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Assessing the Potential for
Successful Translocation and Co-Management
of Two Endangered Aquatic Species

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science
in Biology

by

Samantha Kay Snowden

2021

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ABSTRACT OF THE THESIS

Assessing the Potential for
Successful Translocation and Co-Management
of Two Endangered Aquatic Species

by

Samantha Kay Snowden

Master of Science in Biology

University of California, Los Angeles, 2021

Professor Gregory F. Grether, Chair

Translocations are often necessary to reduce extinction risk for endangered species, but opportunities are usually constrained by limited available recipient habitat. In California, due to drastic declines in freshwater habitat, multiple aquatic species may require translocation to the same streams or ponds. However, interactions such as predation and competition could potentially impede the recovery of species translocated into the same sites. In this study we characterize interactions between two endangered species, the mountain yellow-legged frog (*Rana muscosa*) and unarmored threespine stickleback (*Gasterosteus aculeatus williamsoni*), to assess the potential for their coexistence following translocation. Using controlled laboratory experiments with *R. muscosa* tadpoles and a surrogate stickleback subspecies, the partially-armored threespine stickleback (*Gasterosteus aculeatus microcephalus*), we measured

stickleback predation on tadpoles, tadpole interference in stickleback nesting, and impacts of both species on the habitat use of the other. Stickleback preyed on tadpoles that were small in size, but predation attempts on larger tadpoles were sub-lethal. Tadpoles had little to no impact on stickleback eggs and nesting behavior. In regard to interference in habitat use, when both species were present, stickleback moved to vegetated and shallower areas, while tadpoles shifted to deeper areas. This study is the first to describe interactions between these species and suggests that certain management strategies can facilitate the coexistence of translocated populations. Understanding how interactions among endangered species affect coexistence will inform co-management efforts and support species recovery.

The thesis of Samantha Kay Snowden is approved.

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Thomas Bates Smith

Morgan Winn Tingley

Gregory F. Grether, Committee Chair

University of California, Los Angeles

2021

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INTRODUCTION

Endangered species translocations typically focus on single species, but the dwindling availability of suitable habitat may necessitate translocating multiple endangered species to the same protected areas, in a coordinated manner. In addition to making better use of available habitat and management resources, multispecies translocations could foster ecosystem recovery by re-establishing interactions among native taxa (Akçakaya et al., 2018). However, species interactions that might stabilize coexistence at moderate population densities could have the opposite effect in the early post-relocation phase, when population densities are typically quite low. For example, life-stage restricted predation has the potential to stabilize coexistence between species that compete for common resources, but the predicted outcome depends on initial population densities (Polis & Holt 1992). Species that directly interact with each other might need to be translocated in a particular order, or at particular life stages, for multispecies translocations to be successful.

Translocation has become a necessary conservation strategy for many freshwater species impacted by habitat loss, degradation, and fragmentation (Olden et al 2011). Freshwater ecosystems are changing rapidly under pressure from anthropogenic activities (Vörösmarty et al., 2010), and face alarmingly high rates of extinction (Ricciardi & Rasmussen 1999; Kopf et al., 2015). These changes are particularly striking in California, where water diversion, introduced species, and drought have reshaped aquatic communities (Moyle, 2013). As a consequence, nearly half of California's freshwater species are at risk of extinction (Howard et al 2015). In southern California, streams that used to be perennial have become intermittent, making lotic populations of aquatic animals particularly vulnerable to extinction. For taxa that occur in both

streams and lakes, translocating animals to lentic habitats could help stabilize the species and provide a source for assisted recolonization of lotic habitats.

Endangered species in southern California that might benefit from co-management include the mountain yellow-legged frog (*Rana muscosa*) and the unarmored threespine stickleback (*Gasterosteus aculeatus williamsoni*). Both species have suffered from loss of suitable habitat consisting of montane streams, ponds, and lakes with permanent water (USFWS 2009; Brown et al., 2019). *Rana muscosa* were previously abundant in the San Gabriel, San Bernardino, and San Jacinto Mountain ranges, but currently only nine small, isolated populations occur within the historic range in southern California (Backlin et al 2015; USFWS, 2018). Similarly, *G.a. williamsoni* were historically widespread across the Santa Clara River, Los Angeles River, San Gabriel River, and Santa Maria River watersheds but are currently restricted to one creek in northern Santa Barbara County, the upper reaches of the Santa Clara River drainage in Ventura County, and three ponds in San Bernardino County (USFWS 2021). The overlapping historic ranges and similar habitat requirements of these species indicate that they likely co-occurred in high-elevation streams and lakes before their ranges contracted (Moyle 2002; U.S. Fish and Wildlife Service 2012). The recovery of both species could potentially be enhanced by translocating them to the same protected lakes, but first it is necessary to evaluate whether they would negatively impact each other, and if so, whether these impacts could be managed.

Introduced fish are partly responsible for population decline and range contraction of *R. muscosa* (Backlin et al 2015; Hayes & Jennings 1986; Shier et al 2021; USFWS 2012), and lakes with fish are generally considered poor habitat for this species and other amphibians (Semlitsch 1987; Knapp 1996), but research on how native fish impact *R. muscosa* is lacking. Typical prey

for *G.a. williamsoni* include of benthic insects, snails, and small crustaceans (USFWS 2009), but amphibian eggs are highly vulnerable and often palatable to fish (Gunzburger & Travis 2005; Light 1969), and other subspecies of threespine stickleback (*G.a. aculeatus*) are voracious predators of ranid tadpoles (Laurila & Aho, 2017; Teplitsky 2005). *Rana muscosa* frogs are primarily insectivorous and usually hunt while at least partially above water; tadpoles primarily graze on algae and detritus underwater, but opportunistically cannibalize weak or injured tadpoles. (USFWS 2009). While tadpoles might consume stickleback eggs if they had the opportunity, male stickleback are paternal and can defend their nests against predators more formidable than tadpoles (Wootton 1972). Nevertheless, if tadpole densities were high, the time and energy male stickleback spent repelling tadpoles might interfere with other components of paternal care, such as egg fanning (Lissaker & Kvarnemo 2006). Juveniles of both species seek shelter from predation in shallow and vegetated areas (Babbitt & Tanner 1998; Brown et al., 2019; USFWS, 2009). Tadpoles also aggregate in shallow areas to bask, which promotes development and resistance to disease (Brown et al. 2019; Robak et al 2019; Davenport et al 2020). Thus, competition for space between tadpoles and fry could potentially reduce the survival and recruitment rates of both species.

