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Monday, December 06, 2010

Hybridization of Cave and Surface *Gammarus minus*

Introduction

Culver et al. (1995) argue for the importance of cave-dwelling organisms for the study of evolution for several reasons. Cave and surface populations can be easily compared and the drastic differences between them provides for an excellent model of regressive evolution. Additionally, they argue, species that have invaded caves multiple times provide "natural replication."

Gammarus minus is one such species. *G. minus* is an amphipod crustacean that can be found in cave streams and surface springs from eastern Pennsylvania westward to Missouri (Cole, 1970). Both cave and surface populations live in cold, hard and relatively alkaline water and consume leaf litter and the bacteria it harbors (Culver et al., 1995). As is common in species found in both cave and surface habitats, substantial morphological differences are readily visible between subterranean and surface *G. minus* populations. Such differences include reduced pigmentation, enlarged appendages, including antennae and the olfactory bulb, and reduction or loss of eyes in cave populations (Culver et al., 1995; Fong, 1989). As a number of studies have shown, populations are more closely related to other populations living in connected streams than to populations in similar habitats but non-connected streams. This suggests that the morphological commonalities between cave populations are a result of convergent evolution following numerous unique introductions into cave environments (Fong, 1989; Kane et al., 1992; Carlini et al., 2009). In addition to these differences, *G. minus* in some springs have

considerably smaller body size due to predation by fish—while cave populations of *G. minus* do experience some predation by salamanders and possibly crayfish, this predation is not widespread and does not have a major effect on *G. minus* body size (Culver et al., 1995).

The reduction in eyes has been well studied, but whether the loss of functioning eyes is the result of selection or neutral drift is still up for debate (Fong et al., 1995; Carlini et al., 2009). Nevertheless, some linkage with a negative correlation has been suggested between the number of ommatidia and antenna size (Fong, 1989).

Studies have shown that cave populations of *G. minus* tend to have less genetic variability than surface populations (Kane et al., 1992; Carlini et al., 2009). The role of bottlenecks in this phenomenon is under debate. While Kane et al. argue that the levels of variability in cave populations do not suggest that populations experienced a bottleneck or founder's effect (1992), Carlini et al. (2009) suggest these bottlenecks as one of their two possible explanations. These bottlenecks could result from temperature fluctuations in the water (Carlini et al., 2009). Cave streams are more susceptible to changes as a result of an influx of snowmelt or summer rainwater, while the water in surface springs has a more stable temperature as a result of travelling underground for a considerable length of time (Culver et al., 1995; Carlini et al., 2009). In addition, droughts tend to affect caves more than they do springs, as springs often are supplied by water from several cave streams (Carlini et al., 2009).

Water from cave springs frequently passes through extremely small cracks and channels in the rock before reaching surface springs. Much of this is impassable to *G. minus* and creates a barrier between even closely-related cave and surface populations that prevents movement between them (Kane et al., 1992). While barriers exist between surface populations in the form of surface streams and rivers (Carlini, et al., 2009), the small amount of gene flow that does

occur between surface populations is greater than the gene flow through the interstitial channels of rock between surface and cave populations (Kane et al.).

To gain insight into the genetic relatedness of the cave and surface populations we hybridized individuals from a cave population—Organ Cave—in Greenbrier County, West Virginia with a nearby surface spring, Taylor Spring. While hybridization has been used as a technique to determine taxonomic position for populations of other *Gammarus* species (Pinkster and Scheepmaker, 1994), Kane et al. (1992) suggested that morphological differences resulting in mating incompatibilities could stand as a barrier to gene flow between populations of *G. minus*. To test this, we mated surface individuals with cave individuals to determine their ability to reproduce and the viability of their offspring. We then wanted to determine how they morphologically integrated by comparing a number of characteristics, including the number and viability of offspring as well as their total body size, number of ommatidia, and antenna length.

