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Analysis of the Large-Scale Distribution of Quasars Within the *Sloan Digital Sky Survey Quasar Catalog*

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ABSTRACT

Quasars, which were discovered in the early 1960s, have presented astronomers with countless new and exciting questions about the universe. We now know that they are some of the most luminous objects in the sky, and can be used to probe the universe at its earliest stages of development due to their enormous redshifts. This paper investigates the large- and small-scale distribution of quasars in our universe. Focusing on a variety of scaling ranges, we examine quasar clustering with respect to both magnitude and redshift. By exploring these aspects, we hope to form insights into the formation of galaxies and into the structure and development of our entire universe. To study these objects, we employ the fourth edition of the *Sloan Digital Sky Survey (SDSS) Quasar Catalog*, which contains detailed physical properties on over 77,000 quasars. We use the pointwise dimension technique (a mathematical technique, based on fractal geometry, that quantifies the scaling of objects around a specified position) to explore environmental relationships between quasars. Analysis of our data shows that the evolutionary structure of quasars varies on all but the smallest scales. We also find that luminosity and redshift are significant factors in quasar clustering on various scales.

INTRODUCTION

Observationally, astrophysicists detect numerous structures throughout the universe. On small distance scales, we find stars grouping together to form galaxies. Almost all galaxies form clusters, with each cluster containing tens, hundreds, or thousands of other galaxies. In the early 20th century, it was thought that clusters were the largest structures in the universe and that they were evenly distributed throughout space. However, studies in the 1950s detected the presence of "superclusters," or clusters of clusters. It was not until the 1970s, with the study of the Coma supercluster, that the real structure of superclusters became visible as filamentary wisps with great voids with little galactic activity between them. Superclusters are found to extend tens of millions of light-years (Silk 1989).

Residing in the centers of some galaxies, quasars belong to a class of objects known as active galactic nuclei, or AGN. Quasars are observed as star-like points that emit electromagnetic radiation in all wavelengths, which is often highly redshifted. Since redshift is directly related to object distance, we can conclude that such massively redshifted objects reside at significant distances from Earth. With an appreciation of our distance from these objects, we realize that they must be emitting 10-1000 times the luminosity of large galaxies. Astrophysicists observe that a quasar's brightness fluctuates rapidly, sometimes in only a few months: such rapid fluctuations prove that these incredibly bright objects are relatively small, perhaps on the order of a planetary system (Smith and Hoffleit 1963).

A number of explanations were developed to explain the unusual combination of small size and incredible luminosity. Some attributed the energy to the annihilation of antimatter and matter, while others credited the energy to a chain reaction of densely situated stellar explosions (Golden 1976). However, the presence of a supermassive black hole at the core of a quasar best explains the observed structure. Through observation we can deduce some characteristics of this central black hole. Because light exerts pressure on matter (the same phenomenon responsible for the solar winds), a quasar's structure is dependent upon the gravitational force pulling inwards being greater than the luminosity pressure expanding outwards. Considering the amount of light emitted by these objects, astrophysicists conclude that the supermassive black holes residing in quasars can contain more than 1 billion solar masses (Courvoisier 2001).

Previous studies and surveys concerning the distribution of quasars have determined that mid-luminous quasars are the most common, with their number density decreasing at high and low luminosities. Furthermore, there seems to be a significant decrease in the number of quasars at redshifts greater than z=3 (Courvoisier 2001). Studies concerning the large-scale distribution of quasars can address a wide range of observational effects. By examining the structural evolution across various redshift ranges, we can develop insights into how the large-scale distribution of quasars has changed over time. Additional analysis of quasar clustering enables comprehension of quasar lifetimes (Martini and Weinberg 2001), clustering according to baryon density and the Cosmological Constant (Yahata 2005), as well as the effects quasars have on galactic structure (Porciani et al. 2004; Siemiginowska et al. 2007). Since the study of quasar distributions has a variety of implications, a quantification of this distribution is crucial to our understanding of the universe (Thompson and Best 2006).

