Honors Capstone: Exploring the Basic Biology of the Freshwater Amphipod *Stygobromus tenuis potomacus*

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ABSTRACT

This study employed the Schnabel and Schumacher-Eschmeyer (S-E) methods to estimate the population size of the freshwater amphipod *Stygobromus tenuis potomacus* at each of two seepages springs (seeps B and C) draining hypotelminorheic habitats near Washington, DC. Data was collected with a multiple mark-multiple recapture design, over two sample periods of different sampling intervals. The first period involved weekly sampling for eight weeks. The second period involved daily sampling for four days. Data was also collected on the temperature of the seeps and other physical aspects. Ovigerous females were kept in captivity and the head length of each was noted, along with the size of her brood. For seep B, the Schnabel analysis of the weekly sampling data produced a population estimate of 9207, with a 95% confidence interval of 4545 to 45021 individuals. The same data produced an S-E estimate of 14533, with an undefined 95% confidence interval. The Schnabel population estimate obtained from the daily sampling data was 5498 individuals, with a 95% confidence interval of 3002 to 14728 individuals. The S-E estimate obtained from the same data was 6367 individuals, with an undefined 95% confidence interval. Low recapture rates made population estimates for seep C impossible. Brood size was found to be positively dependent on female head length, with an R^2 value of 0.26, significant at the 0.001 level. This study represents the first attempt to use mark-recapture methods to estimate *S. t. potomacus* population sizes, as well as the first in vivo analysis of fecundity.

INTRODUCTION

Stygobromus tenuis potomacus Holsinger, 1967 is a freshwater amphipod crustacean common to hypotelminorheic aquatic habitats in the Washington, DC metropolitan area. Its range extends north to Pennsylvania and south through Virginia (Culver and Pipan 2011; Culver *et al.* 2006; Holsinger 1978). The hypotelminorheic is a surface layer of loose rocks and hummus that sits atop a layer of clay. The clay prevents rainwater from penetrating vertically down to the local water table, forcing it instead to percolate horizontally through the hypotelminorheic layer. Seepage springs, or seeps, often form at low spots in the hypotelminorheic, where water wells out from underground. A wide diversity of subterranean aquatic invertebrate fauna can be collected at these seeps (Culver and Pipan, 2011; Culver *et al.* 2006). This paper presents the results of a population size estimate of *S. t. potomacus* living in two hypotelminorheic seeps, near the Potomac River outside of Washington, DC. Population estimates were obtained using a multiple mark-multiple recapture design and both Schnabel and Schumacher-Eschmeyer (S-E) analytical methods. Also included are the results of a fecundity study correlating female head

length (as an indicator of overall body size) to brood size.

Like all members of its genus, *S. t. potomacus* exhibits troglomophy, lacking eyes and pigment and posses an attenuated body and appendages (Holsinger 1978, Pipan and Culver 2012). *Stygobromus* species occupy many subterranean aquatic habitat types, including hypotelminorheic seeps, epikast groundwater, cave streams, and phreatic reservoirs (Culver *et al.* 2006; Fong *et al*. 2012; Holsinger 1978). The genus shows wide diversity, and includes at least 93 species (Holsinger 1978). Beyond these few details, however, little is known about the basic biology of these organisms, including features of their population structure and fecundity (Fong *et al.* 2012).

The basic assumption of any mark-recapture design is that all individuals within the population stand an equal chance of being captured and recaptured. Thus, mark-recapture assumes that:

- there are no changes to the population size (by birth, death, or migration, i.e. the population is closed); and
- marks do not wear off or affect the chance of recapture by altering behavior or survival.

If these assumptions hold true, then the total population size can be derived from the overall number of individuals marked and the rate of recapture (Krebs 1999).

Because of both the wide diversity of and the lack of knowledge pertaining to the genus *Stygobromus*, it is important to conduct basic research to gain a better understanding of these organisms. More broadly, it is important to understand the biota of the hypotelminorheic, itself a poorly understood habitat. As the only endangered invertebrate in the District of Columbia, *S. hayi* illustrates the importance of understanding both the genus *Stygobromus* and the hypotelminorheic habitat where it lives (Culver *et al* 2006). If we are to protect this habitat and those species that dwell within it, we must first generate basic knowledge about the habitat and its biota.