Methods

We collected the first specimens from a cave (Organ Cave) and a spring (Taylor Spring) in Greenbrier County, West Virginia in October, 2010 and more specimens from Taylor Spring were collected in January, 2011. Specimens in Organ Cave were sucked from the stream in turkey basters and placed in water-filled Ziploc bags. Taylor Spring specimens were collected by kicking up sediment from the bottom of the spring and holding a net downstream to collect the disturbed *G. minus*, which were then placed in water-filled Ziploc bags. Several hundred specimens were collected from each location in October, and thousands more from Taylor Spring were collected in January. The specimens were transported in the Ziploc bags in an ice-filled cooler to the lab, where they were placed into shoebox-sized plastic tubs with the water from the spring. These tubs were then placed in an incubator that was kept at 10 °C, although the

temperature was lowered to 6°C in November and left at that temperature for the duration of the study. After allowing the temperature to stabilize overnight, we changed the water, filtering out the *Gammarus* with small nets and replacing the water with about 2000 ml of distilled water that had also been kept in the incubator. Twice a week, we changed the water and added elm and maple leaf detritus from a tank of tap water that was continuously aerated. Before breeding the *Gammarus*, we separated males and females into different containers, and allowed them to remain separate from each other for at least two weeks before breeding to ensure that the females were not already carrying fertilized eggs. To separate the *Gammarus*, we located mating pairs and removed them from the water until they separated, place each in its respective container. Sometimes, we used paper towels or gently blew on the specimens to help them separate.

Amplexing pairs were also used to see if there was any connection between habitat and how rapidly the pairs split when disturbed. To perform these tests, we removed single pairs from the water and placing them in nets until they separated. Paper towels and blowing were not used in these tests.

When we were not able to locate enough mating pairs for use in the breeding experiment, other criteria were used to determine *G. minus* sex. Very large specimens were separated as males, and obviously ovigerous individuals were separated as females. We also used the gnathopod size to separate males from females, as males tend to have larger posterior gnathopods (Hume et al., 2005). Females who were found to be carrying fertilized eggs were further isolated in individual containers, where the eggs could hatch. We later analyzed these females' fecundity and their offspring's viability as a control.

The isolated males and females which were not ovigerous were used in the breedings. We crossed cave males with cave females, cave males with surface females, surface males with

cave females, and surface males with surface females. For each breeding, we used three males and ten to twelve females, depending on availability. To mate the *Gammarus*, we simply put the males and female being mated into a tub together. These tubs were about half as large as the shoebox-sized tubs the populations were kept in, and were filled with about 1000 ml of distilled water. Each type of the matings was performed four times (Table 1). The tubs were checked every four to seven days, and the pairs, ovigerous females, and offspring were counted. Individuals were replaced when necessary.

		MALE	
		Org. Cave	Tay. Spr.
FEMALE	Org. Cave	3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females
	Tay. Spr.	3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females
		3 males x 12 females	3 males x 12 females

Table 1: This chart shows the breedings performed during the course of this study

We intended to count the number of offspring produced and determine viability by counting how many survived to adulthood. We then intended to look at a number of different physical characteristics (Table2).

Characteristic	**Cave/surface difference**
Body size	Larger in cave populations and spring populations without fish
Pigmentation	Paler in cave populations
Number of ommatidia in eyes	Fewer in cave populations
Antennae length	Longer in cave populations

Table 2: Characteristics observed or measured in offspring

Results

The specimens collected from Organ Cave were considerably larger and paler than those collected from Taylor Spring. In addition, the spring population initially seemed reluctant to mate with each other, even when the incubator temperature was decreased, requiring us to sex them by body and gnathopod size. Starting in late January, however, the spring population began breeding more actively and we were able to sex them from amplexing pairs. We also collected more specimens on January 29th. These *G. minus* were noticeably smaller than those collected in October, even from the spring.

Amplexing pairs collected from Taylor Spring appeared to separate much more quickly and easily than those from Organ Cave. Our tests to determine the difference between the two populations confirmed these observations (Table 3; Table 4).