METHODS AND MATERIALS

Through observational evidence, we see structure manifesting itself similarly on both large- and small-scales. However, this structure is complex and irregular, making its quantification difficult. Fortunately, the 'self-similarity' of galactic and quasar distribution implies that the mathematical concept of fractal geometry may apply (Mandelbrot 1983; Peebles 1993).

Fractals are the mathematician's concession that, as Benoit Mandelbrot (the 'father of fractal geometry') once said, "Clouds are not spheres, mountains are not cones, coastlines are not circles, and bark is not smooth, nor does lightning travel in a straight line" (Mandelbrot 1983). Fractals are used to describe shapes in nature that cannot be expressed using simple Euclidean terms. Objects such as coastlines, blood vessels, and lightning are not simple spheres, cylinders or squares: traditional geometry would be a limited tool in describing these phenomena.

Another aspect of fractals is scale-invariance, where changes in the observational scales have no bearing on the general shape of the object: the fractal looks similar to itself on every scale. Figure 1 is the common fractal known as the Mandelbrot set. One can note the self-similarity and scale-invariance of this fractal, observing how the same shape is repeated over various scales.

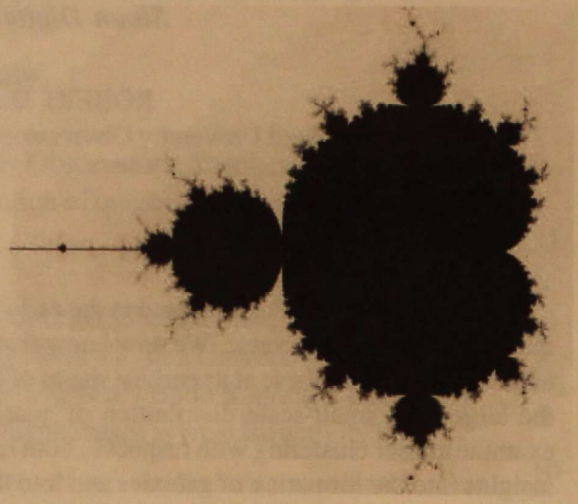

Figure 1: The Mandelbrot Set

Applications of fractals have grown significantly over the years. The examination of viscous fingering (generally the result of combining a highly viscous material with one of low viscosity) resulting from oil moving through earth can be better understood through fractal mathematics, enabling better methods of extracting such oil. Further applications include mapping of complex river systems, and even examining stock price changes (Takayasu 1990).

Our specific methodology applies the pointwise dimension (described in Best et al. 1996) to study the distribution of quasars using fractal-based geometry. We first consider the function $N(r)$, which is the cumulative count of the number of quasars within a radius r from a specific quasar at reference point x. By increasing (r_{max}) and decreasing (r_{min}) this radius, we can create effective bounds over which d (the pointwise dimension) represents the quantification of the environment of the quasar. The specific equation we use to calculate the pointwise dimension is as follows:

$$d_{\bar{x}m} = \frac{\log(N_{\bar{x}m}(r_{max})) - \log(N_{\bar{x}m}(r_{min}))}{\log(r_{max}) - \log(r_{min})}$$

For each quasar, we plot the cumulative number of objects counted versus the radius from the quasar. The least-squares fit to this plot in log-log space is the pointwise dimension, which serves as the quantification of the environment around a specific quasar.

THE DATA: *SLOAN DIGITAL SKY SURVEY*

The *Sloan Digital Sky Survey* (SDSS) is in the process of developing the most comprehensive survey of the universe to date. The survey will create a three-dimensional map encompassing over one-quarter of the sky, focusing on half of the northern celestial hemisphere and a smaller portion of the southern celestial sphere. The 2.5-meter Sloan telescope, housed at the Apache Point Observatory in Sunspot, New Mexico produces the data for the survey. Due to its isolation from light pollution and atmospheric contaminants, this telescope is able to acquire details for millions of objects, revealing their positions, luminosities, spectra, redshifts, and many other fundamental properties (SDSS 2008). Of all the quasars identified in the catalog, nearly 96% were discovered for the first time by the SDSS (Schneider et al. 2007). The first phase of the survey, SDSS-I, was completed in June 2005 and identified nearly 200 million celestial objects. SDSS-II will expand the number of detected objects to provide an even greater understanding of the universe (SDSS 2008).

