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

PROPOSAL #138-14-HCP

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Background and Significance

The Edwards Aquifer Habitat Conservation Plan (HCP) calls for the establishment of refugia (captive 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 this HCP, captive rearing, life history, and environmental requirement 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. Although the San Marcos Aquatic Resources Center (SMARC) maintains 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 substantially large 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 are being proposed. The anesthetization of test organisms is crucial to be able to determine their gender, developmental stage, length, weight, and to mark or photograph. The organism's response to light could affect its stress level and health in captivity. 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 stage also must be determined.

This document provides Tasks 1 and 2 under the Scope of Work for the Refugium Project. Task 1 provides a review of the literature related to several of the important methods and questions associated with maintaining captive breeding populations and Task 2 is an outline of the proposed methodology related to the research that will be conducted. Specifically, we will address four main research topics in this project: (1) methods for anesthesia for invertebrates held in captivity, (2) responses of epigean and/or hypogean invertebrates held in captivity, (3) mating behaviors of amphipods (specifically the Peck's cave amphipod) held in captivity, and (4) methods for holding Peck's cave amphipods in captivity.

Literature Review

1. Review of anesthesia methods for aquatic Coleoptera and other Crustacea

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 & Mosley, 2012). Amphipods and rifle 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 (Graham, 1990). However, adult rifle beetles respire through a plastron which means aqueous solutes may not be an effective method for delivery of anesthetic to adult rifle 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 would likely be a more effective method. Because water breathing organisms tend to have a larger exchange surface with water than air breathing organisms have with air (Graham, 1990), it is likely that air breathing organism will also require a higher dose of anesthetic because of smaller absorptive surfaces and therefore less chemical exchange with the environment when compared to water breathing organisms.

Specific information on Coleoptera

Very little work has been done on the anesthesia of 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 & 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 both adult and larval respiratory mechanisms; however, CO_2 has been shown to cause permanent changes in behavior of anesthetized coleopterans (Lizé et al., 2010) and effect survivability and fertility of other insects (Barron, 2000; Champion de Crespigny, F. E. & Wedell, 2008). Another method that has been used to anesthetize insects is cold induced anesthesia, but this has also been shown to affect survivability (Barron, 2000; Champion de Crespigny & Wedell, 2008) and is likely inappropriate for spring/aquifer adapted organisms that experience little to no thermal fluctuations *in situ*. Volatile chemicals 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).

Specific information on Amphipoda
Both soluble chemical and gaseous chemicals bubbled into solution have been used as anesthetics for aquatic crustaceans. The efficacy and survival rate does seem to vary appreciably between these two general 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. 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 much higher than what would be considered acceptable for refugium purposes. In contrast, Venarsky & Wilhelm (2006) found that that there was a positive relationship between CO_2 dose and mortality within different size classes *Gammarus minus*. Cumulatively, these studies suggest that mortality associated with CO_2 anesthesia may be avoided at proper dosages. Clove oil is another commonly used anesthetic, but the duration exposed 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 & 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 & 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 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 & Mosley, 2012), and if used properly can have low mortality rates (Ahmad, 1969). Like clove oil, concentration of dose 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 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 anesthetic which have been used on crustaceans are dilute chlorobutanol and ethanol (EtOH) thought these have not been tested on amphipods nor have its 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 anaesthetize hypogean amphipods and isopods from the Edwards Aquifer, but have not conducted a systematic study to determine tolerance limits and dosages.

Concluding recommendations for anesthesia methods
Based upon the literature review, we recommend that CO_2 only be tested as an anesthetic on riffle beetles and not amphipods, due to the high mortality and long-lasting effects observed in previous experiments. If CO_2 is tested as an anesthetic on riffle beetles, larvae and adults (preferably siblings in both cases) should be reared with and without anesthetic under otherwise identical conditions to determine if this anesthetic has a long term effect on fecundity. MS-222 shows the most promise as a crustacean anesthetic and if tested should also have the long term effect on fecundity studied. Finally, we recommend that there be a series of experiments with EtOH due to preliminary observations and the overall low cost and availability of EtOH.

