REFUGIA RESEARCH: DEVELOPMENT OF HUSBANDRY AND CAPTIVE PROPAGATION TECHNIQUES FOR INVERTEBRATES COVERED UNDER THE EDWARDS AQUIFER HABITAT CONSERVATION PLAN

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EXECUTIVE SUMMARY

The primary objectives of this research were first to thoroughly review relevant literature, and secondly to assess and evaluate various methods and techniques that might successfully be used to study and establish refugium for the federally-listed invertebrates found in springs and wells of the Edwards Aquifer and in the Edwards Aquifer Habitat Conservation Plan. All experiments initially used surrogate species closely related to endangered species before actual testing on the endangered species. This minimized the chance of harm to the troglobitic amphipod *Stygobromus pecki* and the spring-adapted riffle beetle *Heterelmis comalensis* during testing.

Four sets of experiments were conducted. Each was designed to answer specific questions related to handling and detailed study, holding, basic reproductive traits, and culture of endangered organisms. All experiments were designed to address substantial gaps in current knowledge that might present obstacles to the successful holding and culture of refuge populations.

Anesthesia techniques that do not affect long- and short-term survival are required so that individual invertebrates can be identified, imaged, and studied at high magnification. This is essentially impossible while animals are moving. Our results showed that a dilute solution of ethanol (EtOH) was the most effective anesthetic and had almost no short- or long-term detrimental effects on any of the tested organisms. High concentrations of dissolved CO_2 were also effective for anesthesia of beetle larva, but logistics were more difficult that use of EtOH.

Responses of aquifer-adapted organisms to light exposure was evaluated to determine how it might alter behavior and/or metabolic rates (i.e., organism stress). Responses varied between five different amphipod species, but results indicate that successful culture of the amphipod *Stygobromus pecki* will probably require complete darkness and short observation periods under low-intensity long-wavelength (red) light in order to minimize changes to behavior, metabolic rates, and/or stress levels.

Amphipod reproduction experiments were performed on *Stygobromus pecki* to assess reproductive rates and sexual maturation rates. No changes in female reproductive development were observed during a two-month study period, other than release of young from a single female that was gravid at the beginning of the study. More work is needed to determine the time required for ovaries to develop and mature in female *Stygobromus* amphipods. This experiment did show that pairing equal- or larger-sized males with equal- or smaller-sized females resulted in the lowest incidence of cannibalism, and that pairing large females with smaller males should be avoided for this reason.

Experiments to assess the best methods for holding and growing groups or individuals of *Stygobromus pecki* proved that amphipods fed and grew measurably over a two-month period. Holding amphipods in groups was shown to be effective, provided there is habitat available for young and smaller amphipods to hide and avoid cannibalism.

1. INTRODUCTION

The Edwards Aquifer Habitat Conservation Plan (EAHCP) calls for the establishment of captive refuge populations for the species of concern associated with the Edwards Aquifer and the springs emerging from the aquifer. To develop successful captive propagation programs for the invertebrate species covered under the EAHCP, captive rearing, life history, and environmental requirements research needs to be conducted. Five aquatic invertebrates: the endangered Comal Springs riffle beetle (*Heterelmis comalensis*), Comal Springs dryopid beetle (*Stygoparnus comalensis*), and Peck's cave amphipod (*Stygobromus pecki*); and the petitioned Edwards Aquifer diving beetle (*Haideoporus texanus*) and Texas troglobitic water slater (*Lirceolus smithii*) are covered under the HCP. The San Marcos Aquatic Resources Center (SMARC) operated by the U.S. Fish and Wildlife Service maintains some of these species on site and has performed a variety of research projects on maintaining captive populations and propagating them for long-term refuge purposes. However, there are still several substantial questions and issues associated with many of these taxa which currently impede the ability of resource managers to maintain captive populations. For example, the USFWS can successfully hold Comal Springs riffle beetles and Peck's cave amphipods in captivity, but has experienced difficulties in refugium establishment with low numbers of beetles successfully pupating into adults and reduced survival of amphipods likely due to cannibalism. Additionally, very little is known about life history and the environmental requirements of any of the covered species.

In order to develop methods for captive propagation, four preliminary studies were executed during 2015. These studies looked at anesthesia, light response, mating behavior, and different holding systems for some of the protected species. The anesthesia of test organisms is considered crucial to be able to determine their sex, developmental stage, length, weight, and to individually mark or photograph organisms. Organisms' response to light could affect their stress level and health in captivity. Thus, understanding the mating behavior of Peck's cave amphipod and the role cannibalism plays in reproduction, if any, is essential to management of the refugia. The role of cannibalism and methods to limit it during all life stages also must be determined if populations are to be maintained in substantial numbers.

This document contains the results and methods we used to address the four main research projects: (1) methods for anesthesia for invertebrates held in captivity, (2) responses of epigean and/or hypogean invertebrates to light, (3) reproduction of amphipods (specifically the Peck's cave amphipod) held in captivity, and (4) methods for holding Peck's cave amphipods in captivity.

2. LITERATURE REVIEW
2.1. Anesthesia

Typically, invertebrate anesthetic agents rely on the respiratory system of organisms to gain entry into the nervous system of invertebrates where chemical disruption of synapses anesthetizes the organism (Cooper, 2011; Lewbart and Mosley, 2012). Amphipods and riffle beetle larvae respire through gills (Graham, 1990), thus water soluble chemicals are an effective

delivery method of anesthetics because of direct chemical exchange with water. However, adult riffle beetles respire through a plastron which indicates that aqueous solutes may not be an effective method for delivery of anesthetic to adult riffle beetles. This is because there is no direct chemical exchange with water but rather with the trapped gas bubble which is reliant upon atmospheric exchange of gasses with the surrounding water. Therefore, for adult riffle beetles, a gaseous anesthetic bubbled into solution is likely be a more effective method. Alternatively, use of a chemical anesthetic with a low vapor pressure, such as ethanol or ether, may be effective. Because water breathing organisms tend to have a larger exchange surface with water than air breathing organisms have with air (Graham, 1990), air breathing organisms may require higher concentrations of anesthetics because of smaller absorptive surfaces and therefore less chemical exchange with the environment when compared to water breathing organisms.

2.1.1. Specific information on effects of different anesthesia methods on Coleopterans

Very little work has been performed on the effects of different anesthesia agents and methods on aquatic beetles. However, a fair amount of work has been done on the anesthesia of terrestrial beetles, where CO_2 treatments are the widely utilized anesthetic of choice (Lewbart and Mosley, 2012). This may be an effective method for anesthesia of both adult and larval riffle beetles because CO_2 will be readily taken up by respiratory mechanisms, but CO_2 has been shown to cause permanent changes in behavior of anesthetized coleopterans (Lizé *et al.*, 2010) and affect survivability and fertility of other insects (Barron, 2000; Champion de Crespigny, F. E. and Wedell, 2008). Another method that has been used to anesthetize insects is cold induced anesthesia, but this has also been shown to also affect survivability (Barron, 2000; Champion de Crespigny and Wedell, 2008) and is likely inappropriate for spring- or aquifer-associated organisms which are likely to experience limited *in situ* environmental temperature variation and are thus likely adapted to these conditions (i.e., stenothermal). Volatile chemicals agents such as ethers or ethyl acetate have also been used for anesthesia and have been shown to have effects on survivability and behavior comparable to CO_2 (Loru *et al.*, 2010).

2.1.2. Specific information on different anesthesia methods on Amphipods

Both soluble chemical and gaseous chemicals bubbled into solution have been used as anesthetics for numerous species of aquatic crustaceans in a diversity of settings. The efficacy and survival rate varies appreciably between these two general anesthesia methods. For example, Cothran (2008) found that CO_2 treatments had 44% mortality when used on one species of *Hyalella* and almost complete mortality when used on another smaller species of *Hyalella*. In the same study, clove oil was used on the smaller *Hyalella* species and mortality was only 26% (Cothran, 2008); however, these mortality rates are likely much higher than what would be considered acceptable for refugium purposes. In contrast, Venarsky and Wilhelm (2006) found that that there was a positive relationship between CO_2 dose and mortality within different size classes of *Gammarus minus*. Cumulatively, these studies suggest that mortality associated with CO_2 anesthesia may be avoided at proper dosages.

Clove oil is a commonly used chemical anesthetic, but the duration of exposure to clove oil also had a positive relationship with increased mortality while time to anesthesia was shown to be negatively correlated with dose, and time to recovery was shown to be positively correlated with dose (Venarsky and Wilhelm, 2006). Ultimately, it appeared that a size appropriate dose and exposure duration could be elucidated that would effectively anesthetize the organisms while minimalizing mortality to an acceptably low rate (Venarsky and Wilhelm, 2006). However, no information has been collected on the effect of clove oil on the long term survival and fecundity of anesthetized organisms.

A more commonly used chemical anesthetic, which has also been tested on a diversity of vertebrates and invertebrates, is tricaine mesylate (a.k.a., tricaine methanesulfonate, TMS, and MS-222). MS-222 has been demonstrated to be effective at anesthetizing a diversity of invertebrates, including amphipods (Lewbart and Mosley, 2012), and if used properly can have low mortality rates (Ahmad, 1969). Like clove oil, dosage concentration is negatively correlated with time to anesthesia and positively correlated with time to recovery (Ahmad, 1969). Temperature was also shown to be negatively correlated with time to anesthesia (Ahmad, 1969), though as previously stated, we do not recommend that temperature be manipulated as it is unlikely that aquifer-adapted organisms can tolerate temperature fluctuations because they are likely to have evolved in physicochemically stable environments. The relationship between size of amphipod and concentration of dose has not been studied but it is likely that these two factors are positively correlated. Unfortunately, the long term effects of MS-222 on survivability and fecundity has not been studied.

Other anesthetics that have been used on crustaceans are dilute chlorobutanol and ethanol (EtOH) though these have not been tested on amphipods nor have their long term effects on survival or fecundity been studied (Ross *et al.*, 2009). However, the investigators in this project have previously used EtOH to short-term anesthetize hypogean amphipods and isopods from the Edwards Aquifer, but have not conducted a systematic study to determine tolerance limits and dosages.