METHODS

Study Site

We located two seeps containing populations of *S. t. potomacus*, along the Potomac River, above Pimmit Run, at Chain Bridge Road, in the Virginia suburbs of Washington, DC. Previous studies suggest that these seeps may also contain *S. pizzinii* and *S. sextarious*. Historically, though, these species are rare compared to *S. t. potomacus*. Furthermore, since it is nearly impossible to distinguish these three species without killing the individuals, we assumed that all *Stygobromus* individuals were *S. tenuis potomacus*. The northern seep was labeled B, while the southern seep was labeled C (seep A existed during previous surveys, but has since ceased flowing). Seeps B and C were 5.8 m apart, with the source of seep B 0.18 m above that of seep C. We sampled both seeps from their sources to a point 2.0 meters downstream. Seep B was 0.80 m wide, with a slope of 1.1%, while seep C was 0.30 m wide, with a slope of 1.4%. The total sampling area of seep B was 1.6 m^2, while that of seep C was 0.6 m^2.

Field Methods

We sampled the seeps weekly, for eight weeks, from January 30 to March 20, 2013. At the end of these eight weeks, we sampled daily for four days, from March 27 to March 30, 2013. Each sampling event lasted 60 person-minutes. We began sampling at each seep's

source, widening our search laterally and downward. To collect organisms, we gently pulled back the leaves and stones covering the water and used plastic spoons to scoop up any *Stygobromus* individuals and the surrounding substrate. We stored individuals in plastic bins containing water from Pimmit Run for transport back to the laboratory. We placed individuals from both seeps into the same bins, but kept a count of the number of individuals collected from each seep. All individuals collected were returned to the laboratory for processing and then released back to the seeps after a one week or one day interval. A VWR brand Traceable Thermometer recorded the air and water temperature at the source of each seep. Visual estimates of seep depth were also made. When the sampling event was completed, we returned the leaves and stones to the seeps, restoring the habitat. During the daily sampling interval period, four Hobo brand Tidbit Waterproof Temperature Data Loggers were used to record the water temperature both at each seep's source and at a point 2.0 m downstream. The data loggers were programmed to sample the temperature at five-minute intervals for the entire four-day period.

Laboratory Methods

In the laboratory, non-ovigerous individuals were counted and placed into a container of spring water with several decaying leaves and a handful of dolomite chips. We noticed that the specimen count in the laboratory was sometimes less than the count in the field, owing to counting errors made in the field. Before release back into the seeps, the non-ovigerous individuals were soaked in 100 mL of a 50 mg/L solution of neutral red dye for four hours, in order to establish a mark. This marking method was inspired by the methods outlined by Drolet and Barbeau (2006). Immersion produced a distinct red coloration (see Fig. 1) on the cuticles of the organisms. Observations of individuals retained in the lab showed that marked organisms remained distinct for several weeks beyond the scope the study. Furthermore, these organisms appeared healthy and to exhibit normal behavior, suggesting that the marking procedure was relatively noninvasive. The following week, the marked individuals were released back into the seeps, after that week's sample had been collected. The marked individuals were released in proportions that reflected the number withdrawn from each seep during the previous week.

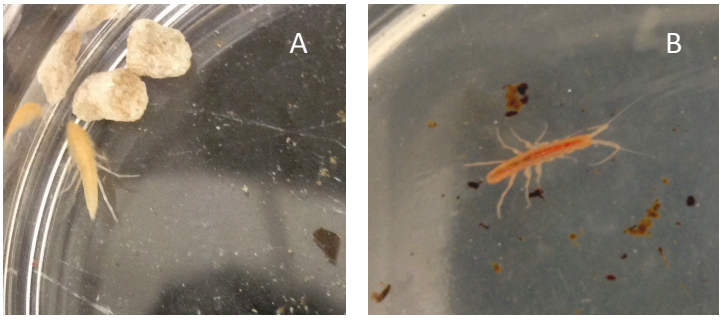

Fig. 1–A comparison of unmarked (A) and marked individuals (B)