Organ Cave			
Collected 8/14/10		Collected 10/2/10	
Date tested	Length (seconds)	Date tested	Length (seconds)
4/16	798	4/12	524
4/16	241	4/12	285
4/17	62	4/12	3
4/17	1	4/17	47
4/17	115	4/17	158
4/17	52	4/17	57
4/17	5	4/17	104
4/17	184	4/17	68
4/17	100	4/17	353
4/17	5	4/17	28
		Additionally, 2 pairs broke before timing began	
Average = 156.3 seconds		Average = 162.7 seconds	
Organ Cave average = 159.5 seconds			

Table 3: Time for amplexing Organ Cave pairs to separate when placed out of water in net

Taylor Spring			
Collected 10/2/10		Collected 1/29/11	
Date tested	Length (seconds)	Date tested	Length (seconds)

4/8	6	4/12	153
4/8	87	4/12	10
4/8	312	4/12	107
4/16	190	4/16	135
4/16	325	4/16	5
4/16	175	4/16	24
4/16	129	4/16	1
4/16	61	4/18	35
4/16	1	4/18	2
4/16	67	4/18	7
Additionally, 2 pairs broke before timing began		Additionally, 8 pairs broke before timing began	
Average = 135.3 seconds		Average = 47.9 seconds	
Taylor Spring average = 91.6 seconds			

Table 4: Time for amplexing Taylor Spring pairs to separate when placed out of water in net

Average separation times for Organ Cave pairs from both samples were longer than separation times in both Taylor Spring samples. The average times for the two cave samples were only 6.4 seconds apart (Table 3). However, At 47.9 seconds, the average time for the spring sample collected in January was only about a third as long as that of the October spring sample, for which the average separation time was 135.3 seconds. Additionally, the January spring sample had a higher number of pairs that broke apart before timing could begin (Table 4).

Breeding sets with cave males mated slightly more actively than those with spring males. However, frequency of mating was not consistent through multiple trials of the same crosses. For example, in one cross between Organ Cave males and Taylor Spring females, mating pairs were observed on most occasions, while in the other cross between Organ Cave males and Taylor Spring females, only one pair was ever observed (Table 6). Similarly, only one pair was ever observed in one of the breedings within the spring population, while pairs were observed on every occasion in the other (Table 8).

Organ Cave male x Organ Cave female									
Date	Cross 1 3m x 12f Started on 12/3			Cross 2 3m x 12f Started on 1/23			Cross 3 3m x 12f Started on 3/21		
	Pairs	Ovig.		Pairs	Ovig.		Pairs	Ovig.	

1/24	0	1		0	0		-	-	
1/27	1	-		1	-		-	-	
2/4	1	1		1	0		-	-	
2/8	1	2		1	1		-	-	
2/15	2	2		0	1		-	-	
2/21	2	2		0	1		-	-	
2/22	-	-		-	-		-	-	
2/26	1	3	Only 10 found	1	1	Only 13 found	-	-	
3/15	1	3	Only 10 found	1	2	Only 13 found	-	-	
3/20	2	3	Only 9 found	0	2	Only 13 found	-	-	
3/30	2	2	Only 9 found	0	2	Only 13 found	0	0	All 15 present
4/6	1	1	Only 7 found	0	2	Only 13 found	0	0	All 15 present
4/12	3	1	8 added, 15 present	0	2	Only 13 found	1	1	All 15 present
4/18	3	1	All 15 present	0	2	Only 13 found	1	1	Only 14 found

Table 5: Pairs and ovigerous females observed in breedings within cave population

Organ Cave male x Taylor Spring female						
Date	Cross 1 3m x 11f Started on 1/21			Cross 2 3m x 10f Started on 2/8		
	Pairs	Ovig.		Pairs	Ovig.	
1/24	1	0		-	-	
1/27	2	-		-	-	
2/4	2	0	One found dead	-	-	
2/8	2	1	One found dead	-	-	
2/15	2	1		0	0	
2/21	0	1	Only 5 found, 10 f added	-	-	
2/22	-	-		0	0	Only 9 found, five f added
2/26	1	2	Only 13 individuals found	0	2	All 14 present
3/15	1	3	Only 12 found, 3 added	0	5	All 14 present
3/20	0	4	All 15 present	0	5	All 14 present
3/28	0	3	Only 13 found, 1 dead	0	6	All 14 present
4/6	0	3	Only 12 found	0	6	All 14 present
4/12	0	2	Only 12 found	0	6	All 14 present
4/18	0	2	Only 12 found	1	6	All 14 present

Table 6: Pairs and ovigerous females observed in crosses between cave males and spring females