To assess the potential for co-managing translocated populations of these endangered species in lakes within their ancestral range in southern California, we studied interactions between *R. muscosa* and a closely related surrogate of *G.a. williamsoni* in aquaria. Specifically, we tested for the types of interactions that seemed most likely to occur based on the species' natural history and the results of research on similar systems: (1) predation of frog eggs or tadpoles by stickleback; (2) predation of stickleback eggs or interference with male stickleback paternal behavior by tadpoles; and (3) displacement of either species by the other along

vegetation or depth gradients. The primary goal was to inform decision-making regarding co-management at translocation sites.

METHODS

Source populations

Mountain yellow-legged frog tadpoles and eggs

We obtained 250 4-week-old *R. muscosa* tadpoles from the captive breeding facility at Omaha Henry Doorly Zoo in June 2020, which we refer to as ‘large tadpoles’ or ‘large tadpoles with hind limbs’ depending on their developmental stage. The tadpoles were produced from a backcross of a male of mixed lineage (San Jacinto x City Creek population) and a San Jacinto female. We also acquired 336 unfertilized *R. muscosa* eggs from San Diego Zoo Wildlife Alliance. In June 2021 we obtained an additional 200 recently hatched San Jacinto tadpoles from San Diego Zoo Wildlife Alliance, which we refer to as ‘hatchling tadpoles’.

Partially-armored threespine stickleback adults, fry, and eggs

Due to the endangered status of *G.a. williamsoni*, we used a closely related subspecies, the partially-armored threespine stickleback (*G.a. microcephalus*), as a surrogate. *G.a. microcephalus* is similar to *G.a. williamsoni* in size, diet, habitat use, and nesting behavior (Miller & Hubbs 1969; Sánchez-González et al. 2001). The subspecies designations are based purely on morphology (plate number), not geography or phylogenetics; genetic differentiation between *G.a. williamsoni* populations in different drainages is probably greater than that between *G.a. williamsoni* and *G.a. microcephalus* in the same drainage (Richmond et al 2015). We obtained a total of 120 *G.a. microcephalus* for this project from California Department of Fish and Wildlife (CDFW), which were a mix of adults and subadults. Subadults were distinguished

from adults by their markedly smaller size and lack of breeding condition. The stickleback were collected from a tributary of the Santa Clara River (below the Piru gap) near Fillmore Fish Hatchery in Fillmore, CA and immediately transported to UCLA. To obtain stickleback eggs, we isolated gravid females in separate tanks for up to 16 hours or until they laid eggs at the bottom of the tank. The unfertilized eggs were stored in an ultracold freezer (-76 C) until being used in Experiment 2 (see below). To induce nesting behavior and to obtain stickleback fry for Experiment 3 (see below), we followed a breeding protocol developed by the Santa Barbara Zoo.

Animal housing and care

All animals were housed in a laboratory at the University of California, Los Angeles maintained on a 12-hour dark-light schedule using automatic timers. When not being used in experiments, animals were kept in 25-gallon plastic holding tanks with MarineLand Penguin 200 Power Filters and artificial plants for cover. All holding tanks were kept between 17 and 20 C using CSXC-1 aquarium chillers. In experimental tanks, water temperature was kept at 16-17 C using Arctica HP 1/10 chillers and water was filtered using Fluval 407 canister filters. Tadpoles were fed algae wafers and spirulina algae gel *ad libitum*. Adult stickleback were fed a diet of thawed brine shrimp and bloodworms at approximately 9:30 am and 5:30 pm daily. Stickleback fry were fed live brine shrimp nauplii twice per day.

The experiments were carried out with permission from the United States Fish & Wildlife Service (Permit No. TE76006B-2) and the California Department of Fish and Wildlife. Husbandry and experimental protocols were approved by the UCLA Institutional Animal Care and Use Committee.

Experiment 1. Effect of stickleback on tadpoles

To examine the effects of adult stickleback on *R. muscosa* tadpoles and eggs, in the summer of 2020, we tested tadpoles and eggs in two treatments: “with stickleback” (n=14 trials) in which they were housed in a tank with 2 male and 2 female adult stickleback, and “without stickleback” (n=14 trials), in which they were housed in a tank without stickleback. The animals were held in the tanks over a 22-hour period to allow them enough time to interact while leaving time to set up for the subsequent trial. Testing tanks were 40-gallon glass tanks (89 cm x 37 cm) set up following the same protocol as housing tanks, including an artificial plant for cover.

In each trial we used 12 tadpoles (1.5-2.5 cm SVL, Gosner stage 25-37) and 12 unfertilized eggs (Gosner 1960). During each trial, we used a unique group of tadpoles or fish, although the same individuals were used repeatedly in different trials. To establish a baseline of existing tail damage on tadpoles, we visually estimated the percentage of tail that remained intact relative to an undamaged tail. At the end of the 22-h trial, we placed tadpoles and eggs into temporary observation containers and returned the stickleback to their housing tanks, ensuring that no animals remained in the tank. We then counted all remaining tadpoles and eggs and again quantified tadpole tail damage to compare to initial estimates. Our sample size of 14 trials for each treatment was determined by constraints on time and resources.

To quantify tadpole and fish behavior during trials, we used a video camera (GoPro HERO7) to record each trial for 5 min in the morning after the animals had been in the tank overnight (ca. 16 h after the start of the trial), and again for 5 min within an hour of ending the trial, to allow the animals enough time to adjust to the tank and interact while the camera was recording. We then quantified the frequency of aggressive behavioral events within 30-s periods. Aggressive behaviors included fish biting, chasing, and approaching tadpoles or other fish.

Since it was possible that stickleback would be more likely to have a greater impact on hatchling tadpoles, we replicated this experiment in the summer of 2021 with hatchling (0.75-1.5 cm SVL, Gosner stage 25) tadpoles. We used the same methods except that we used a larger number of hatchling tadpoles in each trial (n=24) and reduced the number of stickleback to n=3 (1 male, 1 female, and 1 subadult) to simulate more natural densities. We did not carry out behavior analysis on the experiment with hatchling tadpoles.