For the current work, we employ the fourth edition of the SDSS Quasar Catalog, which contains information on the physical properties of 77,429 quasars, an increase of more than 30,000 objects since the previous edition. The current edition of the catalog provides a wealth of information for each individual quasar over an area of the sky covering approximately 5,740 square degrees. Among the information available are the object's designation in the catalog, coordinates (in right ascension and declination), redshift, absolute magnitudes in various wavelengths, and morphological information. The survey contains quasars with redshifts between 0.08-5.41, and incorporates an absolute magnitude range of -22 to -30 (Schneider et al. 2007). Figure 2 is a visual representation of the SDSS Quasar Catalog. The dark areas denote the positions in the sky (in right ascension and declination) of the quasars currently mapped.

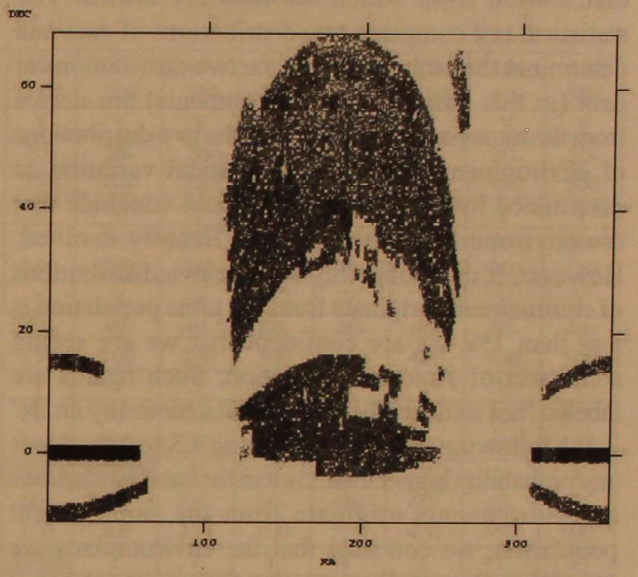

Figure 2: A visual representation of the SDSS Quasar Catalog.

ANALYSES

Using the results from the pointwise dimension calculated for each individual quasar, we are able to quantify the environment around each quasar on numerous distance scales. Specifically, the distance scales (or fitting ranges) we consider for each analysis are 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, and 500 megaparsecs (Mpc). These values correspond to the length of the radius measured around each quasar and were chosen to allow examination on both large and small scales. Additionally, we consider specific redshift ranges (0.00-1.00, 1.00-2.00, 2.00-3.00, 3.00-4.00, and 4.00-5.00), as well as absolute magnitude ranges ($-30 < M < -28$, $-28 < M < -26$, $-26 < M < -24$, and $-24 < M < -22$), to facilitate the comparison of these results with other studies and enable consideration of structural evolution with respect to these parameters. It is important to note that redshift can serve as an age indicator, while magnitude serves as a measure of luminosity. One must also note that a smaller numerical value for magnitude corresponds to a greater luminosity value.

After we have quantified the environment of each object using the pointwise dimension, we use the Kolmogorov-Smirnov (KS) two-sample test to compare

environments and determine structural evolution. The KS test is nonparametric, meaning that there is no assumption made about the underlying parent distribution from which the data are drawn. The statistical test compares two distributions of data and determines the probability that the two distributions of data (in this case, quasar environments) are drawn from the same parent population. If the two distributions of environments show little statistical variation as determined by the KS test, we would conclude that the environments have not significantly evolved. However, if the probability that the two distributions of environments originate from the same population is less than 1%, we are confident that we are seeing some sort of structural evolution. Such results are labeled 'not statistically similar' and denoted by an 'N' in the following tables. If a resulting KS test produces any probability higher than 1% that the two distributions of environments originate from the same parent population, we conclude that the environments are 'statistically similar' to the 99th percent level (represented by a 'Y' in the tables).