2. Response to light 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, hypogean organisms may have preferences or tolerances with regard to light levels and the types of light that are present. Though few studies have addressed light response in stygobitic invertebrates, a multitude of methods have been used that quantify responses of organisms to light. In general, these responses are quantified based on the relative amount of movement in light *vs* dark areas, degree of avoidance of illuminated areas, and rate of respiration in light vs dark conditions. In most cases, it was determined that stygobitic/troglodytic organisms can detect light and put significant effort into avoiding light (R. Gibson, *unpubl manuscript*). In agreement with this, it appears there is selection for the ability to detect light in cave invertebrates as non-synonymous nucleotide substitutions in the rhodopsin molecule are discriminated against equally in cave and surface environments (Crandall & Hillis, 1997). Taking into consideration that most cave organisms tested to date avoid light, it is likely that exposure to light may be stressful and disadvantageous to culturing organisms.

Specific information on Coleoptera
To our knowledge, there have been no published studies examining the light responses of subterranean aquatic beetles. However, it has been demonstrated that terrestrial troglodytic beetles previously thought to be blind can detect light and, not surprisingly, avoid light (Friedrich et al., 2011). However, the beetles in their study have reduced eyes rather than absent eyes, therefore it is not surprising that they would be able to detect some light. Despite these differences, the experimental design used by Friedrich et al. (2011) was effective and simple: beetles were placed in a dish with shaded and unshaded halves and were allowed to move about freely. Beetles were then illuminated at a perpendicular angle from above and the authors noted where the beetles came to rest and it was found that all three species of troglobitic beetles examined in the study tended to avoid the unshaded (illuminated) area. More recently, Cooke (in press) examined the preferences of the Comal Springs riffle beetle for differently illuminated environments and found that beetles preferred dark (non-illuminated) over lighted areas.

Specific information on Amphipoda
Information on the sensitivity to light of the stygobitic amphipods of the Edwards Aquifer has yet to be published (but manuscript in preparation by Gibson et al.). However, the amphipod *Niphargus* is adapted to subterranean groundwater environments, is eyeless (and presumably blind), and has had some aspects of their sensitivity to light studied including phototaxis. Borowsky (2011) studied dorsal light reflex [a response of some organisms whereby they orient their dorsad surface towards the origin of light (Foxon, 1939)] in *Niphargus* spp. and found no evidence that they have this reflex. Borowsky (2011) also examined the relative amount of moment in illuminated and dark conditions and if *Niphargus* would move into areas protected from direct light as well as if the intensity of the effect was proportionate to the intensity of the light treatment. In summary, it seems that *Niphargus* spp. have the ability to detect light and the response to light is proportionate to intensity, and that individuals behaviorally avoid direct light and became more active when exposed to light (Borowsky, 2011).

Simcic & Brancelj (2007) examined 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 their oxygen consumption (respiration) rates. It was found that *N. stygius* had a significantly greater rate of oxygen consumption when exposed to light than it did in the dark and that the rate of oxygen consumption was proportionate to the intensity of light exposed while light vs. dark or the intensity of light was showed no effect on *G. fossarum* (Simcic & Brancelj, 2007). This suggests that exposure to light is likely to be more stressful to hypogean amphipods and causes and increase in respiration and metabolism. Therefore, it is likely that more successful culture of stygobitic amphipods in the refugium may require that organisms rarely if ever be exposed to light.

Concluding recommendations for testing light responses
It has been shown that subterranean organisms that are presumed to be blind can indeed detect light and avoid being exposed to light (Borowsky, 2011; Friedrich et al., 2011). Avoiding being exposed to light is no doubt advantageous to subterranean-adapted fauna as being sightless in an epigean environment is almost certainly disadvantageous. The extremeness with which sightless subterranean organisms avoid being exposed to light was further demonstrated by Simcic & Brancelj (2007), by demonstrating that *Niphargus stygius* had up to a 130% increase in rate of O_2 consumption when exposed to light, suggesting that being exposed to light is highly stressful for this species and the cost of avoiding light is more advantageous than passively accepting it. The most likely explanation of this phenomenon is that once a subterranean-adapted organism is exposed at the surface, it almost certainly faces an evolutionary dead end as finding a mate on the surface is extremely improbable and the falling victim to predation is more probable. Therefore, we preliminarily recommend that the best culture technique for hypogean species 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. 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 portions of the photo-spectrum that are undetectable by hypogean organisms. This solution is certainly within the realm of possibility as it has been shown that blind cave crayfish do not show observable differences in behavior when exposed to infrared light but do when exposed to white light (Li & Cooper, 2002). Therefore, teasing apart what wave lengths hypogean refugium invertebrates are sensitive to would be very useful for illuminating research areas without unnecessarily distressing culture organisms. It would also be advantageous to determine if there is a minimal threshold of light intensity that is perceivable or stressful as it has

been demonstrated that light intensity is proportionate to stress, at least in some hypogean species (Simcic & Brancelj, 2007).