We separated ovigerous females into individual 100 mm Petri dishes containing spring water, decaying leaves (as a food source), and several dolomite chips (as a mineral source), and stored them at 10 ^{o}C. We checked each female twice a week for new births. We also refreshed water and leaves during these checks. When young were found, the brood size and date of

discovery were recorded, along with the head length of the mother in ocular microscopy units (OMU). The young were moved into separate 60 mm Petri dishes (up to ten young in each). The relationship between head length and brood size was analyzed with a linear regression.

Data Analysis

We analyzed the data using both Schnabel and Schumacher-Eschmeyer (S-E) methods, as outlined by Krebs (1999). For the Schnabel method, the population estimate ($\hat{N}$) is given by the equation:

$$\hat{N} = \frac{\sum_{t=1}^{s}(C_t M)}{\sum_{t=1}^{s} R_t + 1}$$

Where:

- *s* is the number of sampling events;
- C_t is the number of individuals captured during the t^{th} sample event;
- M_t is the total number of marked individuals in the population during the t^{th} sampling event; and
- R_t is the number of recaptures collected during the t^{th} sample event.

The 95% confidence interval for $\hat{N}$, or $C.I.(\hat{N})$, is given by the equation:

$$C.I.(\hat{N}) = \frac{\sum_{t=1}^{s}(C_t M_t)}{C.I.\left(\sum_{t=1}^{s} R_t\right)}$$

Where $C.I.\left(\sum_{t=1}^{s} R_t\right)$ is the confidence interval for the total number of recaptures across all sampling events. Because of low recapture rates in this study ($\sum_{t=1}^{s} R_t < 50$), $C.I.\left(\sum_{t=1}^{s} R_t\right)$ was obtained from the Poisson distribution table (labeled Table 2.1) in Krebs (1999).

For the S-E method, $\hat{N}$ is given by the equation:

$$\hat{N} = \frac{\sum_{t=1}^{s} C_t M_t^2}{\sum_{t=1}^{s}(R_t M_t)}$$

The 95% confidence interval for this method is derived from the variance and standard error (*S.E.*) for $\hat{N}$, given by the respective equations:

$$Variance\left(\frac{1}{\hat{N}}\right) = \frac{\sum\left(R_t^2/C_t\right) - \frac{\sum\left(R_t M_t\right)^2}{\sum\left(C_t M_t\right)}}{s-2}$$

and

$$S.E.\left(\frac{1}{\hat{N}}\right) = \sqrt{\frac{Variance\left(\frac{1}{\hat{N}}\right)}{\sum\left(C_t M_t^2\right)}}$$

Finally, $C.I.\left(\hat{N}\right)$ is given by the equation:

$$C.I.\left(\hat{N}\right) = \frac{1}{\frac{1}{\hat{N}} \pm t_\alpha S.E.}$$

Where t_α is the Student's *t*-table value for (100-α)% confidence limits with *s* – 2 degrees of freedom.

RESULTS

Population Estimates

Table 1 summarizes the gross multiple-mark and multiple recapture data obtained over both the weekly and daily sampling interval periods for seep B. Table 2 summarizes the same data for seep C. The most successful sampling events at seep B yielded 47 individuals, occurring on both February 20 and March 20, 2013. The least successful yielded 11 individuals, on March 29, 2013. An average of 38 individuals were captured per sampling event during the weekly sampling period, with a standard deviation of 7.6 individuals. An average of 30 individuals were captured per sampling event during the daily sampling period, with a standard deviation of 16 individuals.