Taylor Spring male x Organ Cave female

Date	Cross 1 3m x 12f Started on 1/21			Cross 2 3m x 12f Started on 2/8			Cross 3 3m x 12f Started on 3/21		
	Pairs	Ovig.		Pairs	Ovig.		Pairs	Ovig.	
1/24	1	0		-	-		-	-	
1/27	0	-	One found dead	-	-		-	-	
2/4	0	2		-	-		-	-	
2/8	0	4		-	-		-	-	
2/15	1	4		0	3		-	-	
2/21	1	4		-	-		-	-	
2/22	-	-		0	3	All 15 present	-	-	
2/26	0	4	Only 13 found	0	2		-	-	
3/15	1	4	Only 13 found	1	0	Only 12 found	-	-	
3/20	1	2	Only 13 found	1	0	Only 12 found	-	-	
3/28	1	2	Only 13 found	2	0	Only 12 found	-	-	
3/30	-	-		-	-		0	0	Only 14 found
4/6	1	2	Only 13 found	1	0	Only 11 found	1	0	Only 14 found
4/12	2	3	Only 13 found	1	0	Only 11 found	2	0	Only 14 found
4/18	2	3	Only 13 found	1	0	Only 10 found	2	1	Only 14 found

Table 7: Pairs and ovigerous females observed in crosses between spring males and cave females

Taylor Spring male x Taylor Spring female						
Date	Cross 1 3m x 12f Started on 1/21			Cross 2 3m x 11f Started on 2/8		
	Pairs	Ovig.		Pairs	Ovig.	
1/24	0	0		-	-	
1/27	0	-		-	-	
2/4	0	0	One found dead	-	-	
2/8	0	1		-	-	
2/15	0	1	One found dead	2	0	
2/21	0	1		-	-	
2/22	-	-		1	3	Only 14 individuals found
2/26	0	2	Only 11 individuals found	1	4	Only 13 found
3/15	0	3	Only 10 found	1	5	Only 12 found
3/20	0	3	Only 10 found, 2 f added	2	6	Only 12 found
3/28	0	3	Only 11 found	1	5	Only 12 found

4/6	0	3	Only 11 found	3	5	Only 12 found
4/12	0	3	Only 10 found	2	4	Only 12 found
4/18	1	2	Only 10 found	1	4	Only 12 found

Table 8: Pairs and ovigerous females observed in breedings within spring population

In every set, except for the second cross between spring males and cave females, an increase in the number of ovigerous females was observed. In the first trial of each type of cross, as well as in the second trial of the crosses containing spring males, reductions in the number of ovigerous females followed (Table 5; Table 6; Table 7; Table 8). However, no young *Gammarus* were ever observed.

All of the breedings experienced some loss of individuals, most of which were female (Table 5; Table 6; Table 7; Table 8). Most of the losses were gradual—just one individual disappearing between observations—both of the crosses with cave males and spring females experienced more rapid decreases. In the first of those crosses, only three of eleven females were remaining after 31 days. In the second, four females disappeared in the first 14 days (Table 6).

Discussion

The lack of observed offspring in any of the breedings, including the within-population breedings, prevented us from drawing any conclusions about whether the cave and surface populations exhibited reproductive isolation. It is unclear why no offspring were ever observed. It is possible that the *Gammarus* were simply unable to breed under the laboratory conditions; for example, they may have been disturbed too frequently. If this were the case, future experiments using aerators or filters rather than semi-weekly water changes may be able to produce more offspring. Another possibility is that offspring, trapped in the relatively confined space of the small tubs where breedings occurred, were eaten by adult *Gammarus* before being observed. This scenario is supported by the fact that in many of the breedings, the number of

ovigerous females eventually decreased, suggesting that their eggs may have hatched. If this is the case, a possible solution would be to place a mesh divider, fine enough to keep adults from passing while allowing juveniles through, in the tub to separate the top and bottom sections. Young *Gammarus* would then be able to remain in the relative safety of the bottom section, along with the nutrient-rich debris on the bottom of the tub.