2.2. Responses to light exposure by subterranean and subsurface invertebrates

Housing of organisms and observation of mating behaviors presents several substantial issues with regard to the conditions in which organisms are held and observed. In particular, subterranean organisms may have preferences or tolerances with regard to light levels (e.g., intensity) and the types of light (i.e., wavelengths) that are present. Though few studies have addressed light-dependent responses of stygobiontic invertebrates, a multitude of methods have been used that quantify responses of a diversity of organisms to light. In general, these responses are quantified based on the relative amount of movement in light versus dark areas, degree of avoidance of illuminated areas, and rate of respiration in light *versus* dark conditions. In most cases, it was determined that stygobiontic and troglodytic organisms have the ability to detect light (even eyeless forms) and put significant effort into avoiding light (Simcic and Brancelj, 2007; Borowsky, 2011). Considering that data indicate that many cave organisms tested to date

avoid light, it is likely that exposure to light may be stressful and disadvantageous to culturing organisms.

Information on the sensitivity of stygobiontic amphipods of the Edwards Aquifer to light has yet to be explored thoroughly and published. In contrast, subterranean amphipods from other geographic regions have been studied to a greater extent. The amphipod *Niphargus* is adapted to subterranean groundwater environments of Europe, is eyeless (and presumably blind), and has had aspects of their sensitivity to light studied including phototaxis (Borowsky 2011). Borowsky (2011) studied dorsal light reflex (a response of some organisms whereby they orient their dorsal surface towards the origin of light; Foxon, 1939) in *Niphargus* spp. and found no evidence that they exhibit this reflex. Borowsky (2011) also examined the relative amount of movement in illuminated and dark conditions and if *Niphargus* would move into areas protected from direct light and found that the intensity of the effect was positively related to the intensity of the light treatment. Simcic and Brancelj (2007) compared the sensitivity of subterranean aquatic amphipods and epigean aquatic amphipods, using *Niphargus stygius* and *Gammarus fossarum*, respectively. To compare the relative effect of light on these taxa, both *N. stygius* and *G. fossarum* were exposed to multiple intensities of light under identical conditions and the authors monitored the consumption of oxygen to infer rate of respiration (potentially indicating a stress response). *N. stygius*, had a significantly greater oxygen consumption rate when exposed to light than in the dark. In addition, the authors found that oxygen consumption rate was positively related to light intensity. In contrast, the authors observed no effect of light on respiration rates of the epigean species, *G. fossarum* (Simcic and Brancelj, 2007). Previous studies have indicated rate of oxygen consumption is correlated to stress levels in organisms (Kedwards *et al.*, 1996; Simcic and Brancelj, 2007). These data suggest that exposure to light is stressful to some species of subterranean amphipods. Therefore, it is likely that successful culture of stygobiontic amphipods in the refugium may require that organisms rarely if ever be exposed to light.

Avoiding exposure to light has been hypothesized to be advantageous to subterranean-adapted fauna, because a sightless condition in an epigean environment is almost certainly associated with a suite of disadvantages. This is likely because once a subterranean-adapted organism is exposed to surface conditions, a suite of disadvantages arise and mate finding and predator avoidance at the surface may be more difficult (Fišer et al. 2014). Indeed, some genetic data are consistent with this hypothesis in that it appears there is selection in cave invertebrates for the ability to detect light (Crandall and Hillis, 1997). Taking into consideration that most cave organisms tested to date (see references above) avoid light, it is likely that exposure to light may be stressful and disadvantageous when culturing organisms in a refuge setting.

2.3.   Reproduction of subterranean amphipods

Like many aspects of the ecology and of subterranean amphipods, information on their life history is not abundant in the literature. In general, it appears that subterranean amphipods (like other subterranean species) have a much slower rate of reproduction than epigean species. Most epigean species of amphipods have multiple generations per year (i.e., multivoltine), while subterranean amphipods typically take at least a year to mature (i.e., univoltine) (Venarsky *et al.*, 2007; Crawford and Tarter, 1979). However, the subterranean hyporheic amphipod, *Niphargus aquilex aquilex,* has been shown to have the capacity to produce up to two generations per year

(Gledhill and Ladle *et al.*, 1969). This suggests that surface species may not be suitable surrogates for trying to study developmental rate, but may still be useful for studying developmental events at an accelerated rate.

The pairing of male and female amphipods and "optimal" sex ratios is not well understood. The sex ratio in *Crangonyx forbesi* was shown to fluctuate on an annual cycle (Crawford and Tarter, 1979). During winter months (*C. forbesi* breeding season), males outnumbered females, while during the summer no males were observed (Crawford and Tarter, 1979). It has been suggested that the greater abundance of males during the breeding season corresponds to females having synchronous pre-copulatory molts (Crawford and Tarter, 1979; Bollache and Cezilly, 2004). Sex ratios may also become distorted due to the mechanism of sex determination in amphipods. That is, amphipods do not have all sex determining alleles located on discrete sex chromosomes; rather, sex determining alleles are distributed across several chromosomes and sex is inherited much like a quantitative trait. Furthermore, it has been shown that certain pairings can lead to exclusively male or female offspring (Sutcliffe, 1992). Environmental factors have also been shown to affect or at least covariate with sex, as well (Sutcliffe, 1992; Watt and Adams, 1993; McCabe and Dunn, 1997) and infection with microsporidians (Bulnheim and Vávra, 1968) and chemical pollutants (Gross *et al.*, 2001) have also been shown to affect sex ratios or the development of sexual characteristics in amphipods suggesting that sex determination in amphipods may behave like a developmentally plastic and quantitative trait. Therefore, it is crucial to maintain proper pedigrees of amphipods and ideal culture conditions ratios in the refugium to ensure against improper development or heavily biased sex ratios which could lead to the collapse of specific or preferred culture lineages. Cumulatively, these data indicate that amphipods which are housed in a refuge context (i.e., *S. pecki*) require that reproduction is thoroughly understood.

Currently, there is no information in the literature on the mating behavior for any stygobiontic amphipod species in the world. However, there is a plethora of information on mate selection and timing of reproduction in epigean amphipods and there is some information on reproductive cycle of wild-caught stygobiontic amphipods. Amphipods are thought to only able to mate after the female molts, because only then is the cuticle of the females' exoskeleton flexible enough to allow the release of eggs through the genital pores into the marsupium (Bollache and Cezilly, 2004). Because females are only momentarily receptive to mating, males typically guard a female prior to her molting to insure that he fathers offspring. Molt cycles in males also appear to have a role in reproductive timing because males approaching a molt tend to not be willing to enter into amplexus because they will inevitably have to release the female upon molting thus never actually copulating with the female; a wasted investment in a mating (Bollache and Cezilly, 2004). Mate guarding comes at a cost to males by hindering their ability to forage, thereby reducing lipid stores and hindering growth (Robinson and Doyle, 1985). In response to this energetic cost, males tend to not enter into amplexus unless they have sufficient amounts of stored lipids and glycogen to wait out the females molt cycle and only if the female is expected to molt before the male (Plaistow *et al.*, 2003). Therefore, it is likely that proper nourishment is necessary to offset the nutritional costs of amplexus. In addition, there appears to be significant cannibalism in captive populations of *S. pecki* (R. Gibson, *pers. obs.*). However, keeping both males and females well-fed likely reduces the tendency of cannibalistic interactions to occur.

It has been proposed that because larger females are more fecund, that there is greater competition among males for access to larger females (Bollache and Cézilly, 2004). The resulting consequence is that larger males preferentially out-compete smaller males for larger females, thus there appears to be size assortative paring between males and females; at least in some species of amphipods (Bollache and Cézilly, 2004; Franceschi *et al.*, 2010). However, male-female pairs in amplexus with smaller females tend to have greater swimming efficiency than pairs in amplexus with larger females, suggesting that males tend to be larger than females in scenarios with predation (Adams and Greenwood, 1983). Selection for larger males is also compounded by female resistance to amplexus (Jormalainen and Merilaita, 1995). The ultimate consequence of these size-specific interactions is that females must have a large enough male suitor if mating is to be successful, but also that the female must be large enough (and thus fecund enough) to be worth the male investing in amplexus. However, under scenarios where selection has been relaxed, size selection will only be effected by choosing for the most fecund (thus largest) female that a male is large enough to restrain.

2.4. Amphipod holding and culturing techniques

In order to protect against loss of individuals held in a refugium setting, it is important that the range of conditions are survivable and mortality is minimized. Ideally, organisms should be housed within an "optimal" range of conditions and within this range, conditions should maximize survival and production of offspring for the refuge. In the context of establishing a refuge for the Edwards Aquifer, the Edwards biota live in environments with presumably little environmental variation and it is therefore expected that organisms will perform best under relatively stable conditions that mimic the physicochemistry of the Edwards Aquifer. In addition, this suggests that Edwards Aquifer organisms, regardless of taxonomy, are likely to have similar environmental requirements. However, this also implies that all Edwards Aquifer organisms have been afforded equal opportunities to become adapted to their environment; which is almost certainly not the case. Therefore, closely related groundwater fauna can be physiologically quite different, as with some *Niphargus* species (Issartela *et al.*, 2005) presumably because more recently distributed species have not been afforded the opportunity to become as "ideally" adapted.

In general, the metabolic rate of stygobionts appears to be low when compared to epigean relatives (Hervant *et al.*, 1997; Mezek *et al.*, 2010). Multiple distantly related taxa have been shown to have the ability to go without food for long durations without depleting energy reserves, while closely related surface taxa have been shown to deplete energy reserves during the same duration of starvation (Hervant *et al.*, 1997; Mezek *et al.*, 2010). Therefore, it is likely that refugium stygobionts will require relatively less feeding when compared to epigean species. However, in order to promote or maintain breeding in stygobionts, it is likely that organisms will need to be fed enough food to offset fitness costs (Plaistow *et al.*, 2003).

Among the amphipods in the hypogean genus *Stygobromus*, similarity in gross morphology alone does not appear to recapitulate phylogenetic relationships (Culver *et al.*, 2010). Within *Stygobromus*, a relationship seems to exist between the pore size of the habitat, the gross morphology, and the overall size of the species (Culver *et al.*, 2010). Other species of hypogean amphipods (i.e., *Niphargus* in Europe) also exhibit the same kind of patterns (Trontelj et al.