The most successful sampling events at seep C yielded 17 individuals, on February 6 and 20, 2013. The least successful yielded one individual on March 3, 2013. An average of 16 individuals were captured per sampling event during the weekly sampling period, with a standard deviation of 11 individuals. An average of 10 individuals were captured per sampling event during the daily sampling period, with a standard deviation of 6.9 individuals. Capture statistics quoted above for both streams include total captures (C_t; i.e. new captures, recaptures, and ovigerous females—see Table 6). Capture rates did not appear to vary with time or between sampling interval periods, at either seep. A t-test reveals that the difference in mean captures per seep was significant at the 0.001 level during the weekly sampling period, but not significant during the daily period.

Table 3 summarizes the population estimates produced by our analysis of the data collected at seep B. A total of 234 marked individuals were released into Seep B during the weekly sampling period, and just three were recaptured. This produced a Schnabel population

estimate of 9207, with a 95% confidence interval of 4545 to 45021 individuals. The S-E estimate for this same data was 14533, with an undefined 95% confidence interval of 5838 (lower limit) to -29687 (upper limit) individuals. During the daily sampling period, an additional 121 marked individuals were released into seep B, but just six were recaptured. The Schnabel estimate for the daily data was 5498 individuals, with a 95% confidence interval of 3002 to 14728 individuals. The daily S-E estimate was 6367 individuals. The 95% confidence interval for this data was not defined, with calculations producing a range of 1860 (lower limit) to -1953 (upper limit).

Table 1–Seep B multiple mark-multiple recapture data

Sampling Interval	Date	New Captures + Ovigerous Females	Recaptures	Released
Weekly	1/30/13	30 + 8	0	0
	2/6/13	27 + 1	0	30
	2/13/13	36 + 0	0	27
	2/20/13	47 + 1	1	36
	2/27/13	39 + 2	1	43
	3/5/13	34 + 2	0	39
	3/13/13	28 + 1	1	34
	3/20/13	47 + 1	0	25
Daily	3/27/13	45 + 3	1	47
	3/28/13	20 + 0	3	44
	3/29/13	11 + 0	2	18
	3/30/13	36 + 0	0	12

Table 2–Seep C multiple mark-multiple recapture data

Sampling Interval	Date	New Captures + Ovigerous Females	Recaptures	Released
Weekly	1/30/13	12 + 12	0	0
	2/6/13	17 + 8	0	12
	2/13/13	12 + 5	0	17
	2/20/13	17 + 16	0	12
	2/27/13	6 + 4	0	17
	3/5/13	3 + 1	0	6
	3/13/13	1 + 0	0	3
	3/20/13	8 + 7	0	1
Daily	3/27/13	16 + 4	0	8
	3/28/13	7 + 2	0	16
	3/29/13	5 + 1	0	7
	3/30/13	3 + 1	1	5

The low recapture rate at seep C made population estimates impossible. During the weekly sampling period, 68 marked individuals were released into seep C, and none were recaptured. During the daily sampling period, an additional 36 marked individuals were released, and just one was recaptured. Because of these low recapture rates, no population

estimates could be made for seep C.

Table 3–Seep B population estimates

Sampling Interval	Weekly		Daily	
Method	Schnable	S-E	Schnable	S-E
Estimate ($\hat{N}$)	9207	14533	5498	6367
95% C. I.	4545 to 45021	5838 to -29687	3002 to 14728	1860 to -1953

Physical Data

Tables 4 and 5 summarize the physical data collected at each seep, including the ambient and water temperatures (as recorded by the VWR thermometer) and the average depth. The average source temperature of seep B, over the entire survey period was 11.8 °C, with a standard deviation of 1.29 °C. The maximum source temperature recorded at seep B was 14.3 °C, on February 27, 2013, while the minimum was 10.1 °C, on March 28, 2013. The average source temperature at seep C was 12.5 °C, with a standard deviation of 0.483 °C. The minimum was 12.1 °C, on both March 3 and 28, 2013. The maximum was 13.4 °C, recorded on January 30th, 2013. Visual estimates indicate that seep B had an average depth of 1.2 mm, with little variation and a standard deviation of 0.39 mm. The average depth of seep C was 2.4 mm, with little variation and a standard deviation of 0.89 mm. A t-test reveals the difference between these means to be significant at the 0.001 level.