Experiment 1 data analysis

To determine whether the amount of tail damage on tadpoles differed between the fish and control treatments, we used a one-inflated beta generalized linear mixed model using the ‘GAMLSS’ package in R Studio Version 1.3.1093 (Rigby & Stasinopoulos 2005; R Studio Team 2020). After verifying that the baseline amount of tail damage did not differ between tadpoles assigned to the two treatments, we modeled the effect of treatment on the final tail percentage estimate with trial number as a random effect. To evaluate if the change in number of eggs or surviving tadpoles differed between treatments, assuming that any missing tadpoles were dead, we used Mann-Whitney U tests. We used Spearman correlation tests to determine whether the frequency of heterospecific and conspecific aggressive behaviors were positively correlated, as would be expected if heterospecific aggression is a byproduct of conspecific aggression (Peiman and Robinson 2012).

Experiment 2. Effect of tadpoles on stickleback

To examine whether tadpoles affect stickleback fitness directly as measured through predation on stickleback eggs in the presence or absence of a guarding male stickleback, or indirectly through changes in male stickleback nesting behavior, we tested nest-guarding male stickleback in two treatments: “with tadpoles” (n=9 trials) and “without tadpoles” (n=9 trials).

In the treatment with tadpoles, 10 large tadpoles (2.5-3.0 cm SVL; Gosner stage 38-41) were added to a tank with a single male stickleback tending to his eggs in the nest at one end of the tank, and a fake nest with 30 unguarded stickleback eggs on the other end at least 60 cm away from the real nest. The treatment without tadpoles was the same in all respects except that no tadpoles were added to the tanks. During each trial and across treatments, we used a unique group of tadpoles, although the same individuals were used repeatedly in different trials. A total of 9 different stickleback males were used in the trials and each fish was used in one trial with tadpoles and one trial without tadpoles.

The experiment was conducted in 40-gallon glass tanks (89 cm x 37 cm). All tanks contained gravel and sand substrate, one plastic and one live plant to serve as cover or nesting material, and one floating plastic dock for tadpoles to rest on. The fake nest was made using an unraveled cotton ball and sand to weigh it down. The purpose of the fake nest was to assess whether the tadpoles would consume stickleback eggs if the male was not present.

We began trials approximately 1 day after the onset of nesting behaviors for each fish. We ran each trial for a total of 34 hours to allow the animals enough time to interact, and for any negative impacts to be measured, while leaving enough time to set up for the subsequent trial. At the end of the trial, we removed the tadpoles from the tank and determined if there were eggs remaining in the fake nest. Since a fish or tadpole accessing the eggs in the fake nest would often destroy the nest and make counting the remaining eggs difficult, we opted to record only the presence or absence of eggs in the fake nest following each trial.

Since stickleback nesting behavior changes over the course of rearing a clutch (Stein & Bell 2012), we counterbalanced the order of control and experimental treatments, such that approximately half of the males (n=5) received the control treatment following the initiation of

nesting behavior (days 2-4 after onset) followed by the experimental treatment later during the nesting period (days 5-7 after onset) and the other half of the males (n=4) received treatments in the opposite order for a total of 9 experimental trials and 9 control trials. We ran no more than 2 trials per clutch and we did not conduct trials more than 7 days after the onset of nesting behavior. Our sample size of 9 trials for each treatment was determined by constraints on time and resources.

We observed the tank during 5-minute observation sessions throughout the course of the trial in order to record nesting behavior and any interactions between the stickleback and the tadpoles. We carried out observation sessions twice each day of the trial, in the morning and afternoon, to ensure an appropriate sample size with each observation session considered an independent sample. Each observation session was broken up into 10 equal 30-second observation periods to quantify the frequency of target behaviors. For each observation period, the observer recorded the presence or absence of nesting behaviors including when the stickleback was observed (1) within 15 cm of nest, (2) farther than 15 cm from nest for the full duration of the observation period, and (3) leaving the nest and returning within the observation period. In addition, the observer recorded the number of observation periods during which tadpoles were seen (1) resting less than 15 cm from nest; (2) resting greater than 15 cm from nest; (3) swimming within 15 cm of nest; and (4) swimming farther than 15 cm from nest.

During each observation period the observer also noted instances of aggressive behavior by the nesting male stickleback toward tadpoles. These behaviors included (1) approaching a tadpole < 15 cm from nest, (2) approaching a tadpole > 15 cm from nest, (3) biting a tadpole < 15 cm from nest, and (4) biting a tadpole > 15 cm from nest.

Experiment 2 data analysis

To test for the effect of tadpoles on the presence or absence of eggs in the fake nest, we used Fisher's exact tests. To test if stickleback were more likely to approach or bite tadpoles when they were present < 15 cm from the nest, we classified each observation session by whether or not an approach or bite occurred, and whether or not tadpoles were present <15 cm from the nest, and used binomial regression with the 'lme4' package in R (Bates et al 2015). To test for an effect of the presence of tadpoles on adult male stickleback nesting behavior, we used generalized linear mixed models, including individual fish as a random effect, to run binomial logistic regression using the 'lme4' package in R (Bates et al. 2015).

Experiment 3. Impact of heterospecifics on habitat use

To determine if and how habitat use by stickleback and tadpoles changes when the other species is present, we carried out an experiment with three treatments: stickleback only (n=20 trials), in which stickleback were placed alone in a tank; tadpoles only (n=20 trials), in which tadpoles were placed alone in a tank; and stickleback and tadpoles together (n=20 trials), in which both species were placed together in a tank. We conducted two versions of the experiment with animals at different developmental stages and densities: a) 30 stickleback fry and 30 large tadpoles (1.5-2.5 cm SVL; Gosner stage 25-37), and b) 20 subadult nonbreeding stickleback and 20 large tadpoles with hind limbs (2.5-3.0 cm SVL; Gosner stage 38-41). Each trial consisted of a unique group of animals, although individual animals were used repeatedly in multiple trials.

All trials were conducted in 75-gallon tanks (119 cm x 29 cm). Within the trial tanks, we used gravel to create a water depth gradient ranging from 0 cm to approximately 40 cm deep across the length of the tank. We used tape to divide the tank into 4 equal sized quadrants representing 4 different depths (Figure 1). We added plastic vegetation to provide shelter within

each depth such that approximately one half of each quadrant had open habitat and the other half included cover.