In our first analysis, we attempted to determine if evolution of structure exists over various scales. By comparing different fitting ranges, regardless of energy or age characteristics, we wish to examine if large- and small-scale environments in general affect quasar clustering. Table 1 presents these results.

In Tables 2a-f, we compare similar environment scales for quasars of different magnitudes. By keeping the fitting ranges constant, we examine if luminosity (as measured by magnitude) of the quasar influences the environments. Tables 2a-2e represent different redshift ranges, allowing us to study the effect of magnitude as a function of time. Table 2f represents quasar distribution over all redshift ranges, examining the effect magnitude has on distributions in general. Our final analysis examines quasar environments as a function of redshift only. Using these data, we can determine if quasar distributions evolve over time, independent of luminosity considerations. Tables 3a-e present these results.

Table 1: Comparisons of the large- and small-scale distribution of quasars with no magnitude or redshift constraints. The horizontal axis represents the two environments being compared (in Mpc).
Y = Statistically similar, N = Not statistically similar

Fitting Ranges	10-20	10-30	10-40	10-50	10-60	10-70	10-80	10-90	10-100	10-150	10-200	10-250	10-300	10-350	10-400	10-450	10-500
	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges		20-30	20-40	20-50	20-60	20-70	20-80	20-90	20-100	20-150	20-200	20-250	20-300	20-350	20-400	20-450	20-500
		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges			30-40	30-50	30-60	30-70	30-80	30-90	30-100	30-150	30-200	30-250	30-300	30-350	30-400	30-450	30-500
			N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges				40-50	40-60	40-70	40-80	40-90	40-100	40-150	40-200	40-250	40-300	40-350	40-400	40-450	40-500
				N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges					50-60	50-70	50-80	50-90	50-100	50-150	50-200	50-250	50-300	50-350	50-400	50-450	50-500
					N	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y
Fitting Ranges						60-70	60-80	60-90	60-100	60-150	60-200	60-250	60-300	60-350	60-400	60-450	60-500
						N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges							70-80	70-90	70-100	70-150	70-200	70-250	70-300	70-350	70-400	70-450	70-500
							N	N	N	N	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges								80-90	80-100	80-150	80-200	80-250	80-300	80-350	80-400	80-450	80-500
								N	N	N	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges									90-100	90-150	90-200	90-250	90-300	90-350	90-400	90-450	90-500
									N	N	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges										100-150	100-200	100-250	100-300	100-350	100-400	100-450	100-500
										N	Y	Y	Y	Y	Y	Y	Y
Fitting Ranges											150-200	150-250	150-300	150-350	150-400	150-450	150-500
											N	N	N	N	Y	Y	Y
Fitting Ranges												200-250	200-300	200-350	200-400	200-450	200-500
												N	N	N	Y	Y	Y
Fitting Ranges													250-300	250-350	250-400	250-450	250-500
													N	N	N	Y	Y
Fitting Ranges														300-350	300-400	300-450	300-500
														N	N	N	Y
Fitting Ranges															350-400	350-450	350-500
															N	N	N
Fitting Ranges																400-450	400-500
																N	N
Fitting Ranges																	450-500
																	N

Tables 2a-f: Comparisons of the same fitting ranges for quasars of different magnitude ranges (separately within specified redshift ranges). The horizontal axis represents the environment examined (in Mpc), and the vertical axis represents the two sets of magnitude ranges being compared.