Table 4–Seep B physical data

Sampling Interval	Date	Water Temp (°C)	Air Temp (°C)	Water Depth (mm)
Weekly	1/30/13	13.1	18.1	2
	2/6/13	10.4	16.2	1
	2/13/13	10.4	8.0	1
	2/20/13	11.1	12.3	1
	2/27/13	14.3	12.8	1
	3/5/13	11.5	12.4	1
	3/13/13	11.8	13.6	2
	3/20/13	12.3	15.7	1
Daily	3/27/13	13.4	23.9	1
	3/28/13	10.1	8.6	1
	3/29/13	11.3	11.1	1
	3/30/13	11.9	12.3	1

The data collected by the Hobo temperature loggers is summarized in Figs. 2 and 3. The temperature of both streams varied with the ambient temperature (i.e. they were warmer during the day and cooler during the night). The temperature at the source of each seep was more stable than was the temperature 2.0 m downstream. Seep B showed more temperature variation than seep C, but was slightly cooler than seep C. The coolest source temperature

recorded at seep B was 10.271 °C, between 6:55 and 7:55 AM on March 28, 2013. The warmest source temperature was 12.775 °C, at 1:30 PM on March 27, 2013. Seep B averaged 11.087 °C at its source, with a standard deviation of 0.60619 °C and a coefficient of variation of 0.0020427.

Table 5–Seep C physical data

Sampling Interval	Date	Water Temp (ºC)	Air Temp (ºC)	Water Depth (mm)
Weekly	1/30/13	13.4	18.7	2
	2/6/13	13.3	16.0	1
	2/13/13	13.1	11.8	3
	2/20/13	12.3	19.9	1
	2/27/13	12.8	15.8	3
	3/5/13	12.1	12.6	2
	3/13/13	12.4	13.8	4
	3/20/13	12.3	17.4	2.5
Daily	3/27/13	12.2	12.8	3
	3/28/13	12.2	10.5	2
	3/29/13	12.1	14.0	2.5
	3/30/13	12.2	13.8	2.5

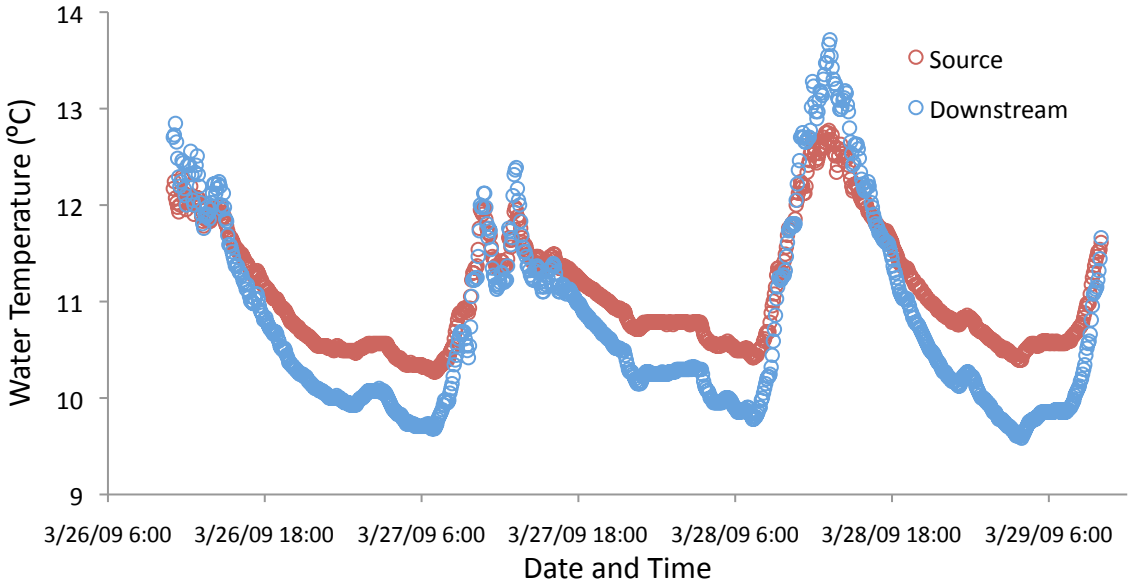

Fig. 2–Four-day temperature profile of Seep B

The temperature at the source of seep C fluctuated by less than 0.010 °C for the duration of the daily sampling period. While the downstream temperature changed with the ambient temperature, the source temperature appeared largely unchanged. Seep C averaged 12.076 °C at its source, with a standard deviation of 0.024656 °C and a coefficient of variation of 0.080096.