We began each trial by transferring all animals from temporary holding containers to the center of the tank simultaneously. Trials were 22 hours in duration which allowed the animals enough time to interact while leaving time to set up and start the subsequent trial. An observer conducted scan sampling approximately 16 hours after the start of the trial (morning) and again at the end of the trial (afternoon) to record the number of individuals of each species in each quadrant and whether they were in the vegetated or open area. The scan sampling consisted of a series of 3 scan samples every 10 minutes to ensure an accurate count after the animals had enough time to adjust to the tank and interact with each other. Our sample size of 20 trials for each treatment was determined by constraints on time and resources.

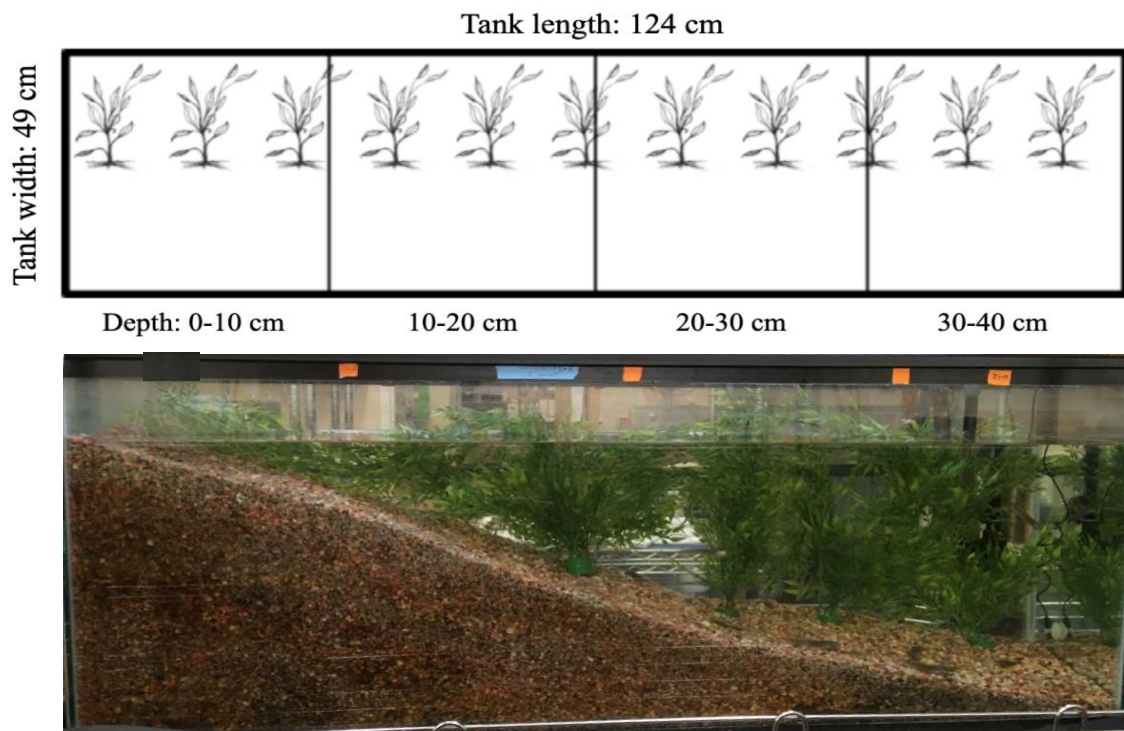


Figure 1. Top and side view of the tank setup for the habitat experiment. A depth gradient was created using gravel along the length of the tank. In the photo, separate quadrants are marked by

orange tape at the top of the tank. Along the width of the tank, half contained plastic plants and gravel, while the other half contained only gravel.

During each trial after the scan sampling, we used a video camera to film each quadrant of the tank for 1 minute following the first scan sample, and again for 1 minute following the second scan sample, to give enough time for the animals to move and interact within the tank. We then used the video to quantify the frequency of tadpole and stickleback behaviors relative to the total number of animals in each quadrant. Stickleback behaviors included (1) shoaling (stationary in a group), (2) schooling (mobile in a group), (3) alone and stationary, and (4) swimming alone. Tadpole behaviors included (1) aggregating (stationary in a group), (2) resting alone, and (3) swimming. Each 1-minute video was divided into 15-second intervals and observers recorded the number of animals seen doing each behavior in that time period, whether the animals were in the vegetation or the open, as well as the total number of animals.

Experiment 3 data analysis

To compare how the distribution of animals differed between treatments, we used negative binomial generalized linear mixed models with treatment, depth, and vegetation as fixed effects, and trial and observation session as random effects in STATA 16.1 (StataCorp, TX). To compare the frequency of behaviors between treatments and microhabitats, we used generalized linear mixed models with negative binomial distribution with treatment, depth, and vegetation as fixed effects, offset by the total number of animals of either species observed, and with trial and observation session as random effects using the ‘glmmTMB’ package in R (Brooks et al 2017). We analyzed the experiments with different size classes and densities separately.

RESULTS

Experiment 1. Effect of stickleback on tadpoles

Large tadpoles and eggs

No large tadpoles died during or after treatments with or without stickleback. However, tadpoles lost more of their tails following the stickleback treatment than the treatment without stickleback (GLMM beta regression: coefficient estimate = -0.58 ± 0.08 , $t = -7.04$, $P < 0.001$; Figure 2), losing $6.4 \pm 5.2\%$ (mean \pm SD) of their tails in the stickleback treatment. In the treatment without stickleback, some individuals' tails regenerated from previous injuries, and the mean percentage of tail remaining increased by $0.26 \pm 0.7\%$ ($n = 14$). The presence of stickleback did not affect the number of frog eggs remaining in the testing tank (Mann-Whitney U test, $W = 88.5$, $P = 0.67$, $n = 28$). An average of 2.07 ± 1.98 of the 12 eggs were lost in the treatment without stickleback, while there were an average of 2.71 ± 2.89 eggs lost in the stickleback treatment.

Behavior	Conspecific		Heterospecific	
	No of Events	% of Trials	No of Events	% of Trials
Biting	45	64	17	57
Chasing	121	92	5	21
Approaching	237	100	95	85

Table 1. Adult stickleback behavior directed toward conspecifics or heterospecifics in the “with stickleback” treatment.

Stickleback were frequently aggressive toward conspecifics and occasionally toward large tadpoles (See table 1). While heterospecific biting was observed 17 times (57% of trials), tadpoles responded to biting by swimming away quickly in 14 of the 17 instances. Across trials with stickleback, there was no significant correlation between the rates of conspecific and heterospecific biting (Spearman correlation, $\rho = -0.05$, $S = 478.09$, $p = 0.86$, $n = 14$), chasing ($\rho = 0.20$, $S = 362.56$, $p = 0.49$, $n = 14$), or approaching ($\rho = -0.2$, $S = 559.42$, $p = 0.43$, $n = 14$).

