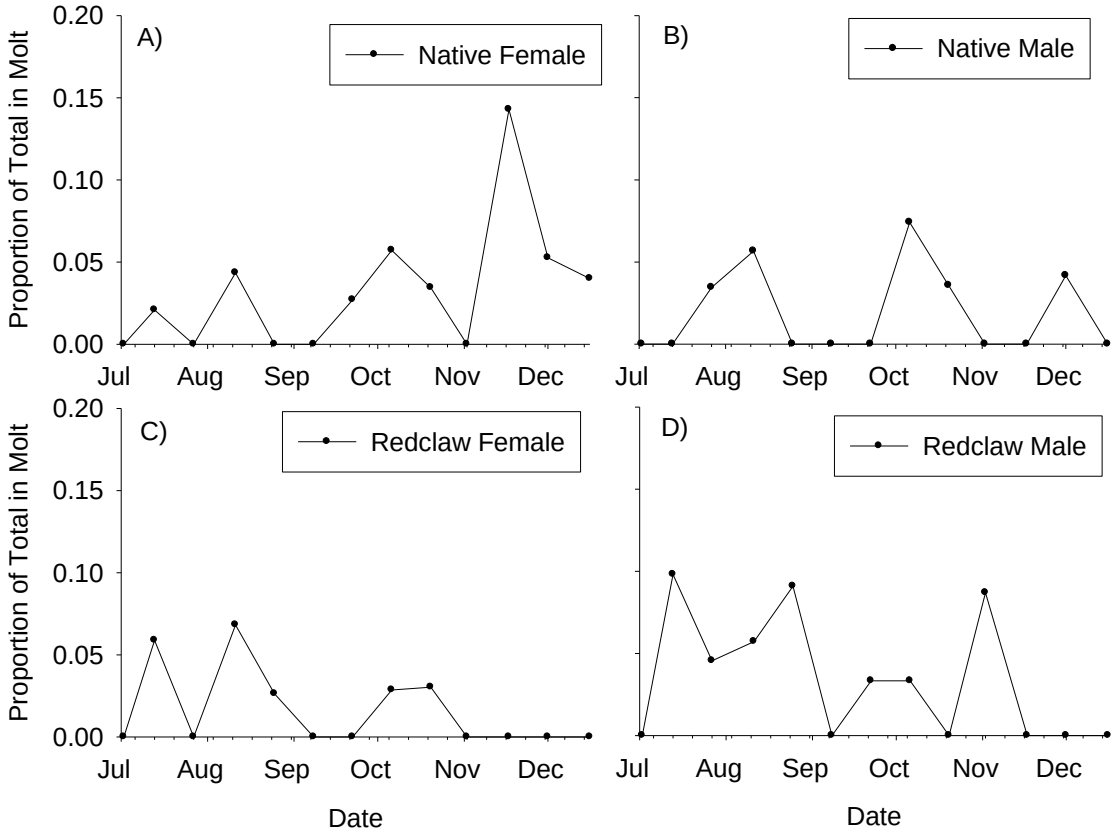

Figure 7. Molting frequency of A) native female, B) native male, C) redclaw female and D) redclaw males expressed as the proportion of the total crayfish of each individual species and gender with a soft carapace and/or obvious gap between the tail and cephalothorax observed during each sampling event.

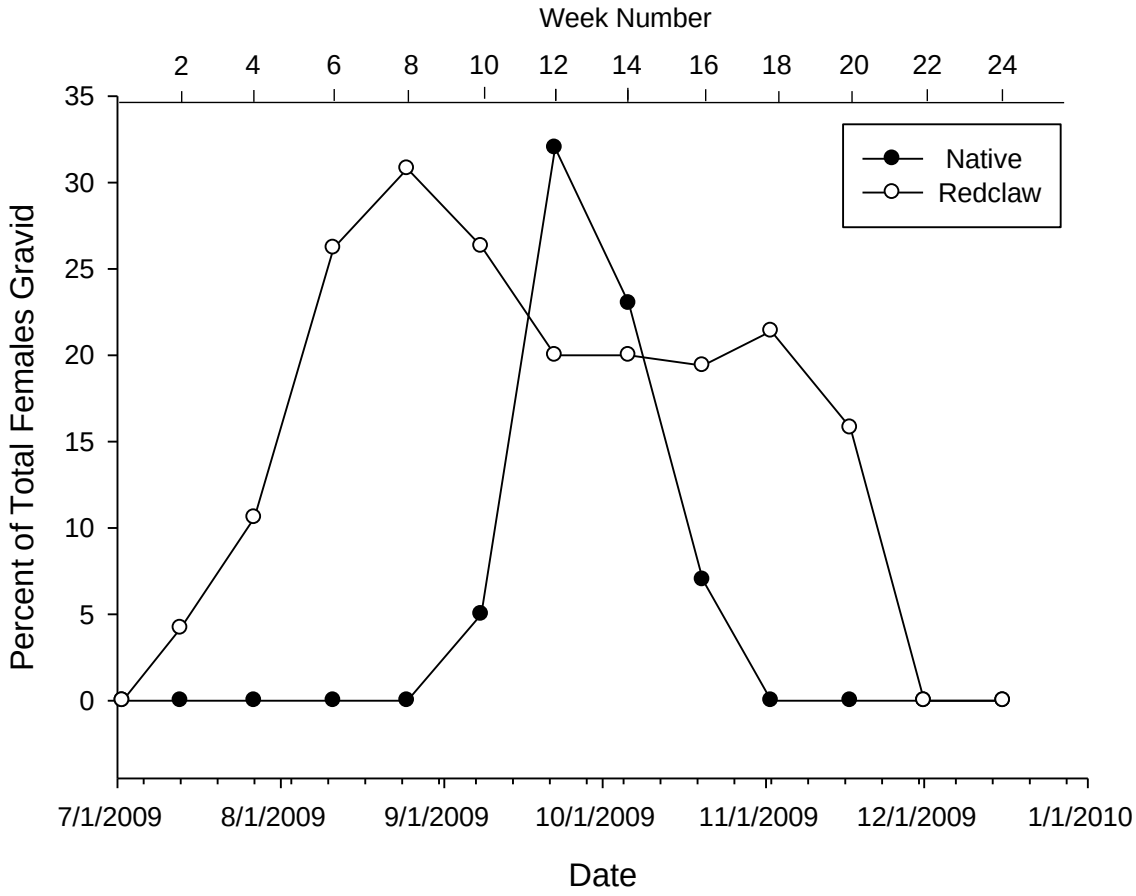

Figure 8. Percent of total surviving female native and redclaw crayfish that were gravid during the study.

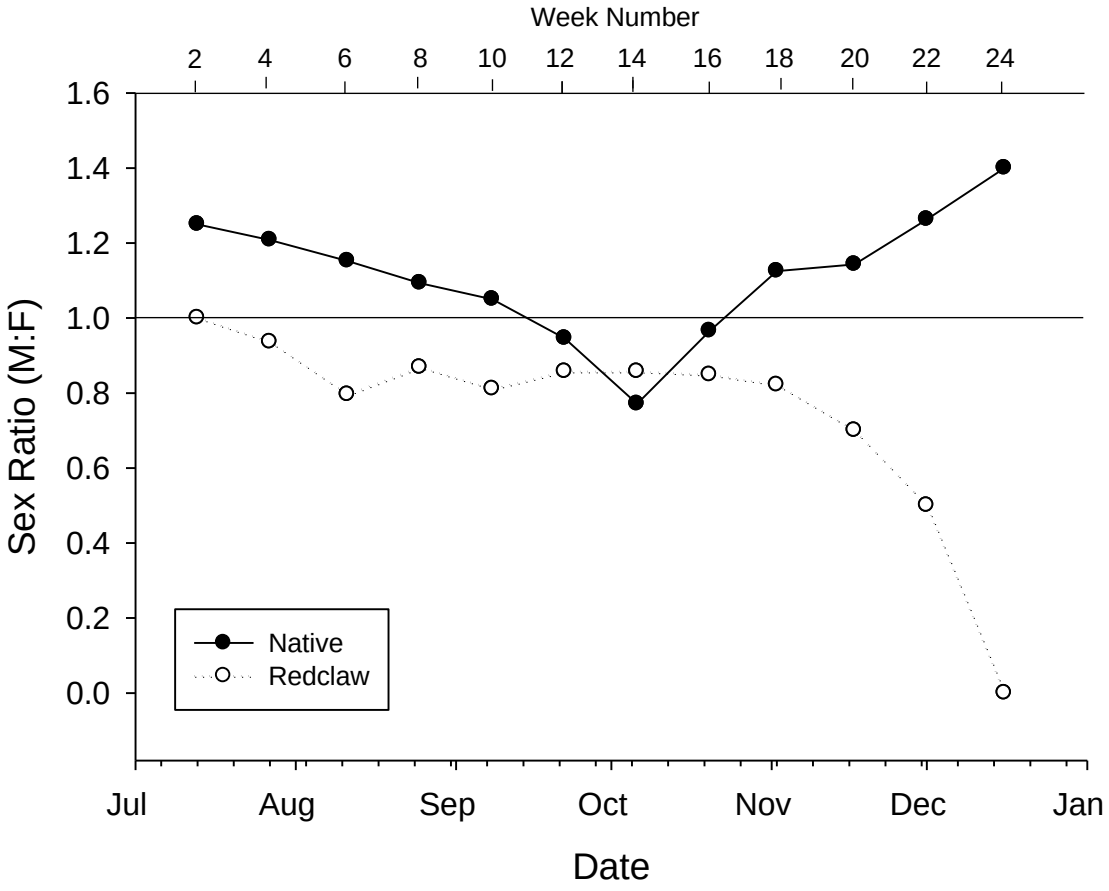

Figure 9. Sex ratios (M:F) of redclaw crayfish (*Cherax quadricarinatus*) and native sharpnose crayfish (*Procambarus acutissimus*) throughout the study. Horizontal line represents a 1:1 sex ratio. Data was calculated from all treatments combined.

III. **Evaluation of anesthetics for juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens), exposed to a physical stressor**

Introduction

The Australian redclaw crayfish (*Cherax quadricarinatus*) was first considered for aquaculture in the 1970s and is quickly becoming an important species for aquaculture worldwide. Redclaw are valued as both a food source and as an ornamental aquaria species. Aquaculture for the redclaw occurs in Australia, Southeast Asia, Africa, Latin America and to a small extent the United States. The redclaw is a subtropical species, native to Australia, with relatively high tolerance to poor water quality. Within a single five to seven month growing season, redclaws will grow to 60 to120 grams, whereas red swamp crayfish (*Procambarus clarkii*), a commercially farmed native species, will only reach 28 to 40 grams (Masser and Rouse 1997, Romaire et al. 2005). The edible tail portion, or dress-out percentage, of the redclaw is considerably higher than that of the red swamp - 30% and 15-20%, respectively (Masser and Rouse 1997). These traits, coupled with its gregarious and non-burrowing nature as well as its relatively large size and palatability makes it a desirable species for aquaculture.