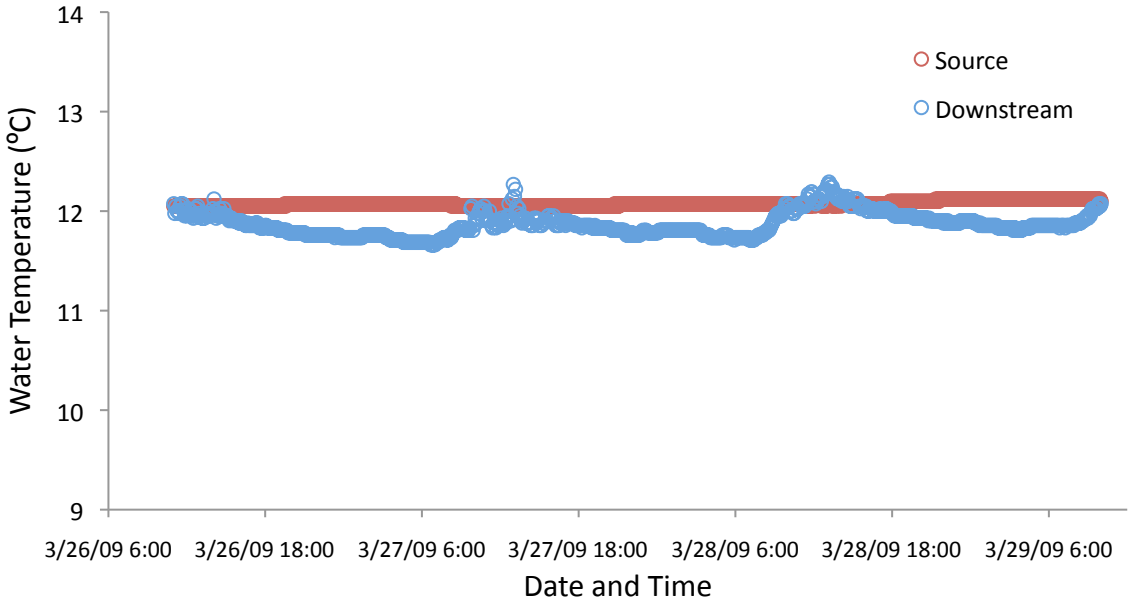

Fig. 3–Four-day temperature profile of seep C

Fecundity

Table 6 summarizes the numbers of ovigerous females collected at each seep. Over the course of the study, 18 ovigerous females were collected at Seep B, representing 4.2% of the total captures at Seep B. The mean proportion of ovigerous females was 3.4%, with a standard deviation of 5.9%. Sixty-one ovigerous females were collected at seep C, representing 36.1% of the total captures at seep C. The mean proportion was 29.2%, with a standard deviation of 15.0%. The difference in mean proportion of ovigerous females captured at each seep was significant at the 0.0001 level.

Table 6-Number of ovigerous collected at each seep

Sampling Interval	Date	Seep B	Proportion of C_t	Seep C	Proportion of C_t
Weekly	1/30/13	8	0.211	12	0.500
	2/6/13	1	0.036	8	0.320
	2/13/13	0	0	5	0.294
	2/20/13	1	0.020	16	0.485
	2/27/13	1	0.024	4	0.400
	3/5/13	2	0.056	1	0.250
	3/13/13	1	0.033	0	0.000
	3/20/13	1	0.021	7	0.467
Daily	3/27/13	3	0.061	4	0.200
	3/28/13	0	0	2	0.222
	3/29/13	0	0	1	0.167
	3/30/13	0	0	1	0.200
Total	—	18	0.042	61	0.361