3. Mating behavior of amphipods

Currently, there is no information on the mating behavior for any stygobitic amphipod species in the world. However, there is a plethora of information on mate selection and timing of reproduction in epigean amphipods as well as some information on reproductive cycle of wild caught stygobitic amphipods. Due to the nature of being an aquatic species with an exoskeleton, female amphipods are only receptive to mating post-molt because only then is the cuticle flexible enough to allow the release of eggs through the genital pores into the marsupium (Bollache & Cezilly, 2004). Because females are only momentarily receptive to mating, males typically guard a female prior to her molting to insure that he fathers the offspring. Molt cycles in males also appears to have an effect as males tend to not be willing to enter into amplexus as they approach molting because they will inevitably have to release the female upon molting if the pair has not copulated yet (Bollache & Cezilly, 2004). Mate guarding may come at a cost to males and can hinder their ability to forage and thus limit their growth (Robinson & Doyle, 1985). In response to this energetic cost, males tend to not enter into amplexus unless they have sufficient amounts of stored glycogen and fats (Plaistow et al., 2003); therefore, it is likely that proper nourishment is necessary to offset the fitness costs of copulation. 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 for 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 & 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 (Bollache & 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, indicating that males tend to be larger than females if the pair is to hope to avoid predation (Adams & Greenwood, 1983). Selection for larger males is also compounded by female resistance to amplexus (Jormalainen & Merilaita, 1995). The ultimate consequence of these size-specific interactions for individuals attempting to breed amphipods in a refuge setting is that females must have a large enough male suitor if mating is to be successful.

Concluding recommendations for testing light responses
At this point, very little is known of the mechanics and timing of reproduction of *S. pecki*. Therefore, it is critical to gain information about the process of mating, the extent of mate guarding and amplexus, and the rates of post copulation cannibalism in the species. We recommend preliminary observations on describing mating behaviors before any additional and more manipulative studies (e.g., examining size-selection influences on mating) are conducted.

4. Culturing techniques previously published for amphipods

To protect against loss of individuals held in the refugium, it is important that the range of conditions that are survivable by culture taxa is understood to insure that more extreme conditions are avoided. Nested within this survivable range should be the ideal range of conditions that taxa thrive in. Edwards Aquifer biota live in environments with little environmental variation, therefore, it is expected that all organisms will perform best under relatively stable conditions that mimic the

physiochemistry of the Edwards Aquifer. This suggests that all Edwards Aquifer organisms, regardless of taxonomy, have similar environmental requirements. However, this also implies that all Edwards Aquifer organisms have been afforded equal opportunities to become adapted to their environment. Therefore, even very closely related groundwater fauna can be physiologically quite different, as with some *Nipharus* species (Issartela et al., 2005).

Metabolic rate of stygobionts appears to be low when compared to epigean species (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 minimal 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 hypogean amphipods in the 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). The survival rate of of *Gammarus pseudolimnaeus* (a 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 range limits of this species. Edward Aquifer water is quite calcium rich, therefore having sufficient calcium for molting amphipods should likely not 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.

Like many aspects of the ecology and of subterranean amphipods, information on aspects of their life history is not abundant in the literature. In general, it appears that subterranean amphipods 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 a year to mature (i.e., univoltine) (Venarsky et al., 2007; Crawford & Tarter, 1979). However, *Niphargus aquilex aquilex* has been shown to have the capacity to produce up to two generations per year (Gledhill et al., 1969). This suggests that surface species may not be suitable surrogates for trying to study developmental rate but may be quite useful for studying developmental events at an accelerated rate.

The sex ratio in *Crangonyx forbesi*, a subterranean amphipod was shown to fluctuate on an annual cycle (Crawford & Tarter, 1979). During winter months (the breeding season for *C. forbesi*), males outnumbered females, while during the summer no males were observed (Crawford & 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 & Tarter, 1979; Bollache & Cezilly, 2004). Sex ratios may also become distorted due to the mechanism of sex determination in amphipods. Amphipods do not have all sex determining alleles located on discrete sex chromosomes; rather sex determining alleles are distributed across many chromosomes, thus sex is inherited much like a quantitative trait and t 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 with covariate sex as well (Sutcliffe, 1992; Watt & Adams, 1993; McCabe & Dunn, 1997) suggesting that sex determination in amphipods may behave like a developmentally plastic and quantitative trait. Infection with microsporidians (Bulnheim & 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. Therefore, it will be 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 culture lineages.