Androgenic gland and eyestalk ablation are surgical procedures performed on crustaceans that affect sexual characteristics and maturation. Androgenic gland ablation is the removal of the androgenic gland, located at the base of the 5th pair of periopods,

which, when performed at an early enough age will lead to the complete sex reversal of some male crustaceans (e.g. Afalalo et al 2006, Rungsin et al 2006). Because androgenic gland ablation must be performed at a very early age, heavy mortality is suspected (Rungsin et al. 2006). Ablation without an anesthetic for *Macrobrachium* resulted in 19.6% mortality from the ablation procedure (Rungsin et al. 2006). This procedure has been successful in the production of neo-females (sex-reversed males), which when mated with a typical male leads to the production of all-male broods (Afalalo et al 2006, Rungsin et al 2006). All-male broods are of particular interest in redclaw aquaculture because mono-sex male cultures exhibit accelerated growth rates and attain greater harvest weights than mono-sex female or poly-sex cultures (Rodgers et al 2006).

Anesthesia has useful applications in handling, transport, and surgery of many aquaculture species. Anesthetics can be used during handling and transport to increase the survival of animals to be stocked into growout ponds (Coyle et al 2005). Sedation has been shown to reduce physical stress from crowding and poor water quality, which is reflected in the reduction of subsequent stress related hormone production (Small 2004). Sedation is sometimes necessary during surgical procedures. Nociception is a reflex reaction occurring in response to tissue damaging stimuli (Barr et al 2007). Surgical procedures on non-anesthetized organisms can cause the nociception reaction, causing that individual to struggle and cause damage to itself, possibly leading to mortality. Ablation of early-stage redclaws is likely to be traumatic and may result in high mortality without the use of anesthesia. However, anesthetizing agents and dosage rates have not been well researched for redclaw (Longley 2008).

Anesthetizing agents are beneficial for some surgical procedures. For example, eyestalk ablation involves the unilateral destruction of the X-organ, located in the eyestalk of crustaceans. Eyestalk ablation is commonly performed on shrimp to induce maturation of the broodstock females in order to produce year-round gravid broodstock (Aktas et al. 2003). Eyestalk ablation has also been widely tested in other crustaceans including the redclaw crayfish, which caused increased breeding in first-time spawners and increased molting frequency (Sagi et al. 1997). Topical anesthetic has been shown to decrease the period of stress-related feeding depression, post-ablation of the eyestalk in *Litopenaeus vannamei* (Taylor et al. 2004). Anesthesia may be beneficial in many other aspects of redclaw crayfish research and culture.

Some chemical anesthetics have been previously tested on freshwater crayfish. Several, such as tricaine methane sulfonate (MS-222) and 2-phenoxyethanol have been found to be ineffective (Brown et al. 1996, Coyle et al. 2005) while others such as lidocaine-HCl, and ketamine-HCl are effective (Brown et al. 1996) but regulated as controlled substances, making them impractical for use by aquaculturists. Anesthetics derived from clove oil (isoeugenol, Aqui-S) are readily available and have been shown to be effective on *Macrobrachium rosenbergii* (Coyle et al. 2005) but not yet tested on redclaw crayfish. Other anesthetics for crustaceans include ethanol, chlorbutanol, 2-phenoxyethanol, isobutyl alcohol, isobutanol, alfaxolone-alphadolone (Saffan), pentobarbitone, procaine HCl, propanidid injection, xylazine, chloroform, ethane disulphonate, and methyl pentynol; however, none of these are reported as effective for redclaw crayfish (Longley 2008). Cold temperature, an alternative to chemical anesthetic, has also been successfully employed as a sedative for crustaceans (Longley

2008) but specific temperature range and exposure times required to achieve heavy sedation in redclaw crayfish have not been reported (Longley 2008).

This study will explore the efficacy of a chemical anesthetic (Aqui-S) and temperature manipulation in improving survivorship of redclaw crawfish subjected to a physical stressor –ablation of the fifth pair of walking legs to remove the androgenic gland. The following hypotheses were tested:

I. Survivorship of the ablation procedure will change with age of juvenile redclaw crayfish.

II. Use of an anesthetic will affect survivorship and allow for surgery at an earlier age.

Materials and Methods

Broodstock for production of experimental animals were obtained from Megar, a redclaw production facility in Tamaulipas, Mexico. The juvenile redclaw crayfish to be used in this set of experiments were produced at Swingle Hall in Auburn, AL, USA. All crayfish were held in a recirculation system with 16 plastic tanks (30x47x15 cm) containing plastic mesh substrate and filled with artificial fresh water (AFW), which was constituted of 0.5 g/L (ppt) of salinity from Instant Ocean™ salt (1L 30 ppt salt water in 60 L deionized water), and 75 mg/L (50 mg/L $CaCO_3$ and 25 mg/L $CaCl_2$) of calcium hardness. Male and female broodstock were held at a 14L:10D photoperiod using a fluorescent lamp on a timer and 28 ± 2 °C using two 100-watt aquarium heaters to induce spawning (Masser and Rouse 1997). Males were removed from each tank upon observation of berried females. A 40% protein sinking feed was provided to berried

females at 3% of female biomass per day until the juveniles were released. Juveniles that were collected post-release were fed a powdered 40% protein feed at 40% biomass daily until they were used in experiments (Masser and Rouse 1997). Water quality parameters, such as total ammonia nitrogen, nitrite nitrogen, calcium hardness and pH, were monitored with a LaMotte water quality testing kit on a weekly basis.

Anesthesia Screening Study

First, a screening study was conducted with Aqui-S and cold temperatures on juvenile redclaw crayfish (average weight = 0.211g ± 0.066g) (Denver Instrument electronic scale, MXX-123, d = 0.001g) to determine appropriate Aqui-S concentration, temperature, and exposure times to test during the ablation procedure.

Aqui-S contains 50% of the active ingredient (isoeugenol) found in clove oil and has a specific gravity of 1.08. Juveniles (20-days post-release;average size = 0.467g ± 0.351 std) were obtained from a single brood and randomly assigned to one of three Aqui-S treatments (5, 10, and 15 ml Aqui-S / L AFW) until each treatment contained 24 individuals. Each juvenile was held individually in a 500 ml beaker containing 100 ml of the appropriate Aqui-S solution at 23.1 ± 1.1° C. Individual crayfish were monitored for up to 30 minutes during the experiment for signs of sedation; longer anesthetizing periods were considered failures because they would be impractical for aquaculture. Each treatment concentration had eight individuals, as well as two control reference crayfish that were not exposed to Aqui-S. Activity was recorded every minute for the first 10 minutes, and every two minutes thereafter. Signs of sedation included reduced claw and tail reaction to stimulus by a wooden stick, reduced walking ability or activity,

and loss of self-righting ability when the crayfish is laid on its side. Light sedation was defined as the loss of the ability to retreat from stimulus and given a rank of 1. Heavy sedation, adequate for surgery, was defined as the inability to self-right and given a rank of 2. These designations were given relative to the non-anesthetized juveniles. Once heavy sedation was reached, a subset (n=2) of the test subjects were moved immediately to an aerated recovery tank. The remaining juveniles continued to be held in the Aqui-S solution for a predetermined period (0, 5, 10, and 30 minutes), with two juveniles held per time period. The hold over period was designed to define the grace period that juvenile crayfish can remain in the anesthetic without mortality. The recovery period was defined as the time needed to regain the ability to perform the previously mentioned activities and was checked at zero, one, and three hours after heavy sedation was reached. Mortalities were recorded at 24, 48, and 72 hours post-sedation.

Cold temperature was also evaluated as an anesthetic. Eight juvenile crayfish (15 days post-release, average size = 0.368g ± 0.115std) were placed in individual beakers containing 100 ml of AFW at 24° C and then placed in an ice bath simultaneously to cool. A temperature probe was used to monitor water temperature of the water in one representative beaker throughout the experiment. Two reference crayfish were also placed in 100 ml of water, but held at room temperature (24° C) rather than the icebath. Crayfish were observed every minute for level of sedation (see previous description). After the appropriate holding time was reached, beakers containing crayfish were removed from the ice-bath, placed on the counter, and allowed to gradually warm back up to ambient room temperature (~ 24 °C).

Efficacy of anesthesia on ablated early-juveniles

Using results obtained from the screening study, the ability of Aqui-S and cold temperatures were tested as a means to increase survivorship of the ablation procedure on three age classes of crayfish: 0-3, 7-10, and 12-15 days after consumption of the yolk sac. During juvenile development, these age classes generally fall prior to, during, and after release from the broodstock female, respectively (Masser and Rouse 1997, King 1993). One hundred individuals were collected from broods at each of the specified age classes, their weights were recorded (figure 12a), and then randomly allocated into 10 groups of 10, held in 100 ml of the appropriate solution in 500 ml beakers. Three groups of ten were then randomly assigned to each of three treatments: ablation + no anesthesia, ablation + 5 ml/L Aqui-S, and ablation + icebath. The remaining ten crayfish were used as a reference group: no ablation, no anesthesia. The anesthetized groups were held in their respective anesthetic until all individuals reached heavy sedation, and then were immediately removed for ablation. Ablations were carried out using fine tipped tweezers and a dissecting scope to remove the fifth pair of walking legs at the base. After ablation, the juveniles were transferred to a recovery vial containing 20 ml of AFW and survival was monitored for 72 hours.

Statistical Analysis

Differences between treatments were determined using a one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test to detect treatment differences (Sokal & Rohlf 1995) at the $\alpha = 0.05$ level. Statistical analyses were performed using SYSTAT 12 (2007) (SYSTAT Software, Inc., San Jose, CA, USA) statistical software.

Results

Water quality parameters measured during the broodstock phase were total ammonia nitrogen (TAN), nitrite, calcium hardness, temperature and pH. TAN was maintained below detectable limits (< 0.02 mg/L), nitrite was below detectable limits (< 0.5 mg/L), and calcium hardness remained >50 mg/L for the duration of the experiment. Temperature in the culture units was maintained at 28 ± 2 °C using 100-watt aquarium heaters during rearing and pH ranged from 6.5 to 7.4. All water quality parameters were within the appropriate ranges for redclaw culture (Masser and Rouse 1997).

Anesthesia Screening Study

Aqui-S was effective as an anesthetizing agent for juvenile redclaw (< 1g) in concentrations between 5 and 15 ml/L. Time to reach light sedation did not significantly differ between 5 and 10 ml/L Aqui-S, but was significantly shorter at 15 ml / L ($p < 0.05$). Juvenile crayfish reached light sedation at ~ 2 minutes at the lower concentrations and ~1 minute at the highest concentration (figure 10A). Average time to reach heavy sedation was significantly higher at a concentration of 5 ml than either 10 or 15 ml/L Aqui-S ($p < 0.05$). Juvenile crayfish reached heavy sedation at an average time of 8 minutes at the lowest concentration and ~3 minutes at the two higher concentrations (figure 10B). Crayfish exhibited 100% survival in all concentrations when exposed for 10 minutes or less beyond heavy sedation. However, crayfish exhibited decreased survivorship or 100% mortality when exposed for 30 minutes beyond heavy sedation. Recovery was immediate when crayfish were removed from the anesthesia as soon as

heavy sedation was reached. However, if crayfish continued to be held in the anesthetic for 10 – 30 minutes after reaching heavy sedation, they required 1-3 hours to fully recover (table 1).

The results of the screening study to assess the effectiveness of the ice bath on sedation are presented in figure 11. The time to light sedation ranged from 3 to 6 minutes with an average of 4.5 minutes, while the time to heavy sedation was 7 to 14 minutes with an average of 11.9 minutes. Survival was 100% at 72 hrs after removal from the ice bath. Light sedation occurred between 19.6 and 18.2 °C, and heavy sedation was reached at temperatures between 18.5 and 13.7°C. The temperature of the water in the beakers submerged in the ice bath changed according to the following equation: $Y=0.009x^2-0.8159x+23.027$($R^2 = 0.9879$); where x is time and y is temperature. The recovery time for the juveniles was short (< 5 min) once the beakers containing the crayfish were removed from the icebath and allowed to warm towards ambient room temperature (~24 °C). However, because we did not record temperature in the recovery beakers, the temperature at which juveniles recovered is unknown.