Fig. 4 displays a plot of brood size with respect to female head length (in ocular microscopy units, OMU) and summarizes the linear relationship between these two variables. This represents the data from just 47 of the 79 females collected. Not included was data from females that had not given birth by the end of the study, had died in captivity, or had not carried their eggs to term. The smallest female had a head of 24 OMU, while the largest had a head of 39 OMU. The mean head length was 32 OMU, with a standard deviation of 3.1 OMU. The smallest brood size was one young, while the largest was 33 young. The mean brood size was 13 young, with a standard deviation of 3.1 young. The smallest brood per unit head length was 0.026 young/OMU, and the largest brood per unit head length was 0.87 young/OMU. The mean brood size per unit head length was 0.41 young/OMU, with a standard deviation of 0.71 young/OMU. The relationship between brood size and head length is linear and significant at the 0.001 level, with an R^2 value of 0.23.

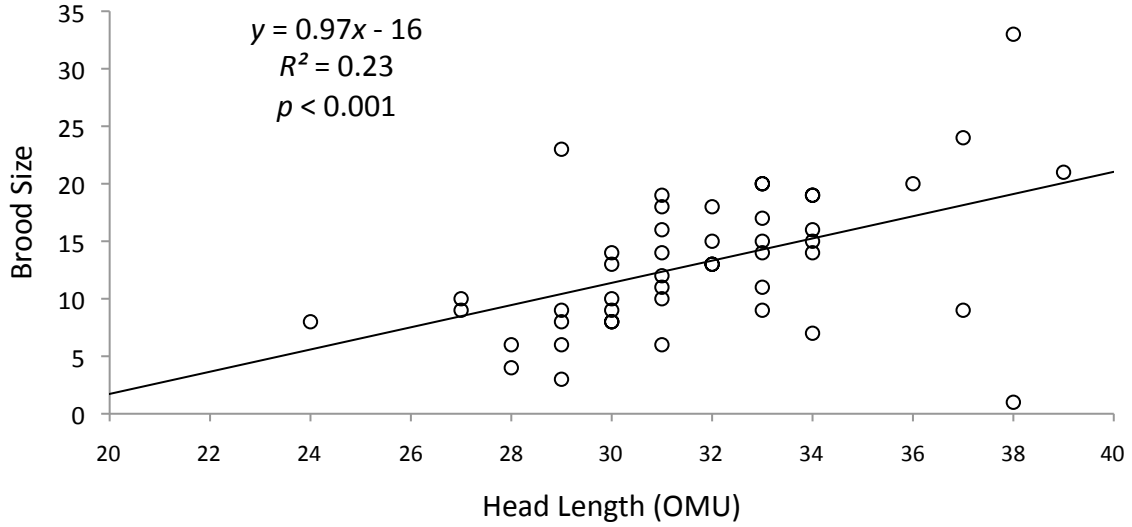

Fig. 4–Brood size with respect to head length (in ocular microscopy units)

Several observations were made regarding the ovigerous mothers and their broods while in captivity. First, young were born live and actively swam from the marsupium (or egg pouch). Next, days or even weeks could pass between the birth of the first and last young of a brood. Finally, it appears that some ovigerous females did not produce offspring. Rather, these females appear to ingest their eggs or dispose of them in some way. It could not be determined by what mechanism or why this happened. However, it is possible that these eggs were not fertilized, and so were terminated.

CONCLUSION

The populations may be effectively open

The population size estimates produced for seeps B and C show that the populations are very large, effectively open populations. This is evident in the low recapture rates. Recapture rates were so low at seep C that no estimate could be made. While estimates were possible for seep B, they varied widely. We take the estimates produced for seep B by the weekly sampling data to be more accurate than those produced by the daily sampling data, because the weekly

sampling interval allowed marked individuals more time to reintegrate into the population. This is reflected in the higher recapture rates during the daily sampling interval period. We also take the estimates produced by the S-E method to be more robust than those produced by the Schnabel method, even though the 95% confidence intervals for the S-E method are undefined. The algebra of the S-E method accounts for more violations of the aforementioned assumptions of a multiple mark-multiple recapture design (Krebs 1999). Thus, our most confident estimate of the size of the *S. t. potomacus* population living in seep B is 14533, with an undefined 95% confidence interval of 5838 (lower limit) to -29687 (upper limit).