2012). The survival rate of *Gammarus pseudolimnaeus* (an epigean aquatic amphipod) offspring co-varies with pore size of habitat (Waters, 1984). Therefore, habitats in refugia will likely require species-specific pore spaces (such as mesh or other structure) in order to optimize refuge habitat suitability and survivability.

In addition to porosity, there is likely a relationship between survival/growth rates and physicochemical conditions. The survival rate of molting amphipods is closely related to the amount of dissolved Ca^{2+} in the water (Zehmer *et al.*, 2002). The same study found that low Ca^{2+} waters were shown to be deadly to most molting *Gammarus pseudolimnaeus*, which appears to be a large factor in determining the geographic range limits of this species. Edward Aquifer water is calcium-rich, therefore having sufficient Ca^{2+} for molting amphipods is not likely to be an issue. However, if refugium stock are moved to different locations, it is important that water at the new locations is rich in calcium; at least during molting.

Determining how to house individuals in order to track individual development and increase survival is critical. *Stygobromus pecki* has a tendency for cannibalism, thus knowing how to hold individuals (in group set ups or in individual containers) is critical to determine how to house individuals.

3. METHODS AND MATERIALS
3.1. Anesthesia
3.1.1. Test subjects and use of surrogates

Initial testing utilized surrogate species to avoid unnecessary mortalities of legally-protected species. When preliminary trials were completed with surrogates, protected species were then tested with relatively small numbers to refine understanding of proper dose of anesthetic. Fortunately, there is abundant locally distributed species in the same family as *S. pecki* (Crangonyctidae), both epigean and subterranean. *Stygobromus flagellatus* and *S. russelli* are the most locally abundant subterranean species and both were used as surrogates for anesthesia testing before *S. pecki* were tested. *Stygobromus flagellatus* and *S. russelli* were collected from drift nets placed over spring outflows at the headwaters of the San Marcos River (San Marcos Springs). The surrogate for the Comal Springs riffle beetle (*Heterelmis comalensis*) was *Heterelmis* cf. *glabra*., collected from Finnegan Springs along the upper Devils River, Val Verde County, Texas. All surrogates were acclimated to captivity for at least two weeks prior to testing to ensure that transport and handling did not confound results. Because it was unknown which anesthetics would be effective on which taxa and what concentration was best to use for each anesthetic, surrogate testing was used as an opportunity to determine the best prescribed doses for anesthesia trials on legally protected species. This was largely accomplished through insight from published literature and trial and error using surrogates species.

3.1.2. Source of legally protected experimental organisms

Adult *Heterelmis comalensis* were collected using poly cotton lures following the methods of Gibson *et al.* (2008) and Huston *et al.* (2015). *Heterelmis comalensis* larvae were produced in captivity by breeding of collected adults. *Stygobromus pecki* were collected by dip netting

sediments in spring openings of Comal Springs using aquarium nets. *Stygobromus pecki* were separated by hand from sediments immediately after collection. All specimens were acclimated to captivity for at least two weeks prior to testing to ensure that transport and handling did not confound results.

3.1.3. Initial test for susceptibility

Taxa were first tested at the highest prescribed dose (based on results from surrogate testing) of each anesthetic to test which anesthetics each taxon was susceptible to. If anesthesia or death occurred it was conclude that the taxon was susceptible to the anesthetic and therefore further testing using that anesthetic was warranted. Subsequent anesthesia trials at different concentrations were attempted to elucidate the most effective and practical anesthetic for each taxa and the most appropriate dose for that anesthetic. All anesthesia trials were maintained at $22^{o}C$ (approximately *in situ* conditions).

3.1.4. Anesthesia trials
3.1.4.1. Experimental groups

Each type of anesthetic was administered to the appropriate taxa at four different concentrations per anesthetic (Table 1 and Table 2), thus there were four treatment groups per anesthetic type. Within treatment groups we replicated ($n=3$) for each taxon within each treatment level; the number and type of test subjects used for each treatment group is presented in Table 3. None of the test subjects used for any of the anesthesia trials were used for any other treatments in order to avoid pseudoreplication.

Table 1. Concentration of anesthetics tested on *Stygobromus pecki* determined from preliminary surrogate testing.

Anesthetic	Treatment Concentrations			
	1	**2**	**3**	**4**
MS-222 (mg/L)	800	1200	1800	2700
(µL Clove Oil/L H$_2$O)	50	100	200	400
% concentration EtOH	2	6	14	22

Table 2. Concentration of anesthetics tested on *Heterelmis comalensis* determined from preliminary surrogate testing. Clove oil was only tested on larvae and CO2 was only tested on adults.

Anesthetic	Treatment Concentrations			
	1	**2**	**3**	**4**
(µL Clove Oil/L H$_2$O)	100	200	400	800
% concentration EtOH adults (larvae)	6 (2)	14 (6)	22 (14)	30 (22)
CO$_2$ (ppm)	130	-	-	-

Table 3. Number of individuals per treatment group by taxon

Anesthetic	Taxon		
	Larval Beetles	**Adult Beetles**	**Amphipods**
MS-222	0	0	12
CO$_2$	0	3	0
Clove Oil	12	0	12
EtOH	12	12	12
Sum=>	24	15	36

3.1.4.2. Preparation of treatment solutions

Clove oil (Eugenol)

A stock solution of 1 part clove oil to 3 parts 100% EtOH was prepared. After preparing the stock solution of clove oil, a 100 mL solution of Edwards artesian well water and clove oil was prepared at the prescribed concentrations (Table 1and2) for each replicate. The solution was

placed in a clean 125 mL Erlenmeyer flask and placed in a water bath of artesian water on a flow through system to insure thermal stability.

MS-222

A 100 mL solution of Edwards Aquifer artesian well water and MS-222 was prepared at the prescribed concentrations for each replicate (Table 1). The solution was placed in a clean 125 mL Erlenmeyer flask and placed in a water bath of artesian water on a flow through system to insure thermal stability.

EtOH

EtOH solutions of 100 mL were prepared at the prescribed concentrations (Table 1 and 2) for each replicate by mixing 100% EtOH with artesian water. The solution was placed in a clean 125 mL Erlenmeyer flask and placed in a water bath of artesian water on a flow through system to insure thermal stability.

CO_2

CO_2 was bubbled into solution in a 2-L sealed container filled with 1 L of Edwards Aquifer artesian well water until reaching saturation. As CO_2 was bubbled into solution, concentration in solution was monitored using a CO_2 meter (OxyGuard CO_2 Analyzer). Despite using a sealed chamber, controlling the amount of CO_2 that went into solution was extremely difficult (see Coyle *et al.* 2004). Therefore, we only assessed one dose level of CO_2 in this study.

3.1.4.3. Experimental procedure

After preparing the experimental chamber with each individual anesthetic treatment, each individual test subject was placed into a separate 100 mL flask of the treatment solution. Upon placement into the solution, a stop watch was started and observation was made until the subject became anesthetized. If 30 min elapsed and the subject was still not anesthetized, this was recorded as not sensitive to treatment dose. If anesthesia occurred, the duration until anesthetized was recorded and subjects were removed from the anesthetic and washed with untreated Edwards Aquifer artesian well water. For *S. pecki*, if thirty minutes of exposure to anesthetic had elapsed and the test subject was still not anesthetized, this was recorded as "not sensitive to treatment dose". For *H. comalensis*, if sixty minutes of exposure to anesthetic had elapsed and the test subject was still not anesthetized, this was recorded as "not sensitive to treatment dose" (except for CO_2 which took appreciably longer for anesthesia to occur; see below). Afterwards, each test subject was photomicrographed at 10x magnification using Olympus Cellcens camera system and software at the standard shutter speed of 3.395 milliseconds. Length was then estimated for each individual using the measuring tool in the Cellcens software system. Following photography, test subjects were placed into individual holding chambers suspended in a flow through system of artesian water. Subjects were then monitored until they recovered and the time until recovery was recorded. If 3 hours elapsed and a test subject was still inactive, this was recorded as a mortality event for the individual.

Surviving test subjects were kept and monitored for an additional month to determine if there was any longer-term effects of each anesthetic on survivability. Anesthesia trials were maintained at 22ºC for the entire duration of the study.

3.1.4.4. Analysis

Multiple single factor ANOVAs were run to determine if size of test subject and/or treatment concentration could explain the variation observed in how long it took for test subjects to become anesthetized and how long it took for test subjects to become revived post anesthesia.

3.2. Light response
3.2.1. Test subjects

Stygobromus pecki Holsinger is federally listed as endangered and is known only from three localities: Comal Springs, Hueco Springs, and a monitoring well in Panther Canyon; all in Comal County, Texas (Krejca 2005; Gibson *et al.* 2008). This species is thought to be exclusively subterranean due to its lack of eyes and is therefore thought to only be encountered incidentally at spring openings. Despite numerous other species of subterranean *Stygobromus* reported or described from across the Edwards Aquifer, the diversity of this genus in the Edwards Aquifer is poorly understood. Therefore, the degree of endemism of this genus is not known, nor is the extent and distribution of most *Stygobromus* species understood.

Despite disparities in knowledge on *Stygobromus*, some species are better understood than others. In this study we tested and compared the relative sensitivity to light of five *Stygobromus* species; the species used depended on availability. All of the species used in this study had some aspects of their habitat preferences understood with the exception of an undescribed *Stygobromus* species that has only been collected from Garden Ridge well in Comal County, Texas. *Stygobromus pecki* is thought to be an exclusively subterranean species that is found in the waters beneath Comal Springs and is therefore expected to encounter epigean waters at an evolutionarily relevant frequency. In the context of this study, we define an evolutionarily relevant frequency as being frequently enough to act as a selection pressure on populations. *S. flagellatus* is thought to be a deep phreatic species that under natural conditions should not be expected to encounter epigean waters at an evolutionarily relevant frequency. *S. bifurcatus* is thought to be an interstitial exclusively subterranean species that is also not expected to encounter epigean waters at an evolutionarily relevant frequency. Finally, *S. russelli* is thought to be an interstitial hyporheic and subterranean species that is thought to incidentally encounter epigean waters at an evolutionarily relevant frequency. If some of these species retained or redeveloped the ability to detect light due to selection, it is hypothesized that this would only occur in the species which are expected to encounter epigean waters at an evolutionarily relevant frequency.