Efficacy of anesthesia on ablated early-juveniles

The different age classes of the ablated juveniles represent different instars, or molts. Post-release (oldest) crayfish were significantly larger than either the pre-release or at-release age classes (p-value < 0.0001); whereas, no significant difference in mean weights was found between the two youngest size-classes (p-value= 0.593) (figure 10a). The temperature necessary to reach heavy sedation was significantly warmer (p-value > 0.05) in the post-release group than in either of the other age classes (figure 12b). The

exposure period required to reach heavy sedation with 5 ml/L Aqui-S was significantly longer (p–value < 0.05) for the post-release group than for the at-release or pre-release groups (figure 12c). Survivorship of non-ablated reference crayfish was high (90 - 100%) for crayfish from all three age-classes (figure 12). Survivorship of the non-anesthetized ablated crayfish was high with averages of 83, 80 and 77 % for "pre", "at", and "post" release age groups, respectively. Survival was not significantly affected (p-value > 0.05) by anesthesia type in any of the three age-classes (figure 13).

Discussion

Anesthetics have been shown to be a valuable tool in crustacean aquaculture and are used to reduce mortality during hauling, handling, and surgery. One of the most promising anesthetics, Aqui-S, has been shown to be a carcinogen in mice and is currently not approved for use in food fish. Therefore, there is also a need to evaluate non-chemical sedation techniques, such as cold temperatures. Ablation techniques are useful in crustacean aquaculture for purposes such as promotion of spawning in shrimp (e.g. eyestalk ablation, see Taylor et al. 2004) and production of all-male broods by freshwater prawns (e.g. fifth leg ablation, see Aflalo et al. 2006, Rungsen et al. 2006). The use of a topical anesthetic prior to eyestalk ablation has been shown to reduce the stress-related, non-feeding period post-ablation. In this study, the efficacies of a chemical and non-chemical anesthetic were evaluated for use on early-stage juvenile redclaw crayfish subjected to fifth leg (androgenic gland) ablation.

The intention of this study was to provide information on the types of anesthetics, concentrations, and exposure periods necessary to sedate juvenile redclaw crayfish, and

whether the use of anesthetic agents enhances ablation survivorship. Although ablation of the fifth pair of periopods was a quick procedure, (~30 seconds per crayfish) more in-depth procedures may require longer sedation periods. In this study, the following characteristics were desired from an ideal anesthetic: heavy sedation achieved quickly (< 10 min), maintenance of heavy sedation for an extended period (> 30 mins for extensive surgeries), and high survival rates (> 90%). The two anesthetics used in this study, Aqui-S and temperature manipulation, are commonly used for other crustaceans and were expected to work well for redclaw crayfish.

Results of this study showed that isoeugenol (Aqui-S™) and temperature manipulation are both effective methods of anesthesia in juvenile redclaw crayfish. Aqui-S was effective at concentrations between 5 ml/L and 15 ml/L. Exposure to Aqui-S at these doses should not exceed 10 minutes beyond heavy sedation because prolonged exposure caused a decrease in survival at all concentrations (table 1). Crayfish remained sedated several minutes- long enough that the ablation procedure could be performed; however a short recovery period (< 1 hr) can be achieved at lower Aqui-S concentrations and shorter emersion periods (table 1).

Coyle et al. (2005) examined the use of Aqui-S as a light anesthetic for long-distance hauling of *Macrobrachium* to improve survival. Groups of five juvenile *Macrobrachium* (average weight 2.1 ± 0.1g) were placed in varying concentrations of different anesthetics and were evaluated based on sedation level and the proportion of juveniles sedated at each time step. Exposure to low Aqui-S concentrations between 100 and 300 mg/L produced light sedation in all treatments, but produced heavy sedation in only a portion of the tested juvenile *Macrobrachium* within the first 30 minutes of

exposure (Coyle et al. 2005). After one hour of exposure these concentrations did not produce heavy sedation in 100% of the juvenile *Macrobrachium* in any treatment except for the 300 mg/L concentration. This is advantageous for transport purposes, but not surgery, since prolonged exposure to Aqui-S may produce mortality (table 1). The goal here was to produce heavy sedation in a relatively short time period (< 10 mins); therefore, the Aqui-S concentrations used were much greater at 2,700 mg/L (5 ml/L Aqui-S), 5,400 mg/L (10 ml/L Aqui-S), and 8,100 mg/L (15 ml/L Aqui-S) of active ingredient. The increased amount of Aqui-S used in our study may have been required because of the size disparity of the juvenile redclaw (0.467 ± 0.351 g) compared to the juvenile *Macrobrachium* (2.1±0.1g). Figure 12 shows that the time to heavy sedation is influenced by age and size, with older individuals requiring more time to reach heavy sedation. Thus, differences in Aqui-S concentrations required for heavy sedation may be influenced by the difference in size between the test subjects. Also, uptake of Aqui-S may be affected by differences in physiology between species.

Temperature manipulation is known to be an effective anesthetic for many invertebrate species (Longley 2008). Semple (1995) found that adult redclaw became inactive at 12 °C, and, if exposed for prolonged periods, mortality would commence from starvation. King (1994) evaluated growth rates of juvenile redclaw (0.04-0.046 g) under multiple temperature regimes, and found that crayfish grown at 15° C did not gain weight and suffered 100% mortality by week six. Roy (1993) evaluated the effects of temperature (14 °C and 20 °C) on the immune response of adult redclaw and found that redclaw that were challenged with *Aphanomyces astaci* (crawfish plague) at 14 °C showed increased susceptibility. Therefore, subjection to cold water temperatures in

order to prolong the sedation period may be problematic, particularly when the crayfish will be immediately transferred to pond culture. However, because King (1994) and Roy (1993) studied temperature effects on redclaw for longer periods, their results may not translate well into short-term exposure scenarios like anesthesia. Patterson (1993) studied the effects of temperature manipulation on Kuriama prawns (*Penaeus japonicas*) and found that heavy sedation could be attained at or below 12 °C. Ice bath heavy sedation for juvenile redclaw occurred at temperatures between 18.5 and 13.7°C, with 50% of the crayfish being heavily sedated at 14.1°C (figure 11). This indicates that an approximate threshold temperature for juvenile redclaw sedation is 14 °C. Unfortunately, ice bath sedation technique does not allow a wide margin of time to complete a surgical procedure once the water temperature surrounding the crayfish is raised. Many of the ablated juvenile crayfish became active during the ablation procedure (~30 sec.) because the crayfish had to be handled out of the cold water bath when using a dissection microscope. Although ice bath anesthesia may not be feasible for juvenile redclaw ablation using this protocol, it may be possible if an ice bath were placed under the microscope to ensure continual exposure to cold temperatures.

For aquaculture species, it is important to consider the retention time, or period of time that a chemical can be transferred to humans through consumption. Other anesthetics used for food-fish anesthesia require a period of days for purging the anesthetic from the consumable flesh. Aqui-S is not considered safe for use on animals to be consumed because it can be carcinogenic. Because ice bath anesthesia does not use a chemical anesthetic, it has no retention time and is safer for use in food-fish than Aqui-S.

For anesthesia to be useful in the applications of handling, transport, and surgery, it should reduce the stress and mortality associated with these activities. Anesthesia, itself, may be stressful to animals; however, the use of an anesthetic ultimately reduces the negative effects of surgery. It was hypothesized that the use of anesthesia would decrease mortality associated with ablation trauma in early-stage redclaws. This study used survival at 72 hours as a measure of stress; however other studies have shown the stress effects of anesthetics through other measures. When an animal becomes stressed, it increases its rate of oxygen consumption (ROC). Taylor et al. (2002) used juvenile *Macrobrachium* (0.1 - 2.8g) to define the relationship between stress and anesthesia via resting oxygen consumption rates. The sedated group was anesthetized for 4 min in a solution of 5 drops of clove oil (95% eugenol in 5-ml 95% ethanol) added to 1000 ml of fresh water. Their findings suggest that anesthesia itself is stressful because anesthesia, with or without an additional physical stressor, increased the ROC significantly above that of the individuals in the absence of that anesthetic (Taylor et al. 2002). However, the benefits of anesthesia may out-weigh the costs of performing surgery without anesthesia. For example, when a topical anesthetic was applied to the eyestalk *L. vannamei* prior to eyestalk ablation, it caused a decrease in the period of stress-related feeding depression post ablation (Taylor et al. 2004). In the interest of reducing pre-ablation stress, the lowest effective Aqui-S concentration of 5 ml/L (5,400 mg/L or 2,700 mg/L isoeugenol) was used during ablation because it provided adequate sedation in a relatively short time period with the lowest incidence of mortality. Additionally, ablation in the ice bath sedation group was done as soon as each group achieved heavy sedation to reduce pre-ablation stress. Minimal effective anesthesia should produce maximum survival and

minimum stress. The use of Aqui-S as sedative yielded a positive relationship between the time necessary to reach heavy sedation and the weight of the animal. The juvenile redclaw sedated with 5 ml/L became sedated based on the following equation: $y = 4.2052 x + 6.8939$ ($R^2 = 0.2769$) where y is time to heavy sedation in minutes and x is weight in grams. For ice bath anesthesia, a similar relationship was made, however the relationship was logarithmic. Once the temperature was transformed by the natural log (ln), the relationship could be described by the following linear equation: $y = 0.9976 x + 2.3098$ ($R^2 = 0.5126$) where y is ln(temperature °C) and x is weight (g).

The creation of neo-females through androgenic gland ablation can produce all-male broods in *Macrobrachium*, a technology that may also prove useful for redclaw crayfish. Aflalo et al. (2006) performed androgenic gland ablation procedure on stage I post-larval *Macrobrachium* to create sex-reversed male (neo-female) *Macrobrachium* at two different age classes - PL_{25-60} (0.15-1g), and PL_{20-30} (0.1-0.3g). 24-hour survival following ablation was 80.92% in PL_{20-30} and was 80.1% in PL_{25-60} (Aflalo et al. 2006). Successful sex reversal was much greater in the younger group; therefore, early-age ablation is crucial (Aflalo et al. 2006). Our ablation survival rates were similar to those reported by Aflalo et al. (2006) across all age classes (figure 13), although our size classes were much smaller (figure 12a). Survival was not increased in any age class with the use of anesthesia (figure 13), which suggests that the use of anesthesia is not beneficial in improving survival of ablation-stressed crayfish. High survivorship of ablated crayfish in the control group suggests that no anesthetic is needed. This may prove beneficial because it will save time and effort during the procedure and preclude

the use of chemical anesthetics, such as Aqui-S, which may be retained by organisms and render them unfit for consumption.