Lastly, determining how to house individuals in order to track individual development and increase survival is critical. *S. 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.

Proposed Methods

1. Anesthesia methods for aquatic Coleoptera and amphipods

Test subjects and use of surrogates

Initial testing will utilize surrogates to avoid unnecessary mortalities of the listed species. When preliminary trials are completed with surrogates, listed species will then be tested at smaller numbers to refine understanding of proper dose of anesthetic. Fortunately, there are abundant locally distributed species in the same family as *S. pecki* (Crangonyctidae), both epigean and subterranean. The most abundant subterranean species is the congener *Stygobromus flagellates* and *Crangonyx pseudogracilis* is an abundant epigean species. Both of these species will be used as surrogates for anesthesia testing before any *S. pecki* are tested. *S. flagellatus* will be collected from the Texas State University artesian well and drift nets on San Marcos. A population of *C. pseudogracilis* will be reared in culture with stock taken from the San Marcos River to insure readily available abundance of crangonyctid amphipods for anesthesia trials.

The surrogate for the Comal Springs riffle beetle (*Heterelmis comalensis*) will be either *Heterelmis vulnerata*, *Heterelmis glabra*, or both. *H. glabra* would likely be a superior surrogate, being closer in life history characteristics to *H. comalensis* than *H. vulnerata*. *H. vulnerata* can be found in the Guadalupe River drainages, and *H. glabra* can be found in springs along the upper Devils River.

Procedure

The anesthetics proposed for testing are MS-222, clove oil, and EtOH. Though many studies have used CO_2, cold treatment, and ether as anesthetics, these methods will not be used for the following reasons: CO_2 has been shown to have immediate and long term effects on fecundity and survivability of anesthetized organisms (Barron, 2000; Champion de Crespigny & Wedell, 2008; Cothran, 2008; Lizé et al., 2010); cold treatment is likely inappropriate for organism adapted to thermally stable environments; and ether is a volatile chemical which could present and unnecessary hazard for refugium workers.

Anesthetics will be administered to organisms individually. For these tests, a single invertebrate test subject will be placed in a closed chamber filled with artesian water maintained at 21 - 23°C. After organisms have come to rest in the test chamber, the anesthetic will be introduced. It is completely unknown what the appropriate dosage for each anesthetic should be and it is almost certain that each species will differ in their sensitivity to anesthetics, therefore, it is difficult to speculate what doses should be administered. However, the goal is to find the minimum effective dosage first with surrogates in order to avoid unnecessary mortality. Therefore, treatment dose will likely be adjusted as information becomes available through experimentation until the proper dose is determined for each species. Because it is almost certain that the size of the organism is related to their sensitivity to anesthetic, each organism tested will have their wet mass measured prior to testing. Organisms will likely be hypersensitive to anesthetics immediately post molting therefore care should be taken to insure that organisms that have recently molted are not used. Response to anesthesia will be quantified by estimating the duration after exposure the organism became anesthetized (not moving and not responsive; loss of response = LOR), the duration to recovery after removing test organisms from test chambers, and the long-term survival rate within treatment groups.

After completing anesthesia experiments it should be possible to calculate the appropriate dosage for all anesthetized organisms for all subsequent refugium work if the tolerances by taxon, age or developmental stage, size of the organism being anesthetized, efficacy by duration, and a

standardized dose can be parameterized for each anesthetic. All of this information should be compiled in reference tables for each factor and used by refugium workers to insure that organisms are properly dosed with the correct anesthetic.

2. Response to light stimuli by subterranean and subsurface invertebrates

Numerous methods have been used to test sensitivity to light in cave adapted animals (see Literature Review above). Behavioral responses are typically used as response variables to estimate sensitivity to light, but noting the occurrence of a behavior can provide insight into the quality of response to light yet fail to effectively quantify the relative degree of sensitivity. The only method used to date which produced a continuous quantitative response variable as an estimate of sensitivity to light is observation on the O_2 consumption (respiratory) rate of organisms (Simcic & Brancelj, 2007). Therefore, both the quality of response to light and O_2 consumption will be used as response variables to estimate sensitivity to light. It is unlikely that momentary exposure to light could be lethal; therefore, surrogate testing should not be necessary. We will examine sensitivity to both full-spectrum and red light.