All *Stygobromus* used in this study were collected using drift nets placed over spring openings with the exception of *S. pecki*, which was collected by dip netting loose sediments of spring openings at Comal Springs, and *Stygobromus spp.*, which was collected by bottle trap form the Garden Ridge Well. Sampling localities and species collected at each is present in Table 4.

Sample size per species varied depending on availability. Prior to any experimentation, all test subjects were acclimated to captivity for at least two weeks prior to any experimentation.

Table 4. Collection localities for *Stygobromus* used in this study.

Locality	Coordinates	Taxa Collected
Big Boiling	30° 56.626'N 97° 32.199'W	*S. bifurcatus*; *S. russelli*
Comal Springs	29° 42.859'N 98° 8.156'W	*S. pecki*
Garden Ridge	29° 38.553'N 98° 18.442'W	*Stygobromus sp.*
Robertson	30° 56.679'N 97° 32.509'W	*S. russelli*
San Marcos Springs	29° 53.596'N 97° 55.864'W	*S. bifurcatus; S. russelli*
Sessom Springs	29° 53.427'N 97° 56.213'W	*S. russelli*

3.2.2. Experimental design

In the initial phase of this portion of the research we wished to determine which taxa, if any, respond to being exposed to any portion of the visible spectrum of light. To do this, all taxonomic groups were initially exposed to full spectrum light. This was done because organisms which only respond to a portion of the light spectrum should also respond to full spectrum light. Therefore, if organisms initially responded to full spectrum light, then further experimentation was performed to determine if there were threshold intensities below which they no longer respond and/or if organisms only responded to certain portion of the light spectrum (Figure 1).
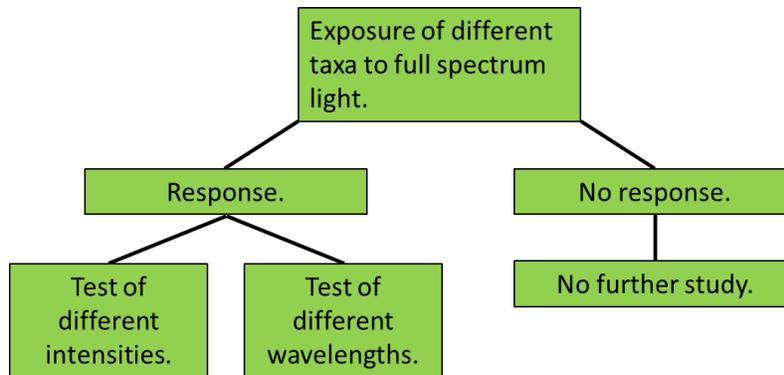

Figure 1. Flow chart of methodology used to test sensitivity of organisms to light.

3.2.3. Lighting apparatus

The lighting apparatus was made by cutting a 15-cm inch diameter hole in the center of the bottom of an inverted 20-L bucket 25-cm in diameter. The bucket was then painted on the

interior surface several times with a non-transparent black paint to insure that as little light escaped or penetrated as possible. A dome shaped metal lamp slightly larger in diameter than the hole in the bucket was affixed face down over the hole so that when the light was turned on the angle of incidence was nearly perpendicular to the horizontal surface. This apparatus was used for all light experiments.

3.2.4. Initial test for light response

A 5.5-W full-spectrum LED light bulb (equivalent to a 40-W halogen bulb) was used for all aspects of this phase of study. Light source was calibrated to 45 µmol m^{-2} s^{-1} of irradiance by adjusting the distance from the test subject and the amount of current to the bulb (via rheostat control). All organisms were submitted to two types of experiments, one that examined if there was a behavioral response to light exposure and another that assessed changes in rate of respiration (O_2 consumption) when exposed to light. Increases in respiration was assumed to be indicative of increased stress.

3.2.5. Behavioral response

A light avoidance experiment was used to determine which taxa preferred to not be exposed to direct light. Test subjects were placed in a 9-cm diameter petri dish with two quarters of its upper surface of the cover painted black with a nontransparent black paint (Figure 2). Light sources were oriented at a perpendicular angle of incidence from directly above. Petri dishes were filled with fresh Edwards Aquifer artesian well water at the beginning of each trial. All individual test subjects were placed in the petri dish and allowed ten minutes to acclimate in the dark prior to making any observations because preliminary trials suggested that ten minutes was an acceptable duration to allow organism to recover from the stress of being handled. After test subject acclimation, the light source was turned on and observations were taken for a 15 minutes period with one individual observation taken at 1 minute intervals. At each observation time, it was noted if the test subject was in one of the light or the dark quarters. At the end of each trial, each replicate had one response variable calculated: proportion of duration spent avoiding direct light. The difference of this proportion from 0.5 was then calculated as the true response variable because it is expected that an organism that shows no preference would be found in the dark and the light quarters half the time each due to randomness alone. Therefore, the null expectation was a value of 0.5 and departure from this value was considered to be a response. The magnitude of this response was tested for significance with a single factor ANOVA using the factor 'species' to predict the relative degree of response to light among the different species tested. A Tukey's HSD was run to determine which species responded in a similar fashion and if there was any general response categories that taxa could be assigned to.
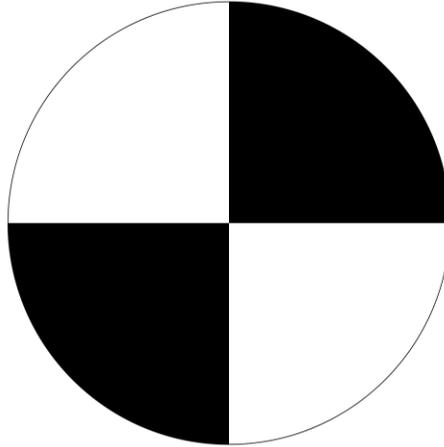
Figure 2. Schematic of a petri dish with two quarters of its upper surface covered in black paint.

3.2.6. Stress response

It is thought that exposure to light may induce stress in some subterranean species. To estimate stress, rate of dissolved oxygen (DO) consumption was compared between test subjects held in the dark *versus* in full spectrum light. We assumed that elevated respiration is related to elevated stress levels. Respiration rates were estimated using Qubit systems OX1LP-30 DO cuvettes with built-in Clark cell type polarographic oxygen sensors. After calibration of the cuvette systems, but prior to testing subjects for sensitivity to light, the DO consumption of the O_2 electrodes was estimated by running the cuvettes filled with 5 mL of water in the dark and full-spectrum light to insure that differences in lighting did not affect the rate of O_2 consumption by the electrodes themselves, and to gain an understanding of the base-line rate of O_2 consumption of the O_2 electrodes.

Using this system, an individual test subject was placed in a cuvette filled with 5 mL of artesian water and was allowed to acclimate to the chamber for 10 minutes to recover from the stress of handling. The test subject was then randomly assigned to an order in which they would receive each of the two treatments: dark or full-spectrum light. The subject was exposed to each treatment for 15 minutes while DO consumption was recorded at 30 second intervals with a 10 minute interval between the two treatments to allow organisms to recover and reacclimatize from any stress that may have been induced from the prior treatment. All experiments were carried out in a dark room. Light incidence on test subjects was perpendicular to the bottom of the cuvette. Cuvettes were jacketed with an artesian water flow through system in order to maintain thermal stability.

Changes in DO measurements over time were converted to a proportion of total DO consumed across all of the treatments for one individual in order to control for variation between test subjects due to size or other physiological differences. The combined data for all test subjects belonging to the same species, or same species from the same collection site, were regressed across elapsed time as the X axis. The slope between the dark and light treatments was then

compared using analysis of covariance (ANCOVA) within and across taxa to determine if species was a valid predictor of response to light and if the response varied within species across collection localities.

Net proportional DO consumption was also calculated for every test subject for both treatments. A two factor ANOVA with the factors 'species' and 'locality' as predictors was run to test if subjects belonging to the same species explained most of the variance or if response varied between populations within designated species.

3.2.7. Further study of taxa shown to respond to light

Species that responded behaviorally and physiologically to light were further studied and analyzed to determine what aspect(s) of full spectrum light they respond. Species that did not show a metabolic response to high intensity full spectrum light were not studied further. Likewise, species that did not respond behaviorally to high intensity full spectrum light were not studied further. Response of any type at lower intensities of full spectrum or at partial spectrum light would unlikely when high intensity full spectrum light failed to produce a response. *Stygobromus pecki* was the only species that showed both behavioral and stress responses to light and was the only species further studied.

3.2.7.1. Study of response to varying wavelengths

Full spectrum light is the combined irradiance of all the wavelengths of visible light, therefore full spectrum light will always have greater luminous flux than only a single wavelength if emitted from a device of the same wattage. In an effort to insure that differences in light intensity were not confounding results and to isolate the variable 'wavelength', it was necessary to calibrate each wavelength tested to the same amount of radiant flux. Therefore, prior to each treatment replicate the light source was recalibrated to 6 μmol m^{-2} s^{-1} of irradiance by adjusting the amount of current for each of the wavelengths tested using a FieldScout LightScout Quantum Light Meter. This was done in an effort to insure that any variation in intensity due to calibration error was spread as evenly as possible across treatment replicates.

Response to the different wavelengths of light was tested using the above described behavioral and respiratory methods. In both cases, the treatment replicates were given 10-mins acclimatization after handling before collecting any data. After the acclimatization period, test subjects were exposed to three wavelengths in a randomized order in an effort to insure that any residual response from the previous treatment would be randomly distributed across treatment replicates so that order of treatment was not a confounding factor. Three wavelengths were tested (red $\approx$ 700 nm; amber $\approx$ 600 nm; and blue $\approx$ 500 nm) on *S. pecki* (*n*=5 for each treatment).