Results show that sedation is not required to obtain high survivorship of ablated crayfish at a very early age. However, Aqui-S and temperature manipulation are effective, seemingly safe, forms of anesthesia for juvenile redclaw crayfish and may prove beneficial for other procedures. Aqui-S was proficient at producing heavy sedation between 5 and 15 ml/L, while the temperature required to induce heavy sedation ranged between 7 °C and 18.5 °C (figure 11, 12a, & 12b). Increased exposure time to Aqui-S, particularly at high concentrations, decreased survival; however, these effects were not evident at less than 30 minutes exposure beyond heavy sedation (table 1). Juvenile redclaw crayfish can be exposed to 5ml/L Aqui-S for 8 minutes to produce heavy sedation because it provides heavy sedation for a period adequate for performing surgery, but has a short recovery period (< 1 hour) and high survival (~100%) (table 1).

Table 1. Data summary for survival and recovery from sedation trials using Aqui-S (active ingredient Isoeugenol) on juvenile redclaw.

Aqui-S™ Concentration (ml/L)	Holdover Period Beyond Heavy Sedation (mins)	Survival (%)	Recovery Time (hrs)
5	0	100	< 1
	5	100	< 1
	10	100	< 1
	30	83.3	1-3
10	0	100	< 1
	5	100	0-1
	10	100	1-3
	30	0	--
15	0	100	0-1
	5	100	0-1
	10	100	1-3
	30	0	--

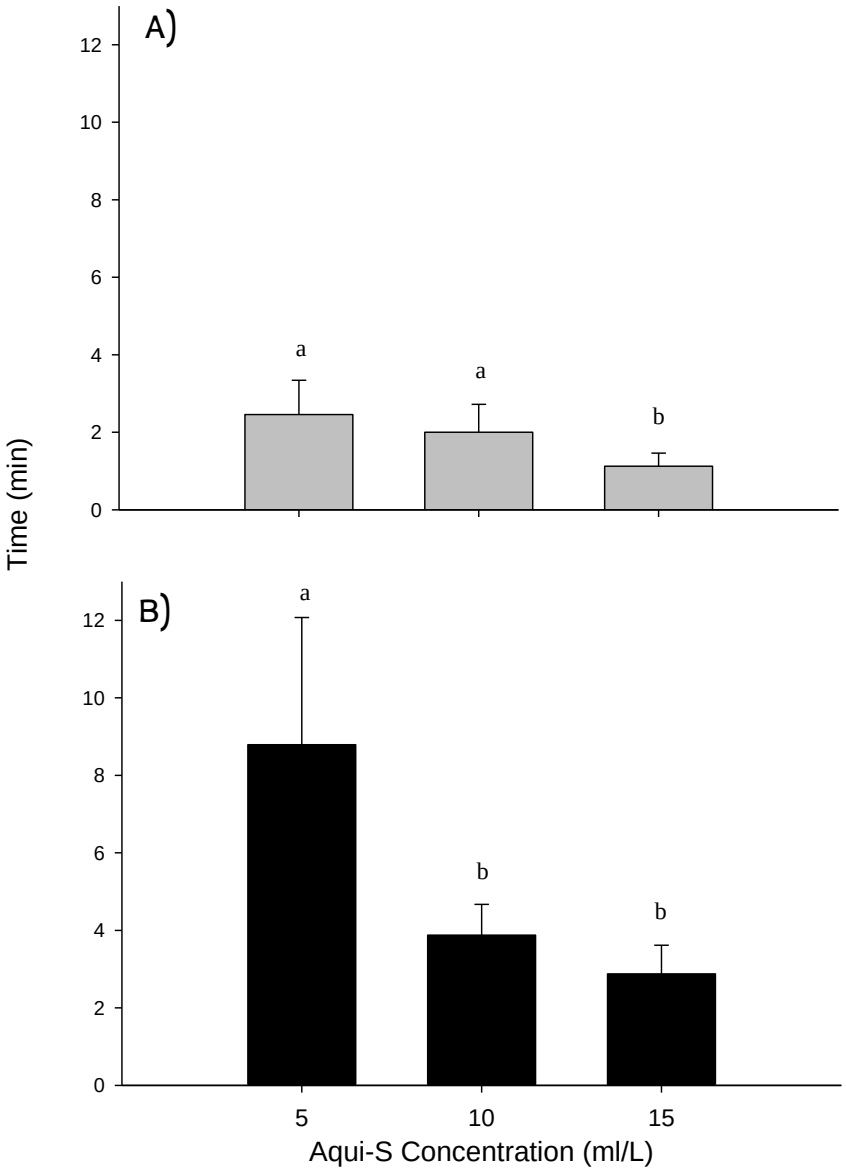

Figure 10. Screening data summary for time to A) light and B) heavy sedation trials using Aqui-S™ Isoeugenol on juvenile redclaw. (Averages are +/- 1 standard deviation) Different letters signify statistically significant ($p < 0.05$) differences.

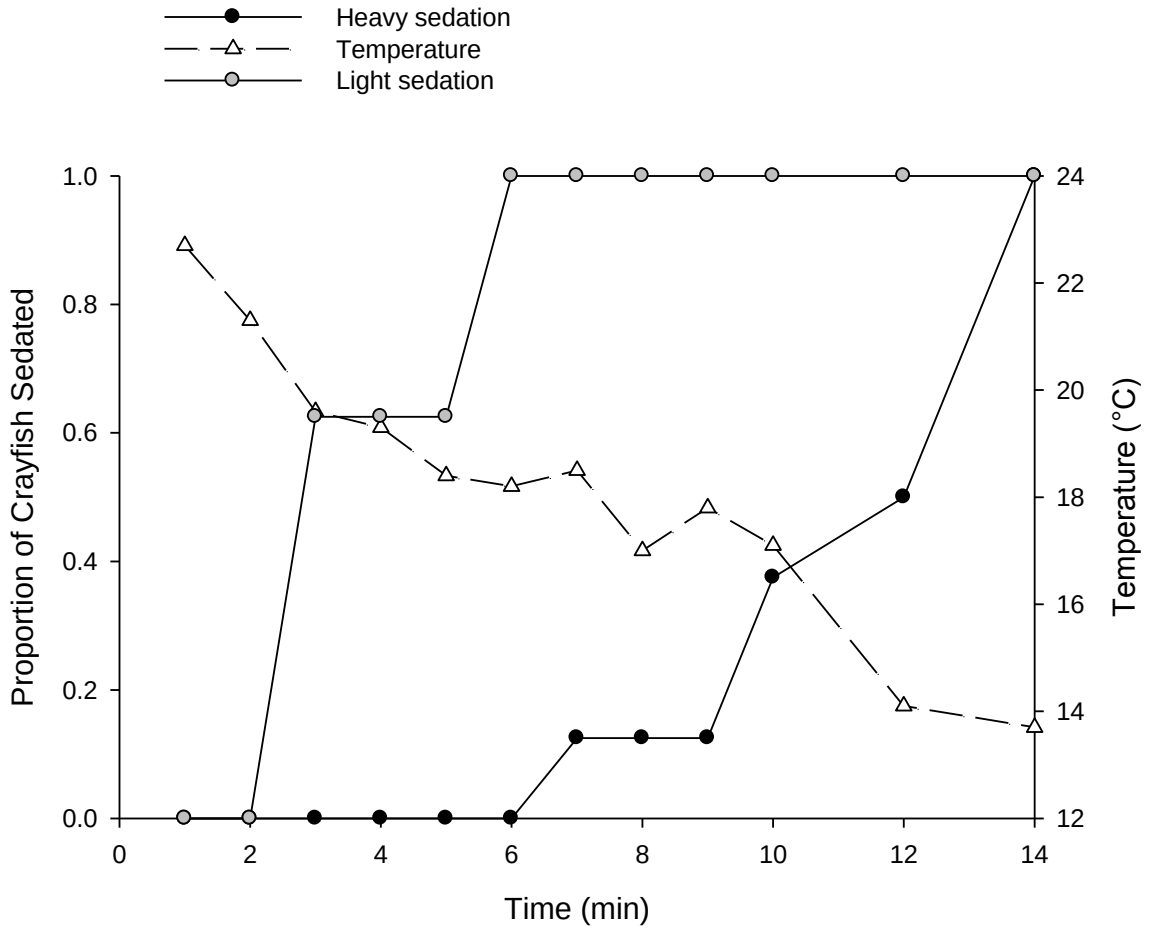

Figure 11. Proportion of individual juvenile redclaw crayfish (average size = 0.368g ± 0.115std) reaching light and heavy sedation in an icebath. Temperature represents the water temperature within the container.

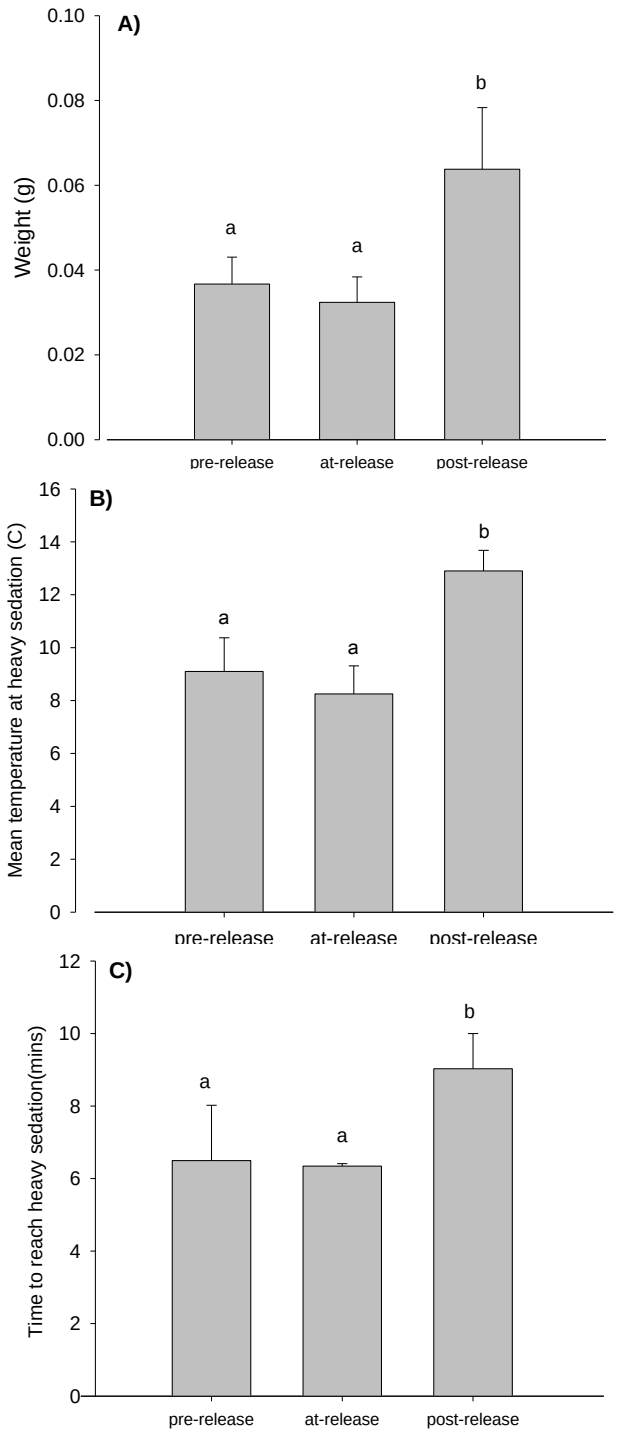

Figure 12. (A) Average weight of the three crayfish age-classes, (B) average temperature at heavy sedation for each age-class held in an ice bath, and (C) average time to reach heavy sedation for each age-class exposed to 5 ml Aqui-S / L AFW. Error bars represent ± 1 standard deviation. Letters designate statistically significant differences between age-classes ($p < 0.05$). Weight was measured on a subset of individuals. Anesthesia data was for ablated individuals only.