Y = Statistically similar, N = Not statistically similar

2422 = -24<M<-22 2624 = -26<M<-24 2826 = -28<M<-26 3028 = -30<M<-28

Table 2a: Comparison of quasars with a redshift range of 0.00-1.00

-	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2826 v. 2422	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N
2826 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2b: Comparison of quasars with a redshift range of 1.00-2.00

-	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2826 v. 2422	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2826 v. 2624	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2422	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y

Table 2c: Comparison of quasars with a redshift range of 2.00-3.00

-	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2826 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2826 v. 2624	Y	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2624	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N	N	Y

Table 2d: Comparison of quasars with a redshift range of 3.00-4.00

-	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2826 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N
2826 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N
3028 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2e: Comparison of quasars with a redshift range of 4.00-5.00

-	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2826 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2826 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2422	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2624	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Table 2f: Comparison of quasars with a redshift range of 0.00-5.41

	10	20	30	40	50	60	70	80	90	100	150	200	250	300	350	400	450	500
2624 v. 2422	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2826 v. 2422	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y	Y	Y	Y
2826 v. 2624	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2422	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2624	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	N
3028 v. 2826	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Tables 3a-e: Comparisons of similar quasar environments as a function of redshift range. The horizontal axis represents the comparison redshift range, and the vertical axis represents the environment examined (in Mpc). Y = Statistically similar, N = Not statistically similar

Table 3a: Comparing all other redshift ranges to 0.00-1.00

-	1.00 – 2.00	2.00 – 3.00	3.00– 4.00	4.00 – 5.00
10	Y	N	Y	Y
20	Y	N	Y	N
30	Y	N	N	Y
40	N	N	N	Y
50	N	N	N	N
60	N	N	N	N
70	N	N	N	N
80	N	N	N	N
90	N	N	N	N
100	N	N	N	N
150	N	N	N	N
200	N	N	N	N
250	N	N	N	N
300	N	N	N	N
350	N	N	N	N
400	N	N	N	N
450	N	N	N	N
500	N	N	N	N

Table 3b: Comparing all other redshift ranges to 1.00 – 2.00

-	0.00 – 1.00	2.00 – 3.00	3.00 – 4.00	4.00 – 5.00
10	Y	N	Y	Y
20	Y	N	N	Y
30	Y	N	N	Y
40	N	N	N	Y
50	N	Y	N	N
60	N	Y	Y	N
70	N	Y	Y	N
80	N	Y	Y	N
90	N	Y	Y	N
100	N	N	N	N
150	N	N	N	N
200	N	N	N	N
250	N	N	N	N
300	N	N	N	N
350	N	N	N	N
400	N	N	N	N
450	N	N	N	N
500	N	N	N	N

Table 3c: Comparing all other redshift ranges to 2.00 – 3.00

	0.00 – 1.00	1.00 – 2.00	3.00 – 4.00	4.00 – 5.00
10	N	N	Y	Y
20	N	N	Y	Y
30	N	N	N	Y
40	N	N	N	Y
50	N	Y	N	N
60	N	Y	N	N
70	N	Y	N	N
80	N	Y	N	N
90	N	Y	N	N
100	N	N	N	N
150	N	N	N	N
200	N	N	N	N
250	N	N	N	N
300	N	N	N	N
350	N	N	N	N
400	N	N	N	N
450	N	N	N	N
500	N	N	N	N

Table 3d: Comparing all other redshift ranges to 3.00 – 4.00

-	0.00 – 1.00	1.00 – 2.00	2.00 – 3.00	4.00 – 5.00
10	Y	Y	Y	Y
20	Y	N	Y	Y
30	N	N	N	Y
40	N	N	N	Y
50	N	N	N	Y
60	N	Y	N	Y
70	N	Y	N	N
80	N	Y	N	N
90	N	Y	N	N
100	N	N	N	N
150	N	N	N	N
200	N	N	N	N
250	N	N	N	N
300	N	N	N	N
350	N	N	N	N
400	N	N	N	N
450	N	N	N	N
500	N	N	N	N