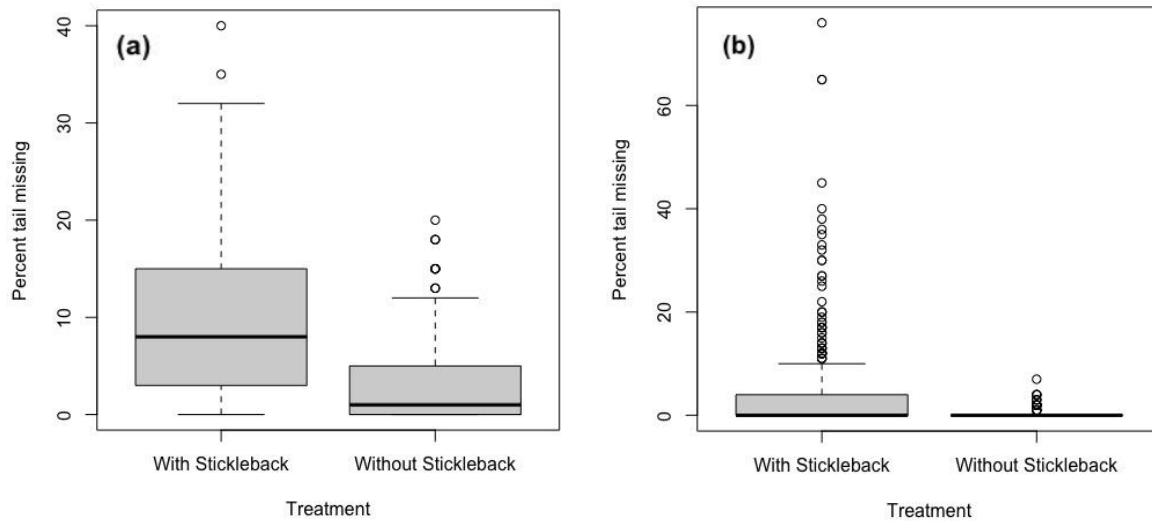


Figure 2. Boxplots of the percentage of tadpoles' tails missing after being housed in a tank with or without stickleback. (a) large tadpoles. (b) hatchling tadpoles.

Hatchling tadpoles

More tadpoles died during stickleback treatment trials than trials without stickleback (Mann-Whitney U test: $W = 42.5$, $P < 0.01$, $n = 28$). A total of 30 tadpoles in the hatchling age class died as a result of the treatment with stickleback, and an additional 14 were not found in the tank, presumably because they had been consumed entirely. In the treatment without stickleback, 7 tadpoles were found dead. Similar to large tadpole trials, hatchling tadpoles lost more of their tails following the stickleback treatment than the treatment without stickleback (GLMM beta regression: coefficient estimate = -0.71 ± 0.21 , $t = -3.34$, $P < 0.001$; Figure 2. On average, hatchling tadpoles lost $5.17\% \pm 5.17$ of their tail following the trials with stickleback.

Experiment 2. Effect of tadpoles on stickleback

There was no evidence of direct fitness impacts of tadpoles on fish eggs. There were eggs remaining in the fake nest after 10 of 14 trials in the tadpole treatment, and 7 of 14 trials in the treatment without tadpoles (Fisher's Exact test: 71.42% versus 50% respectively, $P = 0.44$, $n = 18$). Nesting male stickleback were more likely to behave aggressively toward tadpoles when the

tadpoles were within 15 cm of their nest, as measured by both approaches (binomial GLMM; coefficient estimate = 5.34 ± 2.18 , $P = 0.01$) and bites (coefficient estimate = 3.96 ± 1.58 , $P = 0.01$).

Tadpoles had little impact on the behavior of nesting male stickleback behavior. Nesting stickleback made fewer brief (< 30 s) trips away from their nest when tadpoles were present relative to the control treatment without tadpoles (GLMM: binomial distribution: coefficient estimate = -0.71 ± 0.17 , $P < 0.001$). Stickleback made brief trips from their nest in an average of $18\% \pm 0.16\%$ ($n = 540$) of observation periods in the treatment without tadpoles, and $12\% \pm 0.13$ ($n = 540$) of observation periods in the tadpole treatment. However, the presence of tadpoles did not impact the proportion of observation sessions in which stickleback were near (< 15 cm) their nest (GLMM: binomial distribution: coefficient estimate = -0.1 ± 0.23 , $P = 0.64$). Stickleback were seen near their nest in an average of $90\% \pm 0.15$ ($n = 540$) of observation periods in the treatment without tadpoles, and $93\% \pm 0.15$ ($n = 540$) of observation periods in the treatment with tadpoles.

Experiment 3. Impact of heterospecifics on habitat use

Stickleback fry were not more likely to be observed in any given depth or in the vegetation versus open areas. However, there were fewer fry in the open area and more in the vegetated area when large tadpoles were present (GLMM negative binomial regression; treatment by location interaction: -0.33 ± 0.15 , $\chi^2 = 4.69$, $df = 1$, $P = 0.03$) (Figure 3a). Large tadpoles were more often observed in the vegetated areas (coefficient estimate = 0.91 ± 0.07 , $z = 13.29$, $P < 0.001$), as well as both the deepest and shallowest ends of the tank while avoiding the middle depths (coefficient estimate = 0.90 ± 0.19 , $P < 0.001$). Their distribution shifted slightly to the deepest area when stickleback fry were present (treatment by depth interaction $\chi^2 = 13.60$,

df = 3, $P < 0.01$) (Figure 3b). Subadult stickleback were most abundant in the deepest area of the tank (coefficient estimate = -1.67 ± 0.15 , $P < 0.01$) and shifted to somewhat shallower depths when large tadpoles with hindlimbs were present ($\chi^2 = 11.60$, df = 3, $P < 0.01$) (Figure 3c). Large tadpoles with hindlimbs were most abundant in the shallowest areas (coefficient estimate = 2.20 ± 0.15 , $P < 0.001$) as well as in the vegetation (coefficient estimate = 1.05 ± 0.10 , $P < 0.001$). They were less abundant in the shallowest depth when subadult stickleback were present (treatment by depth interaction $\chi^2 = 12.13$, df = 3, $P < 0.01$) (Figure 3d).