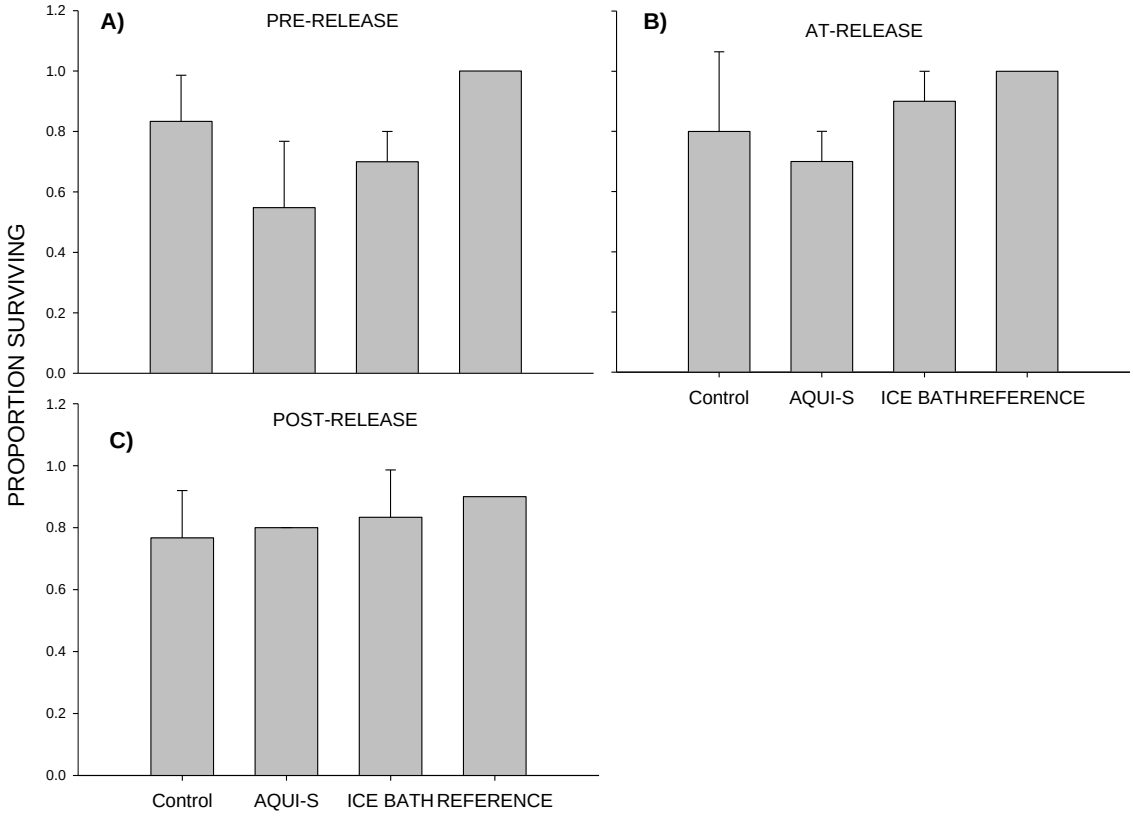

Figure 13. Proportion of juvenile crayfish within each age classes surviving the ablation procedure under different anesthetic regimes. Error bars represent ± 1 standard deviation. No significant differences were found between treatments for any of the three age-classes.

IV. **Effects of Androgenic Gland Ablation on Growth, Survivorship, and Functional Gender of Juvenile Redclaw Crayfish (*Cherax quadricarinatus*)**

Introduction

Mono-sex male culture of redclaw is the fastest and most efficient way to grow large crayfish that will yield a high market price (Curtis and Jones 1995, Naranjo 1999, Rodgers et al. 2006). Mono-sex male cultures produce larger individuals than mixed-sex cultures because males grow faster than females and, in the absence of females, channel their energy towards growth rather than reproduction (Curtis and Jones 1995, Rodgers et al. 2006). When the Australian yabbie crayfish (*Cherax albidus*) was grown in monosex male, female, and mixed sex cultures, the monosex male culture yielded a 70% increase in the market value of the final product when compared to the other cultures (Lawrence et al. 1998). In addition to the money wasted on feed for the slower growing female redclaw, separating the sexes by hand is a time-consuming, laborious process, which needs to be streamlined to improve profitability for farmers.

Concerns about invasive potential must also be addressed and researched when developing a new aquaculture species. Because the redclaw is native to Australia, there is concern about their potential to become an invasive pest in North America. The redclaw has escaped captivity in Mexico and establish breeding populations (Bortolini et al. 2007). However, redclaw are not likely to establish populations in most of the United

States because of cool winter temperatures and fungal diseases like *Aphanomyces astaci* or crawfish plague, to which the redclaw has no immunity (Medley et al. 1993). Production of all-male broods of redclaw crayfish should help to further reduce the chances of establishment of feral populations by keeping females rare to absent.

Ablation Technology/All-Male Brood Production

Male decapods crustaceans possess an androgenic gland attached to the sperm duct, which secretes androgenic gland hormone (AGH). The sperm duct and androgenic gland are located proximal to the 5th pair of periopods in *C. quadricarinatus*. AGH induces the development of the male reproductive organs, and without it, the crustacean becomes a phenotypic female – termed a neofemale (Ford 2008, Barki et al. 2006). The homogametic male theory states that because the chromosome composition of crustaceans is homozygous (WW) for males and heterozygous (WZ) for females, crossing a normal male and a functional neofemale will result in all-male broods (Malecha et al. 1992, Barki et al. 2006, Aflalo et al. 2006). Ablation, or removal, of the androgenic gland at an early age has successfully produced vitellogenic neo-females in giant freshwater prawns (*Macrobrachium rosenbergii*), which have been used to produce all-male broods (Aflalo et al. 2006).

Androgenic gland ablation has been shown to reduce male-like characteristics in redclaw, such as aggressive behavior, copulation with females, and the presence of the distinct red patch on the chelae (Barki et al. 2006). Moreover, the implantation of AG cells into a female redclaw produced behaviors and morphology similar to that of a male (Fowler and Leonard 1999, Khalaila et al. 2001, Karplus et al. 2003). However, true sex

reversal was not observed in these studies, possibly because ablation and implantation procedures were performed on late-stage juveniles.

In this study, the feasibility of using androgenic gland ablation of early-stage juveniles to produce functional neo-females was investigated. Ablation techniques were derived from a similar procedure used on *Macrobrachium rosenbergii*, the giant freshwater prawn, in which successful sex reversal yielded "neo-females" that produced viable eggs (Aflalo et al. 2006, Rungsin et al. 2006). Successful production of functional neo-females required the 5th pair of periopods and their associated vas deferens be removed prior to the development of the appendix masculinae (Aflalo et al. 2006, Rungsin et al. 2006). The procedure was only successful on post larvae < 30 days of age presumably because this period marks the beginning of the sexual development of *Macrobrachium* (Aflalo et al. 2006). Of 87 ablated stage I postlarvae, 80.4% survived the procedure, and of the survivors, 27.14% developed into mature "neo-females", which when mated with males, produced all-male broods in 66% of spawning attempts (Rungsin et al. 2006). In contrast, genetic females paired with normal males produced broods with a sex ratio of 1M:1F. Fecundity of the "neo-females" was similar to genetic females (Rungsin et al. 2006).

Life history differences between crustacean taxa make it difficult to determine the optimum ablation period for crayfish. Crayfish juveniles exhibit direct development on the adult female and are released as juveniles whereas *Macrobrachium* release free swimming larvae that later settle as postlarvae and enter the juvenile stage. Because the ablation procedure was most successful on 20-30 day postlarval stages of *Macrobrachium*, it was hypothesized that only crayfish ablated at a very early age would

develop into neofemales. Juvenile crayfish go through approximately three molts while attached to the broodstock female, and stage III juvenile redclaw crayfish are in the molting stage just before and during release (Levi et al. 1999). In this study, we investigated the feasibility of ablating stage III juvenile crayfish and then looked for subsequent effects on gender expression.

Materials and Methods

Experimental Animals

Adult broodstock were cultured in an indoor recirculation system consisting of 16 plastic tanks (30x47x15 cm) filled with artificial fresh water (AFW) circulating through a common sump, biofilter, mechanical filter and UV filter. AFW was constituted of 0.5 g/L (ppt) of salinity from Instant Ocean™ salt (1L 30 ppt salt water in 60 L deionized water), and ~ 75 mg/L (50 mg/L $CaCO_3$ and 25 mg/L $CaCl_2$) of calcium hardness. Male and female broodstock were held at a 14L:10D photoperiod and 28 ± 2 °C to induce spawning (Masser and Rouse 1997). One adult male and two adult females were held in each tank. A 35% protein sinking feed was provided at 3% of crayfish biomass daily (Masser and Rouse 1997).

Ablation/Growout Methodology

Our goal was to have a population of 30 crayfish per brood for each treatment after a three-month growout period. Therefore, stage III juvenile redclaw were collected from the broods of three female crayfish approximately one week prior to release from the tail. Thirty individuals from each brood were randomly assigned to the control (no

ablation) treatment and 70 to the ablation treatment. From the anesthesia-ablation experiment (chapter 3), it was estimated that the non-ablated group would have 100% survival and the ablated group should have ~80% survival of the ablation procedure. Assuming 80% survival from the ablation procedure, the number of ablated juveniles was further increased (n = 70 per brood) to provide a safety measure to ensure 30 ablated juveniles per brood.

Ablation was carried out by using fine-tipped tweezers to remove the 5th pair of periopods at the base, taking care to remove the attached tissue containing the AG. All juveniles were placed in 20 ml vials for 72 hours to monitor survival. After 72 hours, mortalities were discarded and the control juveniles (n = 30 per brood) and a random subset of the surviving ablated (n = 50 per brood) juveniles were randomly selected and moved to growout containers for three months. The greater number of ablated than control individuals was used in case of excessive mortality in the ablated treatment during the growout period. Growout containers were randomly assigned to, and fully submersed in, one of four, shallow fiberglass troughs (2.5m L x 1m W x 0.15 m H). Water in each of the tanks was recirculated through a common sump and filtration system using a percolation hose to evenly distribute the fresh water to all growout containers. Growout containers (11cm L x 9.5cm H x 8.5cm W) had mesh sides to allow for water exchange. Water quality was maintained via a biofilter equipped with plastic bio-media. Water was recircluated between the troughs and the biofilter with a water exchange rate such that the water was filtered twice per hour. Water quality parameters, such as total ammonia nitrogen, nitrite nitrogen, calcium hardness and pH, were monitored with a LaMotte chemical water quality testing kit on a weekly basis followed by a 10% water

replacement. All crayfish were held at 28 ± 3°C and a 14L:10D photoperiod to stimulate maximum growth rates (Masser and Rouse 1997). Rations consisted of a commercial, 40% protein (Rangen, Inc., Angleton, TX, USA), sinking feed (2mm dia.) at the following rations: Month 1 = 40% of crayfish biomass twice per week, Month 2 = 7% of crayfish biomass three times per week, Month 3 = 3-5% biomass three times per week. (Masser and Rouse 1997). Feed was crushed into a powder during the first two months to facilitate grazing by small crayfish. Wet weight of crayfish was determined as the average of 16 crayfish per treatment every two weeks to determine feeding rations. Survivorship and weight of all crayfish was measured every month to determine growth and mortality rates for each treatment. Sexual development data was collected at the end of the three-month growout period. Sexes were determined based on the presence of either genital papillae (male) or the ovopores (female) (figure 14). Males with genital papillae on only one leg were termed unilateral males, and crayfish that exhibited no external genital development were termed eunuchs (figure 14).