Table 3e: Comparing all other redshift ranges to 4.00 –5.00

-	0.00 – 1.00	1.00 – 2.00	2.00 – 3.00	3.00 – 4.00
10	Y	Y	Y	Y
20	N	Y	Y	Y
30	Y	Y	Y	Y
40	Y	Y	Y	Y
50	N	N	N	Y
60	N	N	N	Y
70	N	N	N	N
80	N	N	N	N
90	N	N	N	N
100	N	N	N	N
150	N	N	N	N
200	N	N	N	N
250	N	N	N	N
300	N	N	N	N
350	N	N	N	N
400	N	N	N	N
450	N	N	N	N
500	N	N	N	N

DISCUSSION AND CONCLUSIONS

Table 1 shows a strong structural evolution on scales larger than 50 Mpc and extending into our largest fitting ranges. It is interesting to note that Thompson and Best (2006) determined an evolution extending into scales smaller than 50 Mpc. However, the data set considered in our research contains over 50,000 additional objects, and the discrepancy may be a result of this greater sampling of quasars.

When examining Tables 2a-f we see that there is some evolution of structure according to redshift and magnitude ranges. If we compare different magnitude ranges for the entire catalog (Table 2f), we find a strong evolution over all scales and magnitude ranges except for the very brightest of quasars (3028 v. 2826). When considering redshift as a limiting factor, we see the most structural evolution in the mid-range redshift quasars (1.00-2.00, Table 2b). This evolution decreases as we examine higher and lower redshift comparisons (Tables 2a, and 2c-e).

A further examination of redshift dependency according to scale can be found in Tables 3a-e. We find the most evolution of structure in high and low redshift quasars (Tables 3a and 3e), clearly manifesting itself on scales above 50 Mpc. We find varying evolution based on redshift when mid-range redshift quasars are compared (Table 3b illustrates this in the middle two columns.) However, once the 100 Mpc fitting range is reached, a strong dependency on redshift is once again found even in these mid-redshift quasars.

Several studies confirm our findings of magnitude-based structural evolution for intermediate magnitude quasars. The works of Croom et al. (2002), Lidz et al. (2006), and Ivashchenko (2007) all argue that quasar clustering depends on magnitude in some way. Our findings at the brightest magnitudes coincide with those of da Angela et al. (2008), who find little dependence of quasar clustering on magnitude in a fixed redshift range. The lack of structural evolution in the brightest of quasars is also in agreement with Thompson and Best (2006). Furthermore, the results of Shen et al. (2007), as well as Thompson and Best (2006), support our findings of redshift-based evolution. We note that while Coil et al. (2007) do not find scale-, magnitude-, or redshift-dependent clustering, the distance scales analyzed in that work are significantly smaller than ours, as is the redshift range chosen. Taken together, these results extend the Thompson and Best (2006) findings, giving us confidence that we are gaining a greater understanding of the evolution of the large-scale structure of the universe.

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Leptin Increases Aromatase Activity in Androgen-independent Prostate Cancer Cells: a Preliminary Study

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ABSTRACT

INTRODUCTION

Obesity is a major health problem negatively affecting prostate cancer. It has previously been shown that the adipokine leptin, produced in adipose tissue, increases proliferation of prostate cancer cells *in vitro*. Also, the enzyme aromatase, which is involved in the malignancy of breast cancer, has been found to be active in the prostate. Aromatase converts androgens to estrogens, and while the effect of estrogen on the prostate is not known, prostate cells do possess estrogen receptors and there is evidence that estrogen can modulate prostate cell activity. Prostate tumors producing aromatase may lead to a worse outcome for the patient. Since it is known that aromatase activity increases in breast cancer cells in response to leptin, we hypothesized that aromatase activity would increase in prostate cancer cells treated with abnormally high concentrations of leptin.