3.2.7.2. Study of varying intensities of full spectrum

In order to determine if response to light is related to the intensity of light, different intensities of full spectrum light were tested. The intensities were 1.5 µmol m^{-2} s^{-1}; 3 µmol m^{-2} s^{-1}; 6 µmol m^{-2} s^{-1}, and 12 µmol m^{-2} s^{-1} for the behavioral study, and 6 µmol m^{-2} s^{-1}; 12 µmol m^{-2} s^{-1}, and 24 µmol m^{-2} s^{-1} for the respiratory study. Each treatment was calibrated to the appropriate intensity of light by adjusting the amount of current and the distance of the light source from the testing chamber. Response to the different intensities of light was tested using the above described behavioral and respiratory methods. In both cases, the treatment replicates (*n*=5) were given 10 min of acclimatization before collecting data. After this period, test subjects were exposed to thre intensities tested in a randomized order in an effort to insure that any residual response from the previous treatment would be randomly distributed across treatment replicates so order of treatments was not potentially confounding results.

3.3. Amphipod reproduction

Nine adult females with visibly gravid ovaries (indicating they would be receptive to copulation after their next molt) and nine adult males were selected at random from a stock culture of *S. pecki* (two females were also already brooding offspring in marsupiums). Each female was then paired with a male and the size of both individuals was measured in length by photomicrographing each individual at 10x magnification using Olympus Cellcens camera system and software at the standard shutter speed of 3.395 milliseconds. Length was then estimated for each individual from its respective photograph using the measuring tool in the Cellcens software system. These measurements were used to estimate the size ratio of the pair. Male-female pairings were adjusted so that there were 3 replicates of each of the following size ratio categories: male larger, male and female relatively equal in size, and female larger. Pairs were then placed in a transparent 1 L container with a mesh substrate set up on a flow through system that received a constant flow of artesian well water 0.5 L per minute.

After establishing mating pairs, initial observations on the reproductive condition of females. Observations were then taken weekly on the reproductive condition of females if amplexus or copulation was observed, and if cannibalism occurred within pairs. Female reproductive condition was categorized into the following categories: visible ova in ovaries, ovaries gravid with eggs, eggs laid in marsupium, linear embryos in eggs, and neonates in marsupium. This data was taken in an attempt to study the developmental rate of the reproduction in females. Observations were made across six weeks unless obvious cannibalism, death for other reasons, or successful reproduction occurred before six weeks elapsed; in which case the duration to one of these events was recorded. At the end of the six weeks each pair was categorized as either successfully reproducing or not. Throughout this experiment all amphipod pairs were fed commercially-available fish flakes *ad libitum*.

It was hypothesized that pairs with males larger than females would have reproductive success more frequently. To test if size ratio of pairs affected mating success, a single factor ANOVA was run using the factor "size ratio" to predict the response variable: reproductive success. A logistic regression was also used to test if size ratio and mating success have a predictable directional relationship.

3.4. Amphipod holding and culture

The current method employed at the SMARC to house and their cultures of *S. pecki* is a "group holding system". These systems utilize plastic containers supplied with a constant supply of Edwards Aquifer well water through a simulated upwelling on the benthic surface which provides dispersed flow. Constant flow limits the development of anoxic areas, while large surface area filtered drainage reduces the likelihood of clogging and flooding. A nylon-net substrate with various pore sizes is provided to increase the three dimensional surface areas for the amphipods to utilize and to limit contact between individuals. Typically large numbers (*n* ranging from 30 – 60) of amphipods are placed together into these containers which has resulted in high mortality, presumably due to cannibalism. In order to reduce cannibalism, group held populations are well-fed.

Using this system, it is impossible to track the growth and development of individual amphipods. Therefore, two other types of holding systems are being proposed which will allow for holding amphipods individually. The first of these systems is the use of individual "flow chambers". The second of these proposed systems is a "suspended static array". Both of these chamber systems were designed to house amphipods individually to eliminate the possibility of cannibalism and also to allow for the study of growth and development of individual amphipods.

3.4.1.   Individual flow chambers

Chambers were made by modifying commercially available single-cup reusable coffee filters (Keurig brand) that were 26 mL in total volume. Chambers were modified to have a water line feeding in while water was allowed to drain out freely through the filter mesh while submerged in a water bath of artesian water in an effort to insure that chambers remained filled to their maximum volume as well as maintained thermal stability. Each system consisted of 5 individual chambers fed from the same water supply and maintained in same water bath.

3.4.2.   Suspended static array

The static array consisted of multiple commercially available single-cup reusable coffee filters arranged into an array with the chambers completely submerged. Each array consisted of 5 individual chambers maintained in the same water bath with a common supply of flow-through artesian water. In contrast to the "flow chambers", this system only had water supplied to the water bath and no water was pumped through the individual chambers. Therefore, in this system, chambers only received water *via* passive diffusion through mesh.

3.4.3.   Group holding system

Group holding systems held 5 organisms together in the same container. These containers were designed as described above and measured 25 by 15 cm on the benthic surface area and were filled to a volume of 2.5 L. Group holding systems received a constant supply of artesian water on a flow through system.

3.4.4.   Experimental design

To compare the efficacy of these systems, an equal number of amphipods were placed into each replicate (5 individuals per replicate); there were three replicates for each type of holding system. Each replicate received the exact same rate of flow to insure that water supply was not a confounding variable; flow rate was calculated by timing the duration required to collect 1L of water from the outflow of each system and then calibrating each system receive 1 to 1.2 L of flow per minute. 36 *S. pecki* were collected by dip netting from Comal Springs in Landa Park on 20 July 2015. These 36 amphipods were randomly distributed into three groups of 12. Because it is only possible to identify *Stygobromus* spp. to species when they are adults only adult amphipods were used from the Landa Park collection. However, in order to properly execute this study, amphipods of all sizes needed to be included. Therefore, 9 immature amphipods that had been hatched and reared in captivity at SMARC (and therefore of known species identity) were selected and randomly distributed into three groups of 3. The three groups of immature amphipods were then randomly added to one of the group of adult amphipods to make for three groups of 15 amphipods that each had a broad range of sizes represented. Each group was then randomly assigned to one of the three treatments. From these three groups, 5 amphipods were selected one at a time and randomly assigned to a replicate within their treatment group until all three replicates had received 5 amphipods.

Amphipods placed into replicates had standard length estimated by photomicrographing each individual at 10x magnification using Olympus Cellcens camera system and software at the standard shutter speed of 3.395 milliseconds. Length was then estimated for each individual from its respective photograph using the measuring tool in the Cellcens software system. Using this length information, mean length and variance was estimated for each replicate. Holding trials were begun on 21 July, 2015 and ended on 11 November, 2015; if any amphipods died in the first week of the holding trials they were replaced. After the prescribed holding period (3 months) length was measured again using the same method for all remaining amphipods; % survival was also calculated. The hypothesis was that in the group holding system, % survival would decrease significantly compared to either of the individual holding systems and that in the group system, the mean size would increase on account of the larger individuals cannibalizing smaller individuals (thus eliminating the effect of smaller individuals on the mean) and the variance would also be reduce significantly in the group holding system when compared to the individual systems because the remaining amphipods in the group systems would all be of similar size (the largest amphipods). All study amphipods were fed commercially available fish flakes *ad libitum*.

4. RESULTS
4.1. Anesthesia
4.1.1.   Clove Oil

During surrogate testing, none of the adult riffle beetles (*H. glabra*) survived anesthesia at any of the treatment doses of clove oil tested. Clove oil showed some promise for use on beetle larvae and *Stygobromus* spp. therefore, we further tested this anesthetic on legally protected *H. comalensis* larvae and *S. pecki*.

4.1.1.1.   *Stygobromus pecki*

Individual length explained a significant amount of variation in time duration until anesthetized ($F=7.008$, $p=0.024$) but not in the duration until revived post anesthesia ($F=0.015$, $p=0.904$). Likewise, concentration was found to explain variation in duration until anesthetized ($F=7.55$, $p=0.021$) but not duration until revived ($F=0.023$, $p=0.883$). No discernable relationship was found between treatment dose and survival rate though there was appreciable mortality across the treatment groups one month post anesthesia.

4.1.1.2.   *Heterelmis comalensis* **larvae**

Individual body length was not found to explain a significant amount of variation in duration until anesthetized ($F=0.369$, $p=0.557$) or variation in duration until revived ($F=0.012$, $p=0.915$). None of the variation in duration until anesthetized ($F=0$, $p=0.99$) or duration until revived ($F=1.322$, $p=0.277$) was explained by treatment concentrations. No discernable relationship was found between treatment dose and survival rate though there was appreciable mortality across the treatment groups one-month post anesthesia. The lack of explainable variation for any of the parameters for this anesthetic suggests that this is not a feasible choice of anesthetic for *H. comalensis* larvae.

4.1.2.   MS-222

During surrogate testing, concentrations of MS-222 orders of magnitude higher than that necessary to euthanize vertebrates showed no effect on riffle beetles. Therefore, it was decided that this was not a practical anesthetic for use on riffle beetles as concentrations necessary to anesthetize beetles would be high enough to be potentially pose a health risk to laboratory workers.