However, even this cannot be taken as a true measure of the size of the population in seep B. Indeed, we envision this population to be very large, perhaps occupying a network of hypotelminorheic reservoirs, though which *S. t. potomacus* individuals freely migrate. Thus the population is effectively open. As a test of this hypothesis, a linear regression was calculated for the ratio of recaptured individuals to captured individuals at time *t* (R_t/C_t) against the total number of marked individuals in the population at time *t* (M_t), for the weekly S-E data. If the basic assumptions of the design are met, then this relationship should be linear, since it follows that the proportion of recaptures should increase with the total number of marked individuals in the population (Bueno *et al*. 2007; Krebs 1999). The regression for our data proved to be insignificant ($p = 0.57$). Thus, the basic assumptions of the design were unmet. We do not think that this violation was the result of deaths, because the habitat was observed to be relatively stable and free of predators. Likewise, births did not skew our results, because young *S. t. potomacus* are easily distinguish from adults and were not included in the data. The application of the mark did not appear to affect the behavior or survival of the organisms. Several marked individuals were retained in the laboratory for monitoring. These individuals still bore their marks and appeared healthy long after the field surveys had been completed. These factors give weight to our hypothesis of an effectively open population in seep B. We entertain a similar hypothesis for seep C, but have no evidence to support this, beyond the extremely low recapture rate ($\sum_{t=1}^{s} R_t = 1$). Unfortunately, we can devise no practical or effective means of resolving the open population issue.

Discrepancies between seeps B and C

In addition to differing recapture rates between the two seeps, we noticed several intrinsic differences between them. These differences lead us to posit that these seeps may be exit points for groundwater from two hypotelminorheic habitats, even though they are just 5.8 m apart. First, it appears that the structure of the *S. t. potomacus* population differs in each seep. The extremely low recapture rate at seep C (see Table 2) suggests that its population is far larger than that of seep B (though no estimates could be made to support this conclusion). Furthermore, seep C yielded statistically significant fewer mean total captures than did seep B. Finally, more ovigerous females, overall and as a proportion of the total captures, were caught at seep C than at seep B. The seeps also showed evidence of physical variation. The Hobo temperature loggers revealed that seep B was slightly cooler on average, but underwent greater temperature variations, demonstrated by the higher coefficient of variation for seep B. While the temperature of each seep cycled with the ambient temperature (warm in the day,

cool at night), seep C's appeared more resistant to change. In fact, the seep C source temperature hardly changed at all over the four-day period. Thus it would appear that these two seeps, though just 5.8 m apart are isolated from one another underground, drawing from separate aquifers. If this were the case, then it follows that their populations are also isolated. We hope to use the analysis of radon content in each seep to resolve their origins.

Brood size correlates positively with female size

Our analysis of female head length and brood size shows that these two factors are positively correlated. If head length is assumed to be a proxy for body length, then it appears that a highly significant ($p < 0.001$) positive relationship exists between female body size and brood size, with an R^2 value of 0.23. This finding appears simplistic, given that we might presumes that larger females should be able to carry more eggs. However, this finding is significant in that it may well represent the first in vivo analysis of fecundity in a *Stygobromus* species. Previous studies of *Stygobromus* fecundity have relied on dissections of preserved specimens (Holsinger 1978). Such studies assume that all the eggs in a preserved specimen would have been carried to term, were the female alive. These studies also assume that preserving the specimen did not damage the eggs nor led to any being lost from the marsupium. Conducting analysis in vivo circumvents such assumptions and results of this nature may thus be more accurate. Furthermore, in vivo methods allowed us to make such observations as viviparous births and pregnancy terminations.

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