To test the quality of response to light, test subjects will be placed in petri dishes with two quarters of its upper surface covered in black paint (
Figure 1). Test subjects will once again be exposed to both red and full-spectrum light with a perpendicular angle of incidence, while control groups will remain in the dark. Both test and control groups will be filmed over an hour period. Footage will then be analyzed to determine if invertebrates avoided direct light and if so how quickly this occurred.
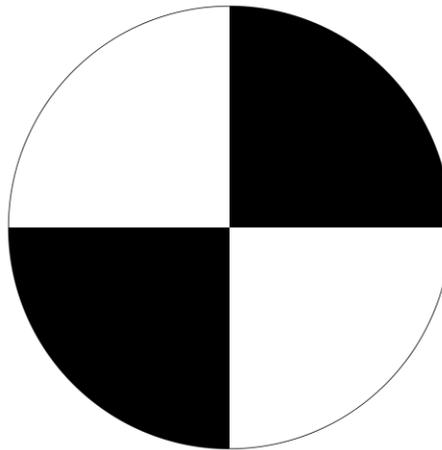

Figure 1. Petri dish with two quarters of its upper surface covered in black paint.

In addition to the aforementioned experiments, light response experiments will be performed by placing a single test subject in a sealed container with a built-in optical DO probe filled with artesian water; one test subject per chamber (4-channel Qbit System). Controls will be kept in the dark while treatment groups will be exposed to red or full-spectrum light at a perpendicular angle of incidence. In order to control for the effect that instrumentation may have on DO, a test chamber in both treatment groups and in the control group will only be filled with artesian water and receive no test

subjects. After the elapsed duration of exposure to light (~1 h), the rate of DO depletion will be determined by plotting DO concentration as a function of time (min). After experiments, wet mass of each individual will be estimated in order to correct for the effect of size on DO consumption.

3. Methods for culturing and holding Peck's Cave amphipods

The current method employed at the SMARC to house and their cultures of *Stygobromus* spp. is a "group holding system". These systems utilize plastic containers supplied with a constant supply of Edwards well water through a simulated upwelling area 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 is provided to increase three dimensional surface areas for the amphipods to utilize and to limit contact between individuals. Typically large numbers 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 also impossible to track the growth and development of individual amphipods. Therefore, there is a need to examine two other types of holding systems is being proposed which will allow for holding amphipods individually. The first of these systems is the use of individual "flow chambers". Chambers are built from PVC pipe or vinyl tubing, with water lines attached to the chamber creating a flow through the chamber. These flow chambers are designed to house amphipods individually, which prevents cannibalism and allows for the growth and development of individual amphipods to be possible.

The second of these proposed systems is a "suspended static array". The array consists of multiple mesh chambers arranged into a suspended/floating array within a living stream system supplied with flow-through artesian water. In this system, artesian water is supplied to just the living stream the mesh containers are submerged in and is not pumped through the individual chambers as above. In the static array system, amphipods are again housed individually, preventing cannibalism and allowing for the growth and development of individual amphipods to be possible.

To compare the efficacy of these systems, an equal number of amphipods will be placed into each replicate of each system. All study amphipods will be fed commercially available fish flakes *ad libitum*. The relative rate of survival (% survival) will be compared within and between types of systems to determine which type is the most effective at preventing cannibalism. It is not known if prevalence of cannibalism varies by species, therefore it may be difficult to draw reliable conclusions about the relative efficacy of the different holding systems using surrogates.

4. Peck's Cave amphipod mating behavior methods

Currently, it is not known how long pairs of *S. pecki* will enter into amplexus for before eventually copulating. In order to study the nuances of *S. pecki* mating, pairs will be placed in small transparent chambers supplied with flow through artesian water. To establish mating experiments, amphipods will have their sex determined, length measured, and females will be checked for visibly gravid ovaries (using microscopy). Only females with gravid ovaries will be used because these females are ready to copulate following their next molt. One male and one female will be distributed to a treatment replicate. The three treatment types will be: male larger, male and female relatively equal in size, and female larger. The ratio of the male and female's lengths will be calculated for each treatment replicate with the hypothesis that replicates with larger males will have greater success unless the male is too large and cannibalizes the female. Pairs will be continuously recorded to

observe behavior. Footage will be analyzed to determine if the size ratio between males and females affects that rate of successful copulation, how long pairs enter into amplexus for prior to mating, if cannibalism occurs within pairs. All amphipod pairs will be fed commercially available fish flakes *ad libitum*. Behavioral data collected from these recordings will then be used to guide breeding protocols for the future of the refuge program.

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