Again, concentrations of MS-222 much higher than what was necessary for the euthanasia of vertebrates were shown to be effective at anesthetizing *Stygobromus* spp. Therefore this anesthetic was further tested in anesthesia trials on *S. pecki*. Length was not found to explain

variation in time until anesthetized ($F=0.452$, $p=0.516$) or time until revived ($F=0.269$, $p=0.615$) across any of the treatment levels. Concentration of dose did explain some variation in duration until anesthetized ($F=20.108$, $p<<0.001$) but did not significantly explain variation in duration until revived ($F=1.687$, $p=0.223$). Differences in duration until anesthetized across the different treatment levels are depicted in Figure 3. Only the lowest concentration varied significantly from the other treatment levels (Figure 3; Table 5). Only one individual died across all of the treatment groups one month post anesthesia, therefore it does not appear that survival varied by treatment dose nor does it appear that MS-222 is lethal to *S. pecki* at any concentration tested.
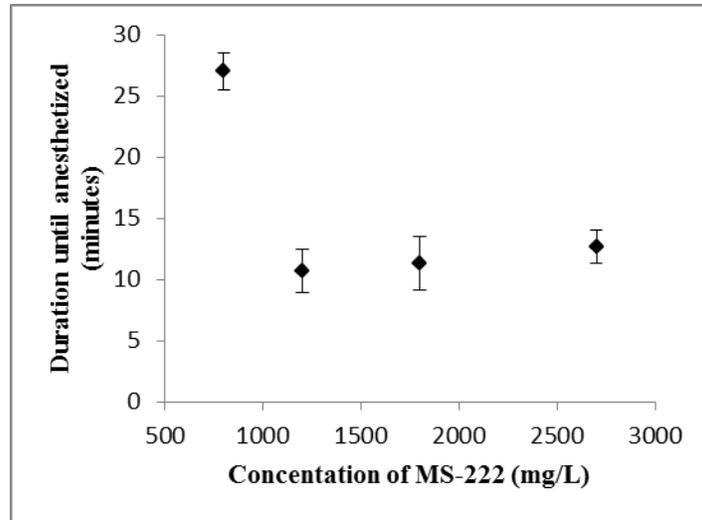

Figure 3. Duration until anesthetized for *Stygobromus pecki* exposed to four different concentrations of MS-222. The three higher concentrations showed no difference in duration until anesthetized. Error bars represent one standard error around the sample mean.

Table 5.) Tukey's HSD indicated that 800 mg/L MS-222 was the only concentration that differed from any of the other concentrations.

Comparison	diff	lwr	upr	p
800-1200	16.33	8.489	24.17	0.001
800-1800	15.67	7.823	23.51	0.001
800-2700	14.33	6.490	22.18	0.002
1200-1800	-0.667	-8.511	7.178	0.992
1200-2700	-2.000	-9.844	5.844	0.845
1800-2700	-1.333	-9.178	6.511	0.946

4.1.3. EtOH
4.1.3.1. *Stygobromus pecki*

Individual amphipod body length was not found to explain a significant amount of variation in duration until anesthetized ($F=0.508$, $p=0.492$) or variation in duration until revived ($F=0.055$, $p=0.819$). However, concentration was found to be a highly significant predictor of variation in duration until anesthetized ($F=67.67$, $p<<0.001$) and duration until revived ($F=42.87$, $p<<0.001$). Only one individual died across all treatment groups one month post anesthesia, thus it does not appear that long term survivability was affected by the EtOH treatment doses used in this study. Duration until anesthetized significantly predicted duration until revived ($F=14.9$, $p<0.001$). Interestingly, EtOH was the only anesthetic we tested as a part of this project that exhibited this relationship.

4.1.3.2. *Heterelmis comalensis* larvae

Boby length was not found to explain a significant amount of variation in duration until anesthetized ($F=1.007$, $p=0.339$) or variation in duration until revived ($F=0.167$, $p=0.691$). Concentration was not found to be a significant predictor of variation in duration until anesthetized ($F=2.695$, $p=0.132$); however, concentration was found to be a highly significant predictor of duration until revived ($F=41.37$, $p<<0.001$). One month post anesthesia, only two individuals had died and the deaths that occurred were correlated to treatment dose, suggesting that the range of treatment doses in this study did not affect long term survivability.

4.1.3.3. *Heterelmis comalensis* adults

Body length was not found to explain significant variation in duration until anesthetized ($F=0.051$, $p=0.827$) or variation in duration until revived ($F=0.005$, $p=0.947$). However, concentration was found to be a significant predictor of variation in duration until anesthetized ($F=24.89$, $p<0.001$) and duration until revived (F=14.0 p=0.004). Three individuals died

immediately following anesthesia; no additional individuals died after one month post anesthesia. Duration until anesthetized significantly predicted duration until revived (F=16.36, p=0.002). Again, as in the results for *S. pecki*, EtOH was the only anesthetic tested which exhibited this relationship.

4.1.4.   CO$_2$

We only tested CO$_2$ on adult *Heterelmis* of both species. Despite using a presumably airtight sealed chamber, we experienced great difficulty establishing specific concentrations of CO$_2$ in solution. Therefore, we were only able to test the effect of CO$_2$ at ≈130 ppm on adults of both species. Our results showed that even at this high concentration, it took a mean duration of 96 minutes (range = 83 − 120 minutes) for individuals to become anesthetized. The duration until revived ranged from 22 to 30 minutes and there was no observed mortality during anesthesia or one month post anesthesia.

4.1.5.   Discussion

Following the initial surrogate testing we concluded that clove oil should not be used at any concentration on adult riffle beetles. Even at the smallest concentrations used in this study, all of the surrogate individuals died. It is unclear why all adult riffle beetle surrogates died after exposure, but it is suspected that clove oil, being hydrophobic itself, interferes with the hydrophobic properties of the plastron (Brown, 1987; White and Roughley, 2008) preventing efficient respiration.

During initial surrogate testing of clove oil on *Stygobromus pecki*, it was observed that surrogates that died during exposure to clove oil turned black in color after death. This is in stark contrast to the opaque white color of live *Stygobromus* spp. It is suspected that at high dosages of clove oil, *Stygobromus* spp. essentially suffer chemical burns and die. Based on these initial findings, it was decided that clove oil should only be tested for efficacy on *Heterelmis comalensis* larvae and *Stygobromus pecki* and only at light dosages. Following experimental testing on *H. comalensis* larvae and *S. pecki*, it was concluded that clove oil is not the most feasible of anesthetics for either of these organisms. This is based on a lack of reliable predictors for duration until anesthetized and until revived, as well as relatively high mortality that was not predictable by treatment concentration. Therefore, it is our recommendation that clove oil not be used as an anesthetic for these species.

After surrogate testing with MS-222, it was determined that riffle beetles are not sensitive to this anesthetic at practically-applied dosages. Therefore, MS-222 was only tested for efficacy on *Stygobromus pecki*. Interestingly, our findings suggest that there is a threshold dosage that above which an increase in concentration of dosage does not increase the anesthesia efficacy of MS-222. This threshold was found to exist between 800 - 1200 μL/L suggesting it may be possible to achieve maximum efficacy with this anesthetic using the minimum dosage above the threshold concentration. The asymptote of efficacy in respect to concentration above 1000 μL/L

concentration of MS-222 in water has been observed in other taxa of amphipods (Ahmad, 1969). Testing of MS-222 on *S. pecki* during experimentation suggests that this anesthetic is not highly lethal to *S. pecki* at any of the concentrations tested. Furthermore, only one individual died across all of the treatment groups one month post exposure; therefore, these results are very encouraging as it does not appear to be possible to overdose *S. pecki* with MS-222 within the concentration range tested, thus reducing concerns of user error in future research.

During both surrogate testing and anesthesia trials on legally protected taxa, EtOH showed the greatest promise as an anesthetic in future studies. EtOH was the only anesthetic that duration until anesthetized significantly predicted duration until revived for any of the taxa tested. Indeed, this relationship was observed for both *S. pecki* and *H. comalensis* adults and likely would have been observed for *H. comalensis* larvae if we were able to obtain larger sample sizes. This relationship is very useful in being able to estimate how long anesthetized individuals will remain anesthetized and how long a window of time is available to work with individuals before they become active again. Recovery time post-anesthetization was significantly predicted by treatment concentration for both *S. pecki* and *H. comalensis* adults, and all other organisms tested. These results once again emphasize the utility of the use of EtOH as an anesthetic because of the level of control possible. Aside from *H. comalensis* adults, there was no appreciable mortality for any of the treatment groups thus suggesting that EtOH may be the ideal anesthetic for *S. pecki* and *H. comalensis* larvae. However, *H. comalensis* adults were relatively sensitive to higher concentrations of EtOH while not at all sensitive to lower concentrations, which failed to anesthetize any individuals in those treatment groups. Conversely, two of the three individuals in the highest concentration treatment group died immediately following anesthesia suggesting that this concentration is above or approaching a lethal threshold. Though there was a predictable relationship for all parameters measured using EtOH as an anesthetic on *H. comalensis* adults, the mortality results suggest that the window of anesthetic dose is quite narrow, suggesting that EtOH may not be the most practical for *H. comalensis* adults.

CO_2 was not used on *Heterelmis* surrogate larvae because other studies have shown CO_2 interferes with proper development of coleopteran larvae (Lizé *et al.*, 2010); nor was it tested on *Stygobromus* surrogates because other studies have shown appreciably high mortality of amphipods anesthetized with CO_2 (Cothran, 2008). Therefore, CO_2 was only tested on adult *Heterelmis*. Anesthesia of surrogates had no mortality, therefore it was determined it was safe to test CO_2 on *H. comalensis*. The difficulty establishing specific concentrations of CO_2 in solution was attributed to great solubility of CO_2 in water. The high solubility of CO_2 in water and difficulties of controlling concentration has also been reported in other studies (Coyle *et al.*, 2004). Because there was no mortality during anesthesia or one month post anesthesia, and there was low variation in anesthesia duration, CO_2 may be the best option for anesthesia of *H. comalensis* adults despite the length of time required to anesthetize individuals and the difficulties associated with controlling concentration.

None of the anesthetics tested showed a predictable relationship between size of organism and sensitivity to anesthetics for any of the taxa. This is likely due to the rate of uptake of anesthetic being directly proportional to the size (volume) of the organism. Therefore, size of organism should not be a consideration when deciding anesthetic concentration. It was observed during surrogate testing that anesthetics at any concentration were lethal to individuals that had recently

molted. This is likely due to the permeability of the post-molt soft exoskeleton. We recommend that great care be taken to ensure that organisms are not anesthetized post-molt until their exoskeletons have hardened.

Our study only examined the effect of concentration on the duration post exposure to anesthetic until anesthetized, the duration post anesthesia until revived, and the immediate and long-term survival of individuals post anesthesia. Although we have some insight into the survival rate of the various organisms at the various concentrations of the various anesthetics tested, we have virtually no information on the long term effects that the different anesthetics would have on the development and fecundity of anesthetized organisms. Therefore, our recommendations for best anesthetic are only based on the factors for which we have data. Thus, it may be determined in the future after further research that certain anesthetics should not be used because of the negative effects they have on the life history of anesthetized organisms. We also anesthetized each individual only once across the entire duration of our study; however, if one was to conduct a long term study on the growth and development study organisms would have to be anesthetized multiple times across their life span, thus cumulative effect may become a factor. Therefore, future research should address the long term effects of anesthetics on development and fecundity, and on the cumulative effect of repeated anesthesia.