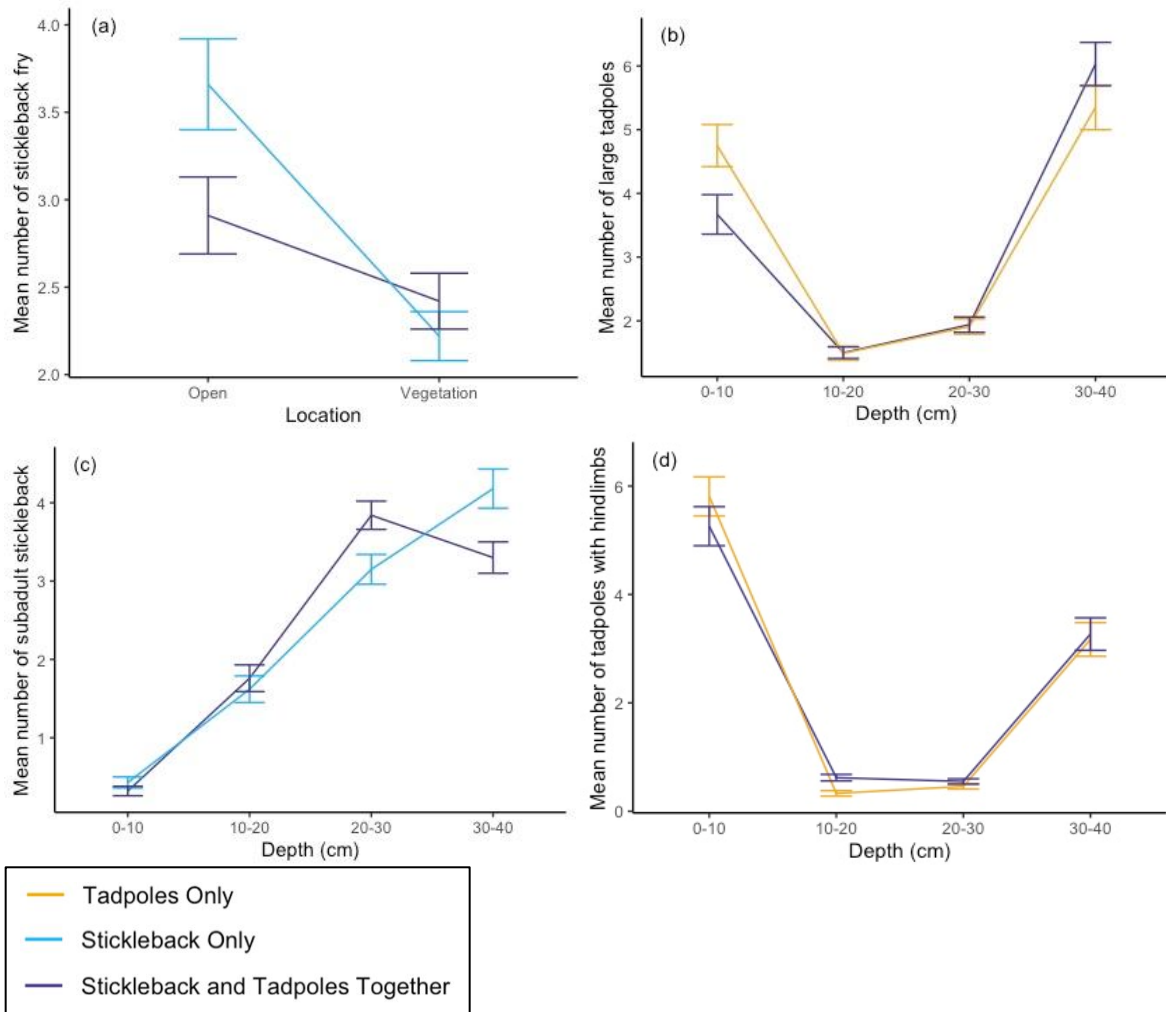


Figure 3. Effect of heterospecific presence or absence on the number of (a) stickleback fry in the vegetated versus the open area; (b) large tadpoles, (c) subadult stickleback, and (d) large tadpoles with hindlimbs across a depth gradient.

The presence of heterospecifics did not impact the frequency of any behavior observed in either size class of tadpoles and stickleback. Regardless of treatment, tadpoles with and without hindlimbs were more likely to aggregate when in the shallowest area (GLMM: negative binomial distribution; large tadpoles with hindlimbs: coefficient estimate (number of animals) = 1.54 ± 0.17 , $P < 0.001$); large tadpoles: coefficient estimate = 0.74 ± 0.16 , $P < 0.001$), while they were more likely to swim when in the intermediate depths (GLMM: negative binomial distribution; large tadpoles with hindlimbs: coefficient estimate = 0.29 ± 0.11 , $P = 0.01$; large tadpoles: coefficient estimate = 0.24 ± 0.05 , $P < 0.01$). In addition, large tadpoles were less likely to aggregate when in the vegetation rather than the open area (coefficient estimate = -0.58 ± 0.1 , $P < 0.001$). Subadult stickleback and fry were more likely to shoal when in the vegetation (GLMM: negative binomial distribution; subadult: coefficient estimate (number of animals) = 0.79 ± 0.22 , $P < 0.001$); fry: estimate = 0.49 ± 0.26 , $P = 0.07$). Subadults were more likely to be alone and stationary (likely hiding) in the vegetation rather than the open as well (GLMM: negative binomial distribution; coefficient estimate = 0.33 ± 0.07 , $P < 0.001$). Subadults were less likely to swim on their own in the shallowest area (GLMM: negative binomial distribution; coefficient estimate = -0.32 ± 0.18 , $P = 0.07$), and were less likely to shoal in the deepest area compared to the shallower depths (GLMM: negative binomial distribution; coefficient estimate = -0.53 ± 0.19 , $P < 0.01$). Fry were less likely to school when in the deepest area (GLMM: negative binomial distribution; coefficient estimate = -0.62 ± 0.22 , $P < 0.01$).