Despite the lack of offspring in any of the breedings, a reproductive barrier between the cave and surface populations may still have been witnessed in the crosses between cave males and spring females. In both of these crosses, a rapid reduction in the number of females was seen (Table 6). Gradual decreases seen in other crosses are likely due to deaths that occur for a number of reasons, potentially including old age. The rapid decreases in theses crosses, however, suggest that there may be other reasons behind the deaths. Because the cave individuals are larger than spring individuals, it is possible that the larger cave males were viewing the smaller spring females as food sources rather than mates. The size differential theory is further supported by the fact that in the second cross, where the females came predominantly from the January population and were smaller, very little mating occurred (Table 6). However, in both of the crosses, the rate of female loss decreased after females were first replaced. Over the course of 56 days, the first cross lost a total of six females, a greater loss than in any of the other breedings, but slower than the initial loss. In the second cave male/spring female cross, however, no females disappeared over the remaining 55 days after the first four losses were replaced. One possible explanation for the lack of disappearances could be that original deaths in that population were caused by something else, such as insufficient food or unclean water. The deaths in the first cave male/spring female cross may have then been due to a specific one or two males that were extra-aggressive.

Size may also have played a role in the length of time it took amplexing pairs in different populations to separate. The times for all populations during these tests (those recorded in tables 3 and 4) were greater than they were when pairs were being separated for use in the crosses. This is because when the pairs were timed, they were placed in a net and remained untouched until they separated, while pairs being separated for use in crosses were actively encouraged to separate. As observed while separating pairs for crosses, cave pairs took longer to separate than spring pairs, and particularly the spring pairs collected in January. One possible explanation for this is that spring individuals, who are more prone to predation, may separate more quickly when disturbed than subterranean individuals, which evolved in the relative safety of a cave. However, the average for even the fastest-separating population was 47.9 seconds, a relatively long time in the face of a predatorial attack (Table 4). Another more likely possibility may have to do with the size of the male. Larger males may have more success holding onto struggling females, which could explain not only why cave pairs had the longest separation times, but also why the small individuals of the January spring population had by far the shortest separation time (Table 4; Table 5). The importance of size on the ability of a male to control a struggling female could be further revealed in a study of separation times for small spring males with large cave females and vice versa.

The slightly higher frequency of mating observed in breedings with cave males may have been a factor of size as well. However, it could also have been caused by the average length of time spent mating: cave males could potentially be mating with similar frequency, but for longer durations. One way to test this would be to record on video the length of time two *Gammarus* spend as an amplexing pair.

Conclusion

The lack of offspring created an obstacle to reaching some of the original goals of the study, but with a few tweaks, future attempts may be successful in determining the level of reproductive isolation between cave and surface populations. The study did reveal interesting insights into the relation between *Gammarus* size and mating frequency, as well as highlight certain barriers to cross-population reproduction. It is likely that size is a primary factor in determining whether crosses would be possible. Too large of males may simply prey on smaller females, while other downfalls may be experienced by males that are too small.

References

Carlini, D. B., Manning, J., Sullivan, P. G., Fong, D. W. 2009. Molecular genetic variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod Gammarus minus. *Molecular Ecology* 18:1932-45

Cole, G. A., 1970. *Gammarus minus*: geographic variation and description of new subspecies *G. m. pinicollis* (crustacean, amphipoda). *Transactions of the American Microscopical Society* 89:514-23

Culver, D. C., Kane, T. C., Fong, D. W. 1995. *Adaptation and Natural Selection in Caves*. London, England. Harvard University Press

Fong, D. W., 1989. Morphological evolution of the amphipod *Gammarus minus* in caves: quantitative genetic analysis. *American Midland Naturalist* 121:361-78

Fong, D. W., Kane, T. C., Culver, D.C. 1995. Vestigialization and loss of nonfunctioning characters. *Annual Review of Ecology and Systematics* 26:249-68

Hume, K. D., Elwood, R. W., Dick, J. T. A., Morrison, J. 2005. Sexual dimorphism in amphipods: the role of male posterior gnathopods revealed in *Gammarus pulex*. *Behavioral Ecology and Sociobiology* 58:264-9

Kane, T. C., Culver, D. C., Jones, R. T. 1992. Genetic Structure of Morphologically Differentiated Populations of the Amphipod Gammarus minus. *Evolution* 46:272-8

Pinkster, S., Scheepmaker, M. 1994. Hybridization experiments and the taxonomy of *Gammarus* (amphipoda): a contribution to the understanding of controversial results. *Crustaceana* 66:2