4.2. Light response
4.2.1. Initial test for light response
4.2.1.1. Behavioral response

The study of the behavioral response of the various *Stygobromus* species tested suggests that species identity is a good predictor of whether or not a species will respond to light, and the magnitude of those responses (Table 6). Only two of the species were found to respond to light and differences in the magnitude of the response of those two species was indistinguishable (Table 7).

Table 6. ANOVA of behavioral response of the various *Stygobromus* species to light. The response variable was proportion of time spent avoiding exposure to direct light. 50% of time spent in light/dark is indistinguishable from random; therefore, magnitude of difference from 50% was the response variable analyzed. Taxon was found to significantly predict response to light.

SOV	df	F	p
Taxon	4	11.79	<<0.001
Locality	4	1.37	0.271
Taxon:Locality	1	0.066	0.799
Residuals	27		

Table 7. Tukey's HSD comparing all possible comparisons of behavioral response between the different *Stygobromus* species tested. *Stygobromus russelli* and *S. pecki* differed significantly from all other species but did not differ from each other.

Taxa Comparison	diff	lwr	upr	*p* adj
S. russelli-S. flagellatus	0.307	0.132	0.482	<<0.001
S. russelli-S. bifurcatus	0.36	0.145	0.576	<<0.001
S. pecki-S. bifurcatus	0.305	0.065	0.544	0.007
S. pecki-S. flagellatus	0.251	0.048	0.455	0.009
GR *S.* sp.-*S. russelli*	-0.227	-0.443	-0.011	0.035
GR *S.* sp.-*S. pecki*	-0.171	-0.411	0.068	0.259
GR *S.* sp.-*S. bifurcatus*	0.133	-0.15	0.417	0.658
S. russelli-S. pecki	0.056	-0.098	0.209	0.832
GR *S.* sp.-*S. flagellatus*	0.08	-0.174	0.334	0.890
S. flagellatus-S. bifurcatus	0.053	-0.2	0.307	0.973

4.2.1.2. Stress response

Table 8. Slopes and R^2s of the regressions of O^2 consumption for each species of *Stygobromus* tested. In the dark treatment none of the species had predictable variation in O^2 consumption except for *S. russelli* ($R^2=0.74$). However, in the light treatments, both *S. pecki* and Garden ridge *Stygobromus* sp. showed highly explainable variation which was also accompanied by a much steeper slope than what had been recorded for the dark treatments for both of these species. Interestingly, taxa was not found to be a significant predictor of variation in net O^2 consumed ($F=1.74$, $p=0.17$) but locality was ($F=3.99$, $p=0.009$) which suggest that niche adaptation may best explain variation in sensitivity to light. Another intriguing aspect of these results in that *S. russelli* did not show a respiratory response though they did respond behaviorally and Garden Ridge *S.* sp. did show a respiratory response despite not responding behaviorally.

Taxon	Locality	N	R^2 (dark)	R^2 (light)	Slope (dark)	Slope (light)
S. flagellatus	San Marcos	5	0.42	0.58	0.08	0.076
S. bifurcatus	Big Boiling, San Marcos	3	0.69	0.69	0.069	0.069
S. russelli	San Marcos, Sessom	10	0.74	0.82	0.074	0.068
Stygobromus sp.	Garden Ridge	3	0.37	0.85	0.03	0.086
S. pecki	Comal Springs	7	0.44	0.93	0.036	0.07

4.2.2. Further study of taxa shown to respond to light
4.2.2.1. Study of response to varying wavelengths

Behavioral

Table 9. Wave length was found to significantly predict the magnitude of behavioral response of *S. pecki* to light.

| SOV	Df	Sum Sq	Mean Sq	F	p
Wavelength	1	0.235	0.235	25.38	<0.001
Residuals	13	0.12	0.009		
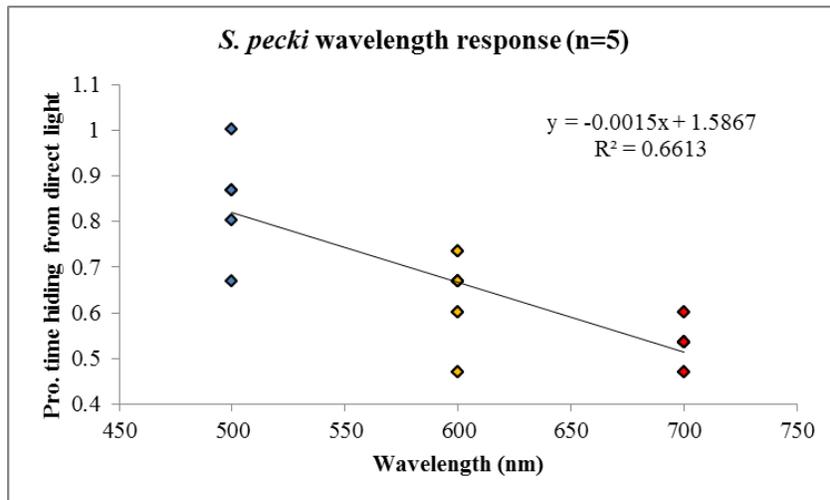
Figure 4. The magnitude of behavioral response of *S. pecki* to light was inversely related to wavelength.

Stress
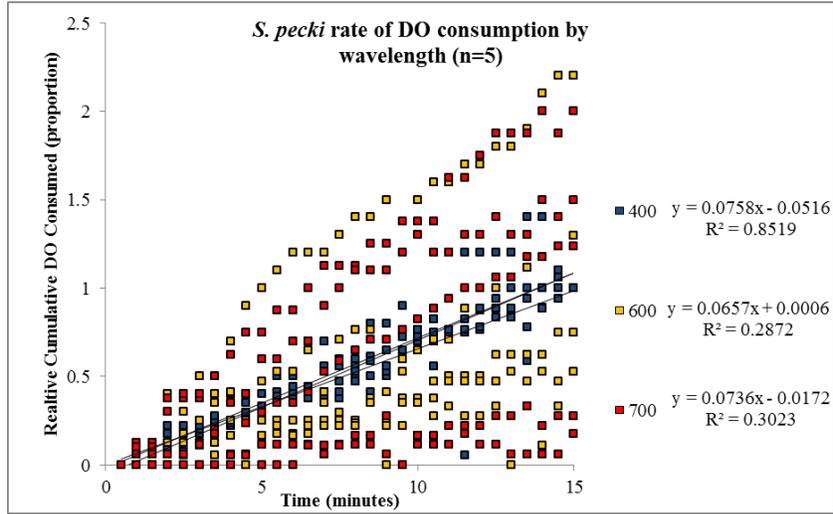
Figure 5. O² consumption of *S. pecki* was only found to be predictable when exposed to blue light (≈ 400 nm) despite all three treatments having similar slopes.

4.2.2.2.   Study of varying intensities of full spectrum

Behavioral

Table 10. The magnitude of behavioral response of *S. pecki* to full spectrum light was found to be positively related to intensity.

SOV	Df	Sum Sq	Mean Sq	F value	P
Intensity	1	0.148	0.148	7.8	0.012
Residuals	18	0.341	0.0189		
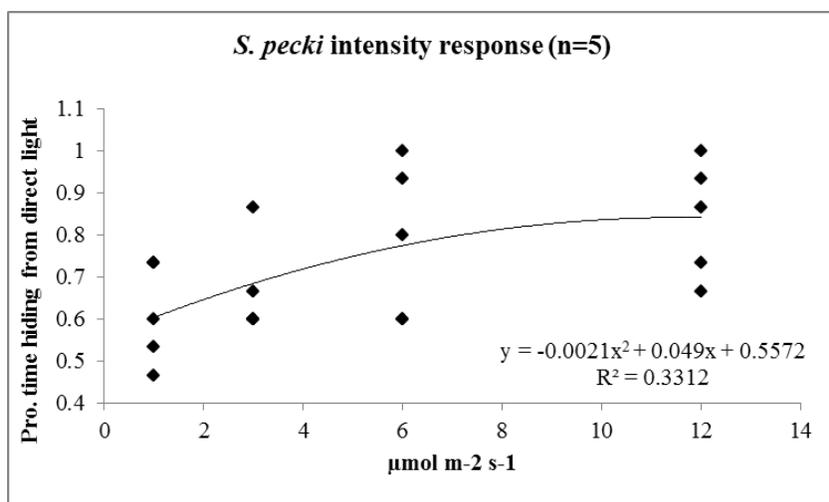
Figure 6. Despite an appreciable amount of variation between individuals, time spent avoiding light was positively related to the intensity of light.

Stress
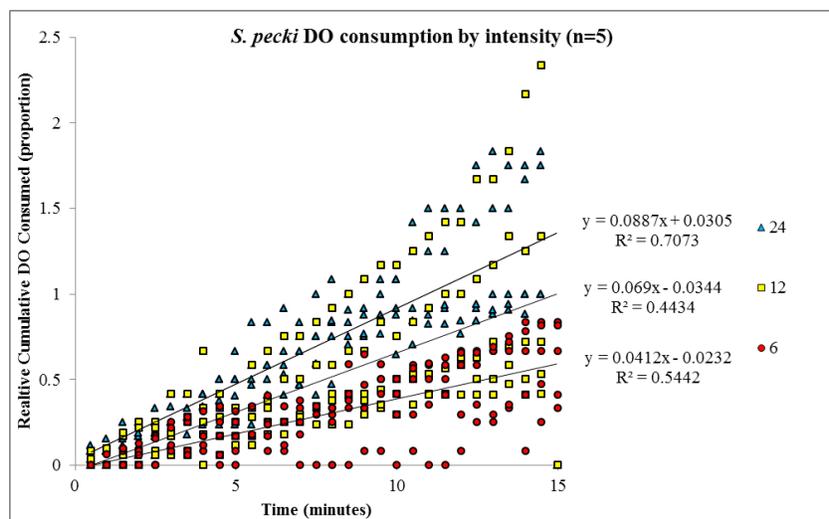
Figure 7. Once again, the greatest observable difference between treatments was differences in explainable variation which was positively related to the intensity of light. Only the highest intensities tested had an acceptable amount of explainable variation (R^2=0.71). Intensities tested were 6, 12 and 24 µmol m² s⁻¹.