Statistical Analysis

Because crayfish were randomly assigned to tanks, with unequal numbers of crayfish from each treatment between tanks, we used a one-way analysis of variance (ANOVA) and a Tukey's post-hoc test to test for differences between treatments (Sokal & Rohlf 1995) at the $\alpha = 0.05$ level. Survival percentages were transformed by the square root of the arcsine to normalize the data before statistical analyses (Sokal & Rohlf 1995). Statistical analyses were performed using SYSTAT 12 (2007) (SYSTAT Software, Inc., San Jose, CA, USA) statistical software.

Results

Water quality parameters measured were total ammonia nitrogen (TAN), nitrite, calcium hardness, temperature and pH. At two months, when the feed size and ration changed, TAN was measured at 3 mg/L. However, during the rest of the experiment, TAN was maintained below detectable limits (< 0.5 mg/L), nitrite was below detectable limits (< 0.02 mg/L), and calcium hardness remained >50 mg/L for the duration of the experiment. Temperature in the culture units was maintained at 27 ± 3 °C during rearing and pH ranged from 6.5 to 8. Generally, all water quality parameters were within the appropriate ranges for redclaw culture (Masser and Rouse 1997).

Survival and Growth

Survivorship of ablated crayfish and control crayfish at 72 hrs was 78% ± 7% and100 ± 0%, respectively. The three-month growout survival rates for the ablated and control groups were 73.3 ± 4.6% and 82.2 ± 7.7%, respectively, and were not significantly different ($p = 0.173$) (figure 15). Water quality did suffer, particularly in trough 1, as a result of overfeeding and interrupted water flow for the week immediately after the feed was changed from powder to pellets at the beginning of month three because the juveniles had not yet adjusted to the new feed, which resulted in the mesh container sides becoming clogged. Mortalities averaged 9% higher in trough 1 than in the other troughs; however, survival did not differ significantly between treatments ($p = 0.173$) or between broods ($p = 0.295$).

The growth of the juveniles is presented in figure 16. At two months, there were significant differences in weight between broods and troughs ($p \leq 0.034$), but not

between treatments ($p = 0.272$). Brood 2 was significantly smaller than broods 1 and 3 ($p \leq 0.002$), but broods 1 and 3 were not significantly different from each other ($p = 0.496$). Weights in trough 2 were significantly different ($p \leq 0.014$) than weights in troughs 3 and 4, but weights in all other tanks were not significantly different ($p \geq 0.342$). At three months the average weight (figure 17) of the juvenile crayfish in the control and ablated groups were 3.247 ± 1.021 g and 3.278 ± 0.924 g, respectively, and were not significantly different ($p = 0.504$). Also, the average weight of the juvenile crayfish did not differ significantly between sexes in either treatment (figure 18).

Sex Ratios

The ANOVA analysis revealed that the gender ratios of the juvenile redclaw differed between the control and ablated groups (figure 19). Although the proportion of females remained consistent between treatments (control 50% ± 7%, ablated 48% ±6%) ($p = 0.746$), the proportion of males were lower in the ablated group (24% ± 15%) than in the control group (49% ± 6%) (Tukey's, $p = 0.04$). Within the control group, males that exhibited male genitalia on only one side, unilateral males, occurred in 3% ± 5% of the males; conversely, the ablated group exhibited a higher (Tukey's, $p = 0.004$) percentage of one-sided male genitalia in 64% ± 17% of the males. Additionally, the percentage of eunuchs increased from 0% in the control group to 26% ± 12% in the ablated group (Tukey's, $p = 0.03$). Proportions of intersex crayfish were not different between treatments (Tukey's, $p = 0.776$).

Discussion

Survival and Growth

Life-history differences between prawns and crayfish make it difficult to determine the optimum ablation period for crayfish. Crayfish juveniles exhibit direct development on the adult female and are released as juveniles whereas, *Macrobrachium* release free swimming larvae that settle as post-larvae. Because the ablation procedure was most successful on pre-juvenile (i.e. post-larval) stages of *Macrobrachium*, it was hypothesized that crayfish will have to be ablated at a very early age to produce neofemales.

In this study, it was shown that early stage (pre-release) juveniles can be ablated without negatively affecting growth or survivorship. No differences in survivorship or average size were found between ablated and non-ablated (control) crayfish three months after the procedure. Growth and survival of crayfish in both treatments were similar to or greater than previous redclaw juvenile indoor growout experiments, suggesting experimental animals were maintained in good health. In an early juvenile redclaw nursery and feeding improvement study, Jones (1995) reported growth rates of juvenile *C. quadricarinatus* over a 50-day period to fit the exponential equation $wt(g)=0.0221*e^{(0.05561*age(days))}$ at temperatures of 24° C to 28° C, whereas our control group growth equation (figure 15) is $wt(g)=0.1306\ e^{(0.03458*age(days))}$ at 24° C to 30° C, yielding a faster growth rate at similar temperatures and water quality. At 50 days post-release from the broodstock female, Jones (1995) juveniles weighed on average 0.36 g, and crayfish from this study weighed on average 0.75 g.

In a redclaw nursery phase improvement study by Parnes and Sagi (2002), the provision of artificial substrate in recirculating aquaculture tanks was evaluated as a factor for increasing juvenile growth and survival post-release from the broodstock female. Parnes and Sagi (2002) reported a final average weight of 0.34g at the end of a 75-day growout trial at similar temperatures (25 to 29° C) for stage III juvenile redclaw; whereas our growth curve indicates at day 75 on average weight was 1.73g. The survival for the control group in this study was 82% at three months (~90 days). Parnes and Sagi (2002) did not measure the survival of all young produced by each broodstock female; however, other research provides basic survival assumptions. If one assumed each broodstock female produced 7, stage-III juveniles per gram of broodstock female body weight (Masser and Rouse 1997), then Parnes and Sagi (2002) should have exhibited approximately 54% survival given the average weight of the broodstock used.

Barki et al. (1996) tested the effects of feed amount, frequency, and placement within a culture tank on growth, survival, and competition between juvenile redclaw. There was a tradeoff between feed ration and frequency of feeding on the growth and survival of juvenile redclaw (Barki et al. 1996). Juveniles fed every fourth day had approximately 10% greater survival than those fed daily; however, daily feeding yielded three times as much growth (Barki et al. 1996), thus our feeding strategy likely helped maintain high survival. A study on compensatory growth during the first 30 days of juvenile redclaw crayfish growth stated that on a 2-day feeding: 2-day starvation feeding schedule that crayfish survival was 75% and the specific growth rate ($SGR=100*(\ln(Wt_f)-\ln(Wt_0))/t$, where Wt is weight in grams and t is time in days) was

4.9 ± 2.9 (Stumpf et al. 2010). Our control group survival for the first thirty days was 90% ± 3% and our SGR was 6.8 ± 1.1.

Manor et al. (2002) evaluated survival and growth of sub-adult (~10g) redclaw grown in the individual cells and found that males grew 1.39 times faster than females. The energetic costs of oogenesis can be quite large, especially for a species exhibiting high fecundity (Tsukimura 2001), which is a likely reason that female redclaw grow more slowly. Over the course of the 206 day growout period the crayfish (starting average weight ~ 10g) reached sexual maturity; this likely occurred in the first 36 days of growout because the average weight of the males (mean wt. 20 g) was significantly higher than that of the females (mean wt. 17 g) (Manor et al. 2002). Vazquez et al. (2008) studied ovarian development of juvenile redclaw using histological assays and was able to define four distinct stages. According to this analysis, ovaries (stage I) can be distinguished early (wt. ~0.1 g); however, full oocyte development (stage IV) typically does not occur until female redclaw exceed 18 g (Vazquez et al. 2008). The average weight of females in this study (3.3 g) suggests that they are probably in stage II of development with immature ovaries. The juvenile redclaw in this study did not differ significantly ($p > 0.05$) in weight between sexes (figure 18) indicating that sexual maturity had not been reached. It is likely that, given time, the average weight of the different sexes (figure 18) will diverge, which will reveal possible benefits of juvenile redclaw ablation. Eunuchs may ultimately exhibit elevated growth rates, particularly in mixed-sex culture, because they cannot likely reproduce.

Sex Ratios

If the ablation procedure was successful in producing functional neofemales, it was expected that fewer males and more females would be observed in the ablated treatment as opposed to the control. Although we found a lower proportion of males in the ablated treatment, the proportion of females did not differ from the control group. However, the combined proportion of males plus eunuchs in the ablated group (50.3 %) was very similar to the proportion of males in the control (49.0%). Thus, it is likely that the external sexual characteristics of males, but not females were affected by the ablation procedure. Approximately half of the ablated males were effectively neutered, exhibiting no secondary sexual characteristics. The remainder of ablated males exhibited either one or two genital papillae by the age of three months, suggesting that they were continuing to develop into functional males. There was no evidence that the ablation procedure was causing some males to develop into functional females. It is possible that the redclaw juveniles were not ablated early enough to ensure the total removal of the androgenic gland prior to development. This is evident because of the presence of unilateral as well as typical males in the ablated groups

Ablation of *M.* rosenbergii was most successful in the early post-larval stages – preceding the juvenile stage (Aflalo et al. 2006, Rungsin et al. 2006). Ablation at a very early age, before external sexual characteristics become apparent, was required for the production of neofemales. The major limitation of crayfish ablation at this early age is that it is difficult to monitor molting phase while the juveniles are attached to the broodstock female. Crayfish do not have a free swimming larval stage, and it was observed that external sexual characteristics are apparent within 26 days after release

(ave. wt. = 0.14g), which is similar to the weight (~0.1g) at which female *C. quadricarinatus* can be differentiated externally as described by Vazquez et al. (2008). Artificial incubation of redclaw eggs may allow for more precise evaluation of egg development, thus allowing for ablation at earlier developmental stages and increasing the likelihood of sex reversal. A study by Gonzalez et al. (2009) compared the survival and growth rates of signal crayfish (*Pacifastacus leniusculus*) eggs incubated both maternally and artificially and found no significant difference in survival (~90%) of eggs; there was, however, significantly faster growth in the artificially incubated group.

Temperature may have also been a confounding factor. A recent study attempting sex reversal of juvenile redclaw utilized culture temperature manipulation (26, 29, and 31 °C) and AGH enhanced feed to produce all-male crayfish (Bock and Greco 2010). Bock and Greco (2010) found that at temperatures above 26° C, the proportion of juvenile females that exhibited partial or complete sex reversion increased from 68% to 90% (Bock and Greco 2010). Vazquez et al. (2004) discovered that it is possible to manipulate the sexual differentiation of early age, undifferentiated juvenile redclaw through temperature manipulation such that increased culture temperature increases the proportion male juvenile redclaw. The culture temperatures used in this study are similar to the ones used by Bock and Greco (2010). It is possible that warmest temperatures experienced in our study (~30°C) could have skewed the population of ablated juveniles to being more male, however, because the proportion of males was approximately equal to the females in the control group, this is likely not the case. The high incidence of eunuchs produced in the ablated group indicates that the androgenic gland was removed entirely from those individuals; however developmental cues for subsequent development

of female reproductive traits may have been lacking at our warm culture temperatures. Results of the current study suggest future studies examining the use of androgenic gland ablation as a means of sex reversal should focus on ablation at earlier molting stages (I and II) and cooler water temperatures.