DISCUSSION

Understanding interactions between endangered species will allow resource managers to assess the viability of relocating them to the same sites, with the goal of maximizing use of limited habitat while recovering the species' functional roles in freshwater communities. We

found that stickleback predation attempts on hatchling tadpoles are often lethal, predation attempts on larger tadpoles are sublethal, and predation on frog eggs was not detected. This suggests that stickleback predation on tadpoles is restricted by the tadpoles' size class and lethal impacts become less likely as tadpoles grow. Tadpoles did not prey on stickleback eggs or impact the amount of time that male stickleback spent at their nest, although they were less likely to make brief trips away from the nest when tadpoles were present. This slight shift in nesting behavior could reflect a tradeoff between nest tending and vigilance against territory intruders. Subadult stickleback were often observed in deep water while tadpoles were more likely to use shallow areas, but the distribution of both species shifted somewhat when the other was present. While these results indicate that the presence of either species could impact the other, we think it should be possible to co-manage relocated populations successfully, as explained below.

Lethal and sublethal effects of fish on tadpoles

Stickleback predation on tadpoles is restricted by the tadpoles' size class and lethal impacts become less likely as tadpoles grow. The study was conducted in aquaria, so it is unclear how often predation would occur in the wild. However, these results are consistent with previous research that has indicated that hatchling tadpoles are more vulnerable to lethal attacks than larger size classes (Anholt et al. 1996; Brodie & Formanowicz 1987; Travis et al. 1985). Since stickleback are gape-limited, the size refuge at which tadpoles can avoid predation is related to the size of the stickleback. Certain behavioral traits such as relatively low activity levels, aggregation, and flight responses could help tadpoles avoid predation until they grow to a larger size (Brodie & Formanowicz 1987; Lawler et al 1989). Complex habitats might also enable tadpoles to survive long enough to outgrow the gape width of predators, by reducing the predator's foraging efficiency (Babbitt & Tanner 1998).

How quickly the size refuge can be reached is dependent on the tadpoles' growth rates. In captivity, *R. muscosa* tadpoles typically reach maximum body size in 4-6 months after hatching (L. Jacobs, personal communication, August 29, 2021). However, variation in natural conditions such as water temperature and food availability could result in slower growth and smaller size in overwintering tadpoles. In theory, faster growth should be favored in amphibians that primarily face threats from gape-limited predators (Urban 2007). However, the opposite effect has been observed in previous studies, in which ranid tadpoles exhibited an extended larval period and smaller size at metamorphosis when raised in ponds with gape-limited predators including stickleback (Davenport et al. 2013; Lawler et al. 1999). This could reflect a tradeoff among other factors that impact larval amphibian growth rates, including activity level and foraging efficiency. For example, larval amphibians often decrease movement when exposed to predators, and species that have a lower base activity level may have higher survival in the presence of fish (Lawler 1989). Tail injuries caused by predation attempts on large tadpoles could also result in reduced growth and developmental rates (Wilbur & Semlitsch 1990). Although tail injury from attempted predation is common and often sub-lethal in tadpoles (Morin 1985), an extended larval period and smaller size at metamorphosis could reduce adult survival and fecundity (Chelgren et al 2006; Pechenik 2006; Smith 1987; Semlitsch et al. 1988).

Interestingly, stickleback did not eat frog eggs in our experiment. Previous research has demonstrated that ranid eggs are palatable to stickleback (Light 1969), so it is possible that the fish in the trials did not eat *R. muscosa* eggs because the eggs were not a preferred food source, or because the fish were unable to detect the eggs. However, the palatability of amphibian eggs to fish can vary across species and populations (Gunzberger & Travis 2005), and has not been studied in *R. muscosa*. In addition, it is possible that the stickleback did not always fully

consume the tadpoles because tadpoles are not a preferred food source, which suggests that predation would be reduced if other prey items were abundant. Invertebrates are sticklebacks' primary food source and amphibians are not commonly found in the diet of wild stickleback (Hynes 1950; Ostlund-Nilsson et al, 2006; USFWS 2009). Preference for invertebrate prey, particularly invertebrates that prey on tadpoles, could explain why the presence of potentially predatory fish does not always reduce tadpole survival and recruitment (Lawler et al 1999). However, stickleback diets shift seasonally according to prey availability (Hynes 1950), and thus predation on small overwintering tadpoles might increase as larval insects become less abundant.

Effects of tadpoles on stickleback reproduction

Rana muscosa tadpoles are generalist grazers that primarily feed on benthic algae and detritus, and have been documented cannibalizing eggs (USFWS 2014; USFS 2014). It is possible that we did not find evidence of tadpole predation on stickleback eggs because the nest structure alone provides eggs protection from potential predators. In addition to the protective nest structure, stickleback males guard their nest and chase away potential predators and territory intruders (Wootton 1976). Consistent with previous observations on nesting behavior, stickleback spent the vast majority of their time at the nest, either fanning water over the eggs or stationed above the nest (Wootton 1972). The presence of tadpoles did not impact the proportion of observation periods that stickleback were observed at their nest. However, the fish were slightly less likely to make brief trips away from their nest when tadpoles were present. This is consistent with other studies that demonstrate nesting stickleback behavior shifts in response to perceived predators (Stein & Bell 2012; Gravolin 2021). It is possible that the fish reduced activity across or outside of their territory in favor of more defensive behavior at the nest in the presence of tadpoles. For instance, nesting males often displayed aggression toward tadpoles

within 15 cm of the nest but rarely when they were farther away. Evidence for the impact of such shifts in time allocation on long-term reproductive success is mixed (Gravolin 2021; Lissaker & Kvarnemo 2006; von Hippel 2000). However, threespine stickleback demonstrate plasticity in their time allocation to parenting behaviors, and can quickly return to their baseline following removal of potential predators (Stein & Bell 2012). In addition, these results suggest that tadpoles can avoid being attacked by nesting male stickleback by moving away from the nest. However, only breeding male stickleback are territorial and not all adults breed each season (Whoriskey & FitzGerald 1985), so spatial avoidance is likely not sufficient to avoid predation or aggression entirely. While tadpoles may require certain conditions to avoid fish, stickleback nest structure and parental behavior appear to be effective in protecting eggs from potential predation by tadpoles.