4.2.3.   Discussion

Darkness is one of the primary environmental factors that affect adaptations of subterranean species, leading to the reduction or disappearance of eyes, loss of pigmentation, and increased sensitivity of non-visual senses that presumably enhance fitness (Aden 2005). The reduction or loss of eyes in most troglomorphic organisms has been attributed to a fitness gain associated with

elimination of these structures (Romero and Green, 2005). Eyes are presumed to be functionally useless in the dark and therefore the relatively high energetic cost of developing and maintaining eyes affords no advantages that could offset the cost of investment and justify maintaining eyes. However, selection also appears to discriminate against non-synonymous nucleotide substitutions in the rhodopsin molecule (a presumably "useless" gene if sight is not important) in cave invertebrates as much as it does against surface invertebrates (Crandall and Hillis 1997). Furthermore, subterranean organisms that are presumed to be blind have been shown to be able to detect light and prefer to avoid exposure to direct light (Schlagel and Breder, 1947; Borowsky, 2011; Friedrich *et al.*, 2011, and this study). These findings are curious and difficult to explain, but it is possible that selection to remain in a cave environment outweighs the fitness disadvantages associated with maintaining light sensitive organs. If this is the case, it is no doubt because it is almost certainly disadvantageous for a sightless organism to find itself in an epigean environment.

Previous studies have shown that increased rates of oxygen consumption are indicative of environmental stress in an organism (Kedwards *et al.*, 1996; Simcic and Brancelj, 2007). The stress induced by being exposed to light was demonstrated by Simcic and Brancelj (2007), who found that *Niphargus stygius* had up to a 130% increase in O_2 consumption rate when exposed to light; suggesting that avoiding light is most advantageous despite the metabolic cost involved because exposure at the surface likely represents an evolutionary dead end as finding a mate on the surface is extremely improbable and falling victim to predation is more probable.

In our study, three of the five species of *Stygobromus* responded to light, but there is no evidence from any study at present that would indicate the presence of photoreceptor nerve cells in the body of any *Stygobromus* species. Even though three of the species tested responded to light, only *S. pecki* had detectable responses in terms of behavior and DO consumption. *S. russelli* only responded behaviorally, and the Garden Ridge *Stygobromus* sp. only responded in DO consumption study. With other subterranean taxa responding to and avoiding light (e.g., Simcic and Brancelj, 2007; Borowsky, 2011), it is not surprising that many of the taxa in this study did, as well. Prior to this study it was not known if the species of concern, *S. pecki,* was sensitive to light or preferred to avoid direct exposure to light. Our findings indicate that *S. pecki* is the most sensitive of the *Stygobromus* species we examined. This is not surprising given the spring associated nature of *S. pecki*. It is difficult to explain why *S. russelli* and Garden Ridge *Stygobromus* sp. responded behaviorally to light, but not in terms of increased DO consumption. However, differences in niche specialization and common ancestry may be able to explain this after further research.

S. pecki response (avoidance of light) was shown in this study to be positively related to the intensity of full spectrum light, and inversely related to wavelength of light at a fixed intensity. Our results show that both light intensity and wavelength significantly predicted the magnitude of the response of *S. pecki* to light. However, wavelength was a better predictor, explaining more variation in the response variable in both the behavioral and stress response studies. This result is not surprising, as water is known to filter out longer wave length light first, therefore only allowing shorter wavelength light to reach an appreciable depth in water. However, there was no wavelength or intensity of light that *S. pecki* did not respond to. Because of these findings we recommend that the best culture technique for *S. pecki* in refugia would be in the

complete absence of light in order to reduce inducing increased stress. However, this condition simply is not continuously feasible if refuge workers are to have interaction with culture stock. Therefore, in order to ensure that organisms are not unnecessarily stressed while still allowing workers to maintain cultures, it may be possible to use illumination with only long wavelength portions of the visible light spectrum at low intensities. Cumulatively, these results lead us to conclude that the best measure would be to use only red light and at very low intensities.

4.3. Amphipod reproduction

Across the two-month study duration, no noticeable development was noticed in the ovaries of any of the females. In fact, the only change observed was the release of young from the marsupium of one of the females that was gravid at the beginning of the study. This showed that the duration of the experiment was not long enough to observe any measurab le development through the reproductive cycle. However, insight into the potential pairing combinations was possible given the disequilibrium of cannibalism by the two sexes (Table 11). This data suggests that males and females should be of roughly equal size to insure that neither are cannibalized and that females should never be larger than males in any of the pairings. Table 11.) Females accounted for a disproportionately greater amount of observed cannibalism, especially when females were larger. However, no instance of cannibalism was observed by the smaller of the two in any of the pairings.

Treatment	n	Proportion of males cannibalized	Proportion of females cannibalized
male larger	2	0	0.5
female larger	3	0.667	0
same	4	0.25	0
total	10	0.333	0.111

4.4. Amphipod holding and culture

Survivorship across the three treatment groups (Table 12) was found to vary significantly between treatments [$F_{2,\,6}$=16.75, p=0.0035, df=2]. However, all that variation could be accounted for in the significantly poorer survivorship of the 'individual' treatment group (Table 13).

Table 12.) Survivorship per replicate of each treatment across three months.

Treatment	Replicate	Surviving proportion (month 1)	Surviving proportion (month 2)	Surviving proportion (month 3)
Group	1	0.8	0.8	0.8
Group	2	1	1	1
Group	3	1	0.8	0.8
Static	1	1	1	1
Static	2	1	1	1
Static	3	1	1	1
Individual	1	0.6	0.4	0.4
Individual	2	0.6	0.2	0.2
Individual	3	0.6	0.6	0.6

Table 13.) Tukey's HSD indicated that survivorship did not vary significantly between the 'static' and 'group' treatments but did vary significantly for all other comparisons.

Comparison	diff	lwr	upr	p
Indi-Group	-0.467	-0.801	-0.133	0.012
Static-Indi	0.600	0.266	0.934	0.004
Static-Group	0.133	-0.201	0.467	0.483

Net growth did not vary significantly between treatment groups regardless of survivorship [$F_{2,}$)= 0.083, p=0.922]

Figure 8).
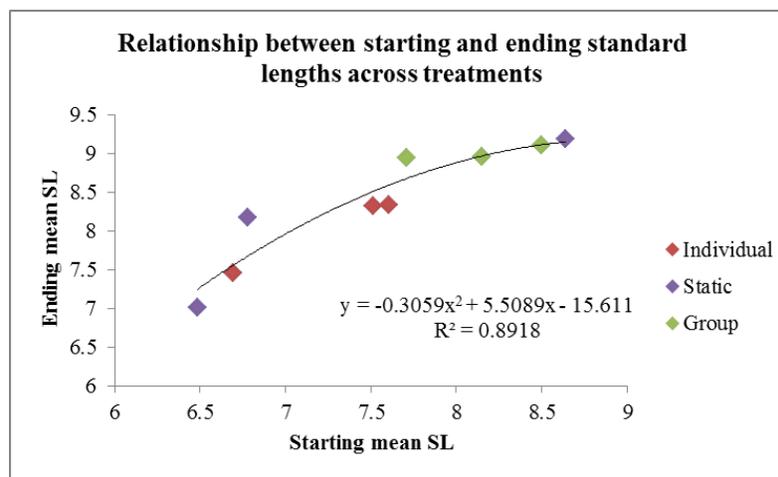
Figure 8. The ending standard length (mm) was predicted by starting standard length with a similar relationship between all replicates of all treatment groups suggesting that treatment had no effect on net growth.

4.4.1.   Discussion

Results from this study suggest that the static array and group holding systems do not differ in survivorship. Although this is well supported by our data, we did not use neonates in this study. Observations made prior to this study suggested that *S. pecki* only cannibalize appreciably smaller individuals. The smallest individual used in one of the group holding treatment groups was 4.28 mm in length, which is more than twice as large as freshly hatched *S. pecki* neonates in length. Therefore, it is believed that if neonates had been incorporated in this study (which were not available at the time of this study) that the static array holding system would have had appreciably higher survivorship.

The individual flow through systems had the lowest survivorship of all treatments. The reason for this is because of the mechanics of the flow through system used at SMARC. The water supply used for all aspects of this study is prone to getting air bubbles in the lines. In the group and static array holding systems, any air fed into these systems through water lines escaped at the surface of the water. However, in the individual flow through chambers, none of the chambers were open to the surface and water that flowed in had to flow out through the mesh side panels. Because mesh size had to be small enough to prevent the escape of organisms, it also prevented the escape of air bubbles due to surface tension, thus gradually filling the individual holding chambers with air. Though effort was taken to routinely check chambers and remove any accumulated air, it was difficult to stay ahead of the rate at which chambers filled with air, thus making this holding system too tedious to be feasible for holding large numbers of organisms. However, flow-through systems could be used if they were re-designed to eliminate the issues of gas bubble accumulation that were encountered in our experimental system.

Therefore, based on prior observations and findings presented here, the recommendations for culturing are to use static array systems for monitored growth and development of neonates and to protect them from cannibalism, and then use group systems for larger organisms to allow them to encounter each other and mate. This would require frequently checking group systems for brooding females and moving any brooding females to isolation. Isolation systems currently used at SMARC for brooding females have proven to be very successful in separating mother from freshly hatched neonates. This system has two chambers stacked on top of each other separated with mesh large enough for young to pass through but too small for adults to pass through. The brooding female is placed in the upper chamber and when she releases neonates from here marsupium they are able to escape through the mesh where she cannot follow. With periodic checks of this system, neonates are able to be removed unharmed and placed in a static array system where they can be grown out to a large enough size to be introduced into group holding systems. Recommendations for use of this proposed method are based on present research and therefore future research is necessary to test the efficacy of the implementation of this proposed methodology.

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