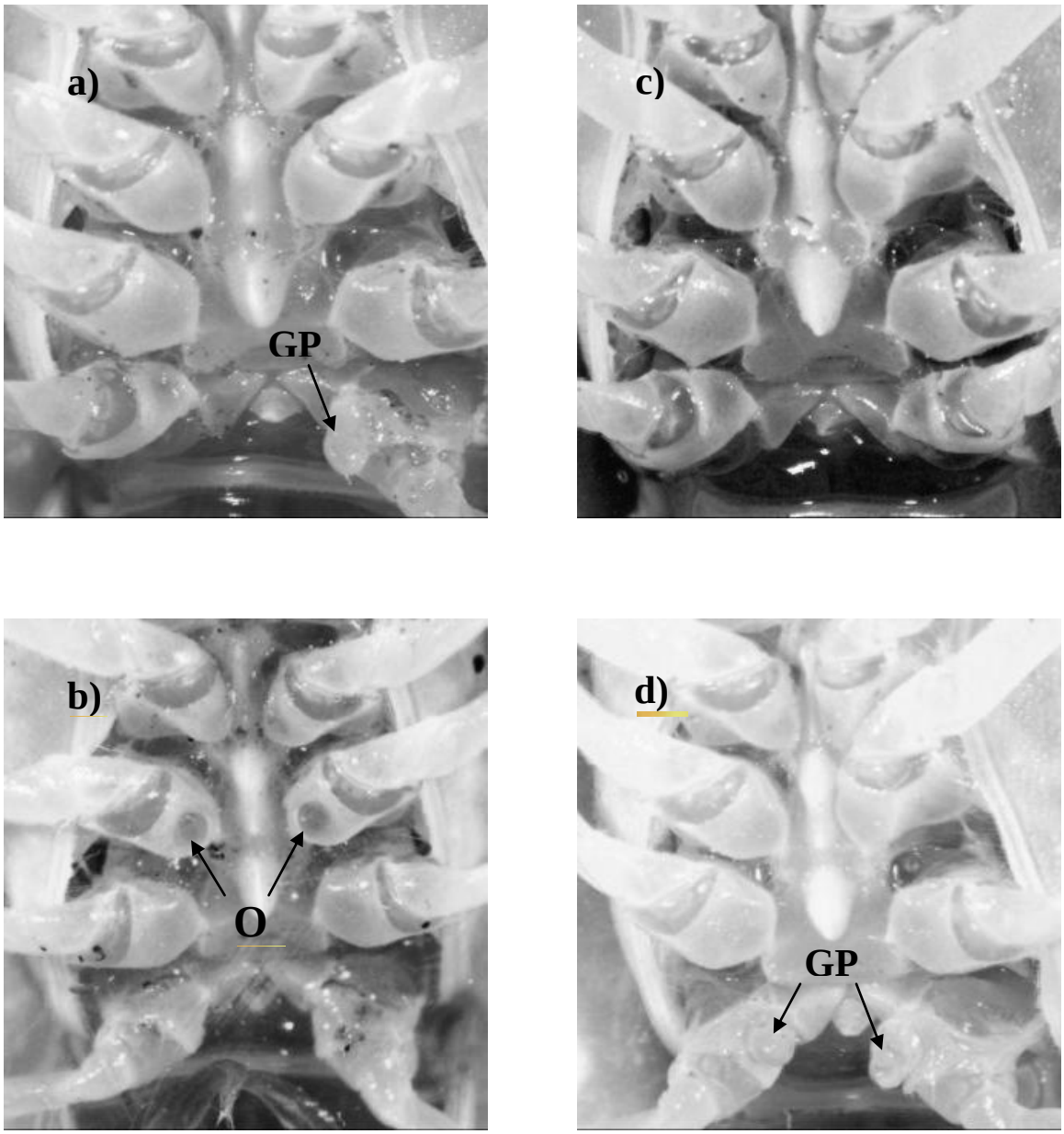

Figure 14. Juvenile *C. quadricarinatus* at three months post-release from broodstock female. a) Unilateral male, b) female, c) eunuch, d) typical male. Arrows depict the genitalia. O = ovopores, GP = genital papillae

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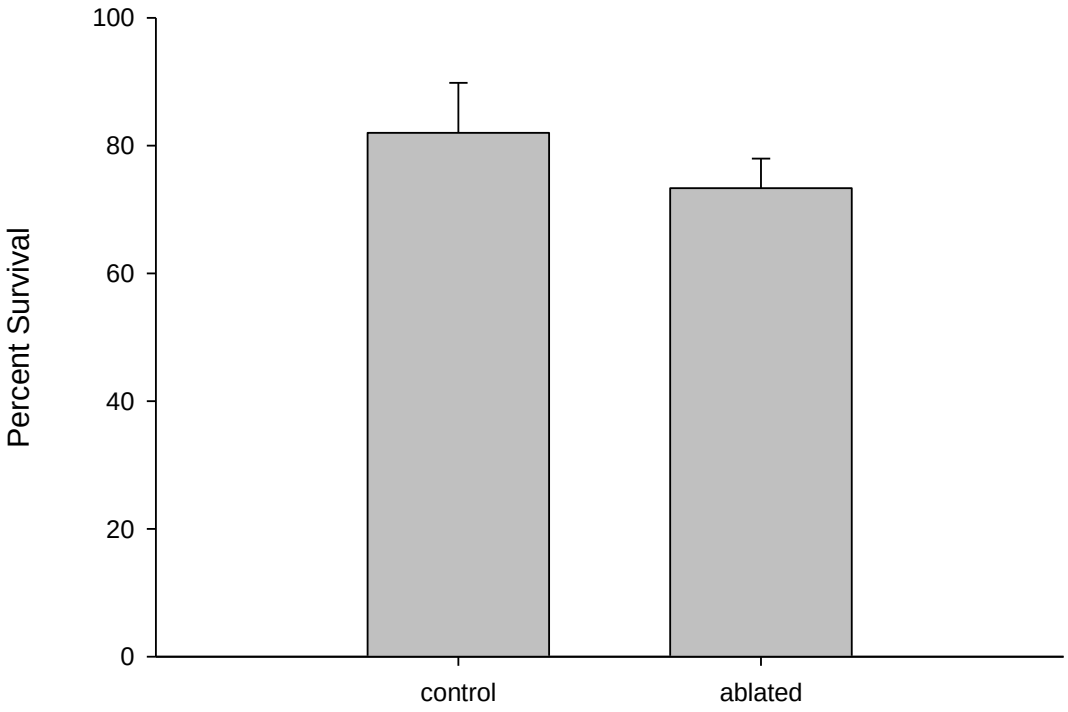

Figure 15. Percent survival of control and ablated redclaw crayfish. Survival was not significantly different (p = 0.173) between control and ablated groups.

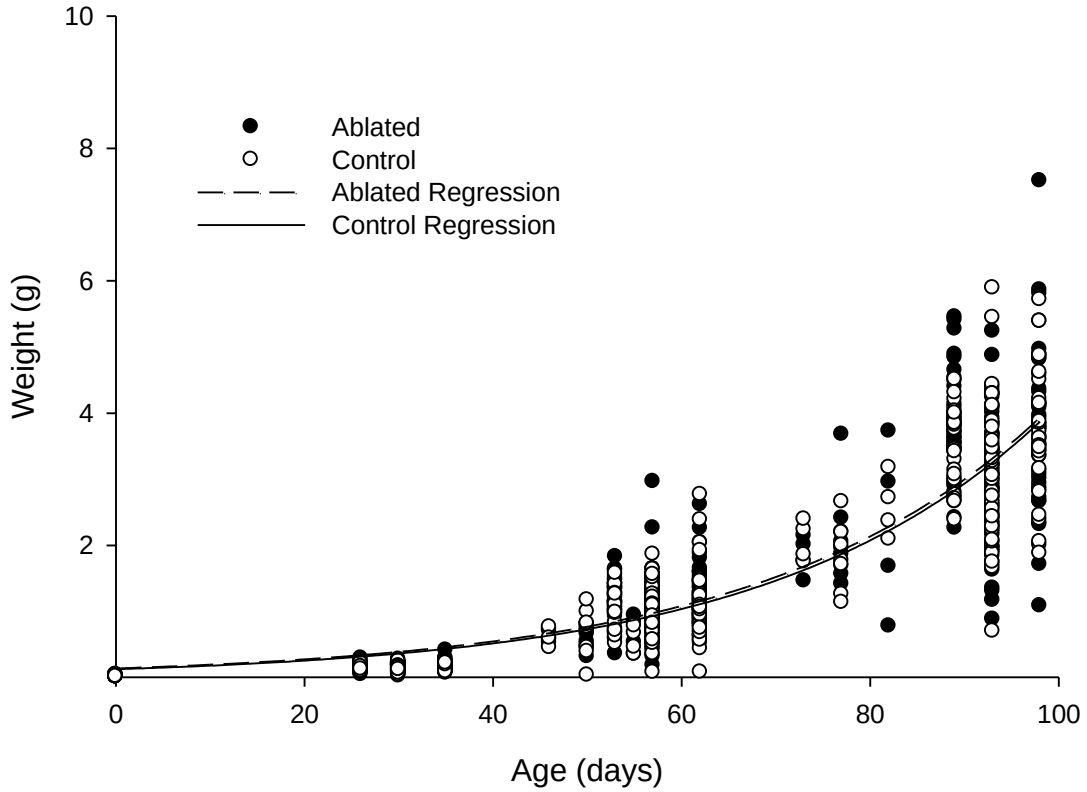

Figure 16. Growth at age for juvenile redclaw crayfish grown in individual containers with and without exposure to ablation stress. Ablated crayfish growth followed the exponential equation $y=0.1422*e^{(0.03387x)}$ ($R^2 = 0.8296$) and control crayfish growth followed the equation $y=0.1306*e^{(0.03458x)}$ ($R^2 = 0.8488$), where y is crayfish weight in grams and x is crayfish age in days.