Heterospecific impact on habitat use

Tadpoles of both age classes were most abundant in the vegetated area of the tank and were observed infrequently in intermediate depths. Large tadpoles with hindlimbs were more abundant in the shallowest area of the tank, while tadpoles without hindlimbs were somewhat more abundant in the deepest area. Preference for shallow areas with more cover is seen in Sierra Nevada yellow legged frog (*Rana sierrae*) tadpoles (Yarnell et al 2019), which are closely related to *R. muscosa* (Vredenburg et al 2007). Warmer water found at lake and stream margins may increase tadpole growth rates, while shallow depths and vegetation may provide refuge from predatory fish (Bradford 1984; Brown et al 2019). When stickleback were present, tadpoles were still relatively abundant in the shallowest area, but more individuals were observed in the deepest area compared to control trials. Changes in microhabitat use may interfere with temperature regulation, since tadpoles use shallow areas to warm their body temperature during the day

(Bradford 1984). If there is a steep thermocline, the colder water in deeper microhabitats may reduce tadpole growth rates and reduce size at metamorphosis (Smith-Gill & Berven 1979; Wheeler et al 2015).

Subadult stickleback were most abundant in the deepest area, while fry showed a similar but non-significant distribution. In the presence of *R. muscosa*, stickleback fry were more often found in the vegetated area, while some subadults shifted away from the deepest area to intermediate depths. It is likely that stickleback habitat use shifted in response to tadpoles occupying the deepest area. In the wild, young *G.a. williamsoni* often inhabit shallow areas of streams and ponds with warmer water and dense vegetation (USFWS, 2009). Adults in the wild are found across a wider range of microhabitat types, from shallow, vegetated areas to relatively deep, open water (USFWS, 2009).

Despite the observed shifts in habitat use, we did not detect any effect of stickleback on the frequency of any tadpole behaviors, or vice versa. However, shifts in microhabitat usage could impact the frequency of certain behaviors. For example, in the presence of fish, some tadpoles shift to deeper water where they are less likely to aggregate. Subadult stickleback and fry shift to shallower areas and vegetation, respectively, in the presence of tadpoles, where they are more likely to shoal. In other words, stickleback may be more likely to reduce their activity and aggregate in the presence of tadpoles, while tadpoles may be less likely to do so in the presence of stickleback fry and subadults. The fish in these trials did not display aggression toward the tadpoles. The tadpoles' microhabitat distribution, and therefore behavior, may differ in the presence of aggressive adult fish that may pose more of a threat. None of the stickleback in the habitat experiment were in breeding condition, but breeding stickleback may prefer to hold territories and build nests in deeper areas with more cover (Kynard 1978; Kynard 1979).

However, given that adult stickleback are aggressive toward tadpoles, and since *G.a. williamsoni* can occupy a wide range of microhabitats in the wild, it is unlikely that the presence of tadpoles would negatively impact their habitat use.

Management recommendations

Based on these results, *R. muscosa* reintroductions are unlikely to have deleterious effects on *G.a. williamsoni* survival, reproductive behavior, or habitat use, but *G.a. williamsoni* might hamper the establishment of *R. muscosa*. Harmful interactions might be mitigated by considering the animals' size class and timing of relocations, as well as habitat characteristics such as size of the water body, abundance of aquatic invertebrate prey, presence of cover, and quality of lake margins (Table 1).

The order of translocations and number of founder individuals could affect the outcome, particularly when introducing competing or consumer-resource species pairs, in which introducing the prey species first is beneficial (Plein et al 2015). Given the direction of impact, the frogs should be introduced first and enough time should pass to measure population growth (Stage 3 recovery) before introducing stickleback (Miller et al 2014). The frogs should reach a high enough density so that they are able to reproduce and replace any individuals lost to stickleback predation. To optimize the probability of establishment of both species into the same receiver site, *R. muscosa* should be reintroduced and allowed to establish, survive and reach population growth, with tadpoles that have grown to greater than 1.5 cm SVL (Gosner stage 25) before *G.a. williamsoni* is reintroduced. These mitigations can support the stability of both translocated populations.

If conditions require that *G.a. williamsoni* be reintroduced in advance of *R. muscosa* or before *R. muscosa* has an opportunity to reproduce, selecting a receiver site with habitat

complexity and an abundance of invertebrate prey could reduce predation pressure on *R. muscosa*. Tadpoles often use shallow areas, while adult stickleback may be more abundant in deeper water. Availability of shallow areas at the receiver site could benefit tadpoles by providing refuge from stickleback. Shallow areas also tend to have warmer water, which could potentially facilitate tadpoles' growth and development (Bradford 1984; Smith-Gill & Berven 1979; Wheeler et al 2015). Both stickleback fry and tadpoles use areas containing aquatic vegetation, which can serve as shelter from predators (Babbitt & Tanner 1998). Relocation sites should have abundant aquatic vegetation where juveniles of both species can seek refuge. Aquatic and terrestrial vegetation can also support invertebrate populations, which form an important part of sticklebacks' prey base (USFWS 2009). Ensuring that the site has an abundance of non-predatory invertebrates could reduce predation pressure on small tadpoles, since tadpoles are likely not preferred prey for stickleback. During the winter, invertebrate prey may become less available, but tadpoles will likely be large enough to avoid stickleback predation. Smaller overwintering tadpoles might rely on submerged cover objects for refuge, particularly if shallow areas are less accessible due to ice during the colder months. In addition, installing a mesh enclosure to serve as a refuge area for small tadpoles could help reduce the impact of stickleback on tadpole survival.

Our findings indicate that while *G.a. williamsoni* pose some risk to *R. muscosa*, the timing of relocations, size and growth of individuals, and quality of habitat could help facilitate their coexistence at the same pond sites. Further work is needed to monitor the long-term impacts of species co-occurrence on the stability of relocated populations.

Table 2. Potential impacts of three spined stickleback (*G.a. williamsoni*) on mountain yellow legged frog (*R. muscosa*) tadpoles, and suggested mitigations.

Observed Interaction	Potential Impact	Suggested Mitigation
Predation	Decreased survival and recruitment	Introduce the frogs before the stickleback; introduce stickleback only after evidence of frog population growth; avoid introducing small tadpoles; choose release site with abundant habitat refuge and numerous shallow areas; ensure alternate prey is available; install an enclosure as artificial refuge
Sublethal predation attempts	Decreased body size and fitness	Choose release site with abundant habitat refuge and numerous shallow areas; ensure alternate prey is available
Territorial or nest defense aggression	Spatial displacement	Choose a release site with abundant habitat refuge and diverse microhabitats
Interference in habitat use	Decreased body size, survival and recruitment	Choose a release site with diverse microhabitats including abundant aquatic vegetation and numerous shallow areas

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