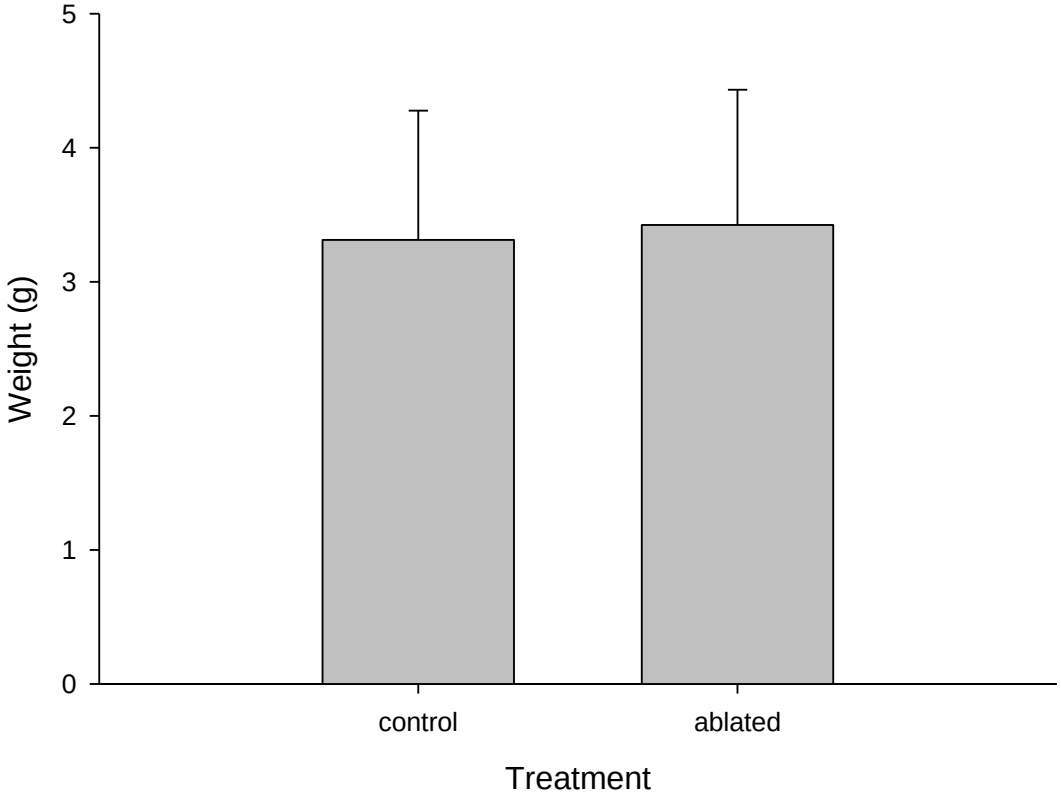

Figure 17. Weight of control and ablated juvenile redclaw crayfish at three months of age. Weights were not significantly different (p = 0.504)

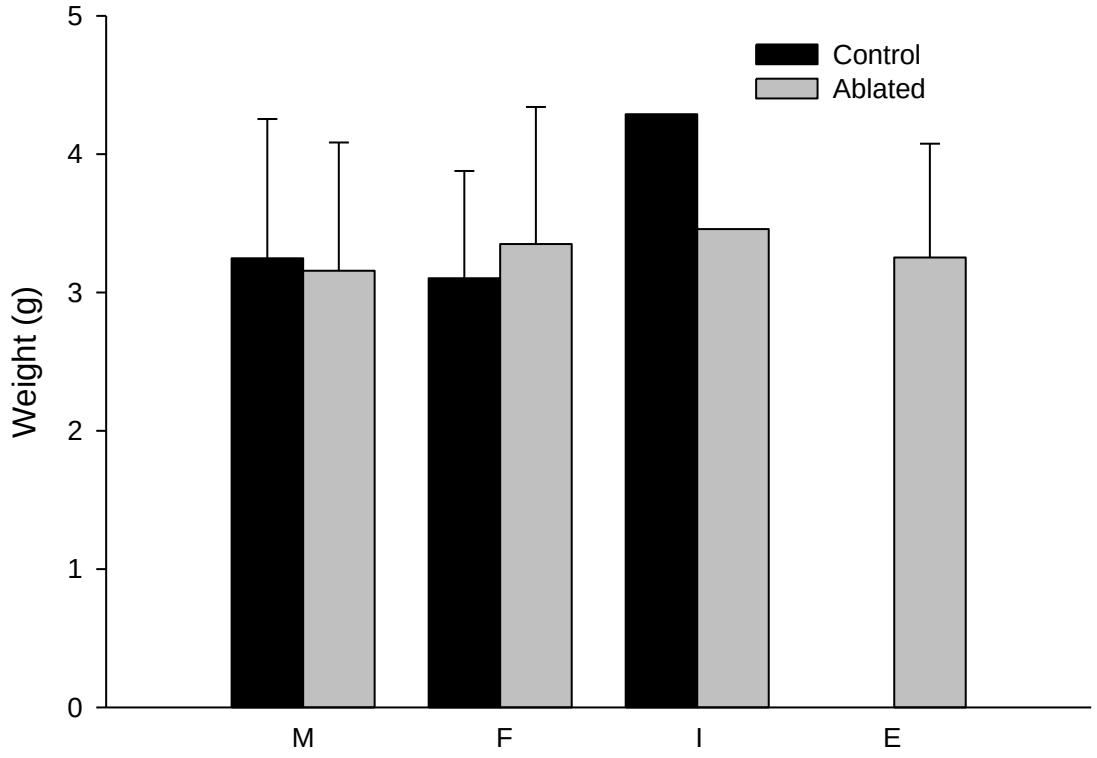

Figure 18. Average weight (±1sd) of juvenile *C. quadricarinatus* grown in separate containers for three months from stage III. E= eunuch, F = female, M = male, I = intersex. Note: There are no error bars of intersex crayfish because only one intersex individual existed per treatment.

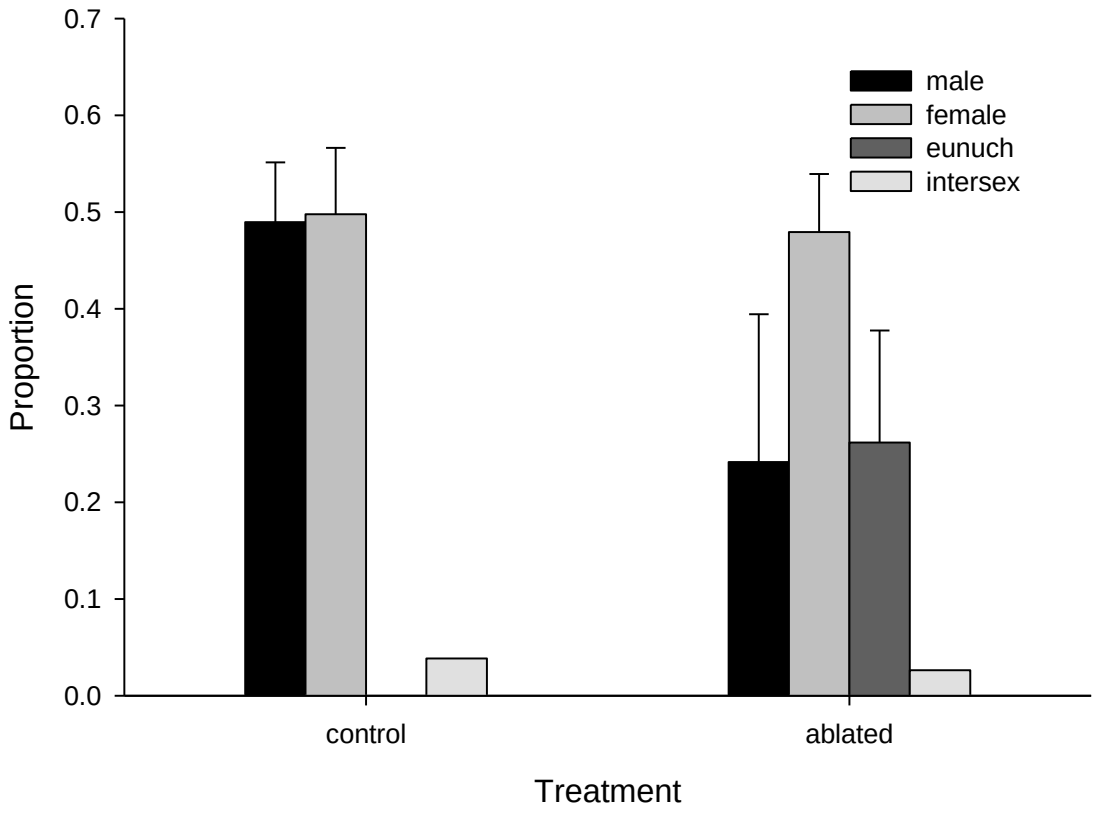

Figure 19. Proportion of individual juveniles in each gender group within the control and ablated treatments. Proportion of males and eunuchs differed significantly ($p < 0.05$) between control and ablated groups.

V. **Summary and Conclusions**

The overarching goal of these studies was to examine potential impacts of introduced redclaw crayfish on native species and to develop and evaluate production techniques (i.e. all-male broods) that reduce the chances of ecological effects while concurrently increasing efficiency and profitability of aquaculture facilities. Because redclaw crayfish 1) are not native to North America, 2) attain much larger sizes than native species, and 3) have established feral populations in Mexico, there is much concern regarding development of commercial redclaw aquaculture facilities in the United States. We found that although redclaws did have a significant, short-term impact on the average size of our model native species, there were also several factors that reduce the potential impact of redclaws on native crayfish populations.

Our results supported the findings of previous studies that redclaws are not likely to successfully overwinter in most of the continental United States due to a variety of factors including immobilization at cool temperatures and suppression of the immune system. Juvenile redclaws were immobilized at water temperatures ≤ 14 °C, which would render them susceptible to starvation and predation in the wild. Adult redclaws in the outdoor mesocosms suffered 100% mortality by mid-December when average daily temperatures were still above 14 °C. The mechanisms behind the die-off are unclear, but may be related to suppression of the immune system at temperatures below 20 °C.

The inability to overwinter suggests that the primary impacts of redclaw escapement will be short term, within the course of a single growing season. Our results

suggest that competition for shelter, and potential increases in cannibalism during molts when shelter is limited, were not mechanisms by which redclaw impacted our model native species. Although both species were observed to use shelters, survivorship of *P. acutissimus* did not increase with increasing redclaw abundance, even when the total number of crayfish in a mesocosm exceeded the number of shelters. Average size of *P. acutissimus* did decrease with increasing redclaw biomass from week 12 onwards. However, although average size of *P. acutissimus* also decreased with increasing total (native + redclaw) biomass, average size of redclaws did not decrease with increasing total crayfish biomass. This suggested that redclaw were either superior competitors for food, or were better able to assimilate the combination of commercial feed and natural food production in the mesocosms. Because *P. acutissimus* females produced broods during this time period, a concern is that egg production, and thus recruitment, of *P. acutissimus* were reduced under food-limiting conditions. However, when we examined male and female *P. acutissimus* separately, we found that only the average size of males declined with increasing redclaw abundance. Thus, although we did not directly measure fecundity, it is not likely that redclaws reduced fecundity of female *P. acutissimus* via competition for food. Future studies examining the relative strengths of intraspecific and interspecific competition for food are warranted as are studies that more directly document the presence or absence of effects of redclaw on fecundity of native crayfish species.

Aqui-S and temperature manipulation are effective forms of anesthesia for juvenile redclaw crayfish. Aqui-S was proficient at producing heavy sedation between 5 and 15 ml/L, while the temperature required to induce heavy sedation ranged between 7

°C and 18.5 °C. Increased exposure time to Aqui-S, particularly at high concentrations, decreased survival; however, these effects were not evident at less than 30 minutes exposure beyond heavy sedation. Recommendations are to expose juvenile redclaw crayfish to 5ml/L Aqui-S for 8 minutes to produce heavy sedation. However, anesthesia did not increase survival of juvenile redclaw when exposed to ablation stress; therefore anesthesia for this purpose is not necessary.

Androgenic gland ablation of stage III juvenile redclaw was not effective for sex reversal. Eunuchs produced from AG ablation should be studied with respect to growth rates, particularly in communal culture to evaluate the effects of de-masculinization on growth rates. Future studies for AG ablation as a means of sex reversal should concentrate on ablation at earlier molting stages (I or II), which may require artificial incubation. Also, growout of ablated juveniles should be tested at lower water culture temperatures to increase the likelihood of producing neo-females.

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