METHODS

Prostate cancer cells (PC3, DU145, and LNCaP) were treated for 24 hours with human recombinant leptin at physiological and obese concentrations, 4 ng/mL and 100 ng/mL, respectively. Cells were then treated for 24 hours with androst-4-ene-3,17-dione (androstenedione), the substrate for aromatase. Aromatase activity was measured by the release of tritiated water from the radiolabled androstenedione precursor.

RESULTS

Aromatase activity was detected in all prostate cancer cells and was increased in response to increasing concentrations of the adipokine leptin in the androgen-independent DU145 and PC3 cell lines.

CONCLUSIONS

These findings suggest that aromatase stimulation via leptin may be a molecular link between obesity and increased prostate cancer proliferation, and that inhibition of this enzyme may be a potentially effective prostate cancer treatment.

BACKGROUND

Recently, prostate cancer prevalence has risen among men of the modern world to become the most common and second most fatal cancer (Zhu *et al.*, 2003). In 2005, 232,090 new cases of prostate cancer were diagnosed, approximately 25% more than the second most prevalent cancer among men: lung cancer, 172,570 cases. Over 30,000 deaths resulted from prostate cancer in 2005 (Jemal *et al.*, 2005). These statistics demonstrate the urgency in the determination of more effective treatments for this disease. The purpose of this paper is to determine the effects of the adipokine leptin on the activity of the enzyme aromatase in prostate cancer cells in order to predict the efficacy of a prostate cancer treatment involving the inhibition of aromatase.

There are two primary classifications of prostate adenocarcinomas, androgen-dependent and androgen-independent. As the names would imply, androgen-dependent prostate cancer cells require androgens for growth, and therefore express androgen receptors (AR) on the cell surface. To the contrary, androgen-independent prostate carcinomas do not require androgens, and thus do not express AR. Interestingly, in benign prostate cancer cells, AR serve as tumor

in the normal AR during malignancy lead to tumorogenic cells expressing AR (androgen-dependent) and tumorogenic cells not expressing AR (androgen-independent). Regardless of the early classification, all prostate cancer cells eventually become androgen-independent due to selection for this phenotype (Litvinov *et al.*, 2006).

Androgens are essential to the prostate for correct growth and maintenance; however, high levels of androgens can increase prostate cell proliferation, increasing the chances of a mutation in the DNA, and ultimately acting as a carcinogen. Long-term studies in both rats and humans have shown that prostate cancer risk increases with exposure to high levels of androgens. In addition, certain prostate cancer cell lines have shown a particular need for testosterone as an essential factor for growth and differentiation (Zhu *et al.*, 2006).

Prior to metastasis, prostate tumors are androgen-dependent, and treatable; however, once the cancer metastasizes it is usually fatal (Zhu *et al.*, 2003). Currently, androgen deprivation therapy (ADT), or androgen ablation, is used to successfully treat cancers that exhibit androgen-dependence. Significant tumor growth reduction and improved patient survival beyond three years have been noted following ADT (Diamond *et al.*, 2004). ADT may involve either surgical removal of the testes (orchietomy) or the administration of a gonadotropin-releasing hormone agonist. Over time, gonadotropin-releasing hormone agonists down-regulate the normal gonadotropin-releasing receptors on the pituitary, which prevents luteinizing hormone production, leading to halted testosterone production (Sharifi *et al.*, 2005).

There are two major drawbacks to ADT. First, even though ADT is a successful approach to combating prostate cancer, the median response time for patients receiving the treatment is less than two years (Smith *et al.*, 2002). This is because all prostate cancer cells eventually become androgen-independent, regardless of early stage androgen dependence. Also, it has been found that men undergoing ADT are more prone to osteoporosis and loss of bone density (Ross and Small, 2002). Abrahamsen *et al.*, (2007) found a significant increase in the likelihood of a bone fracture due to androgen deprivation, especially of the hip. Another study found that 20% of a group of men receiving ADT for 10 years had an osteoporotic fracture (Oefelein *et al.*, 2001). These drawbacks of ADT offer support for research seeking a more effective prostate cancer treatment.

At the present time, only age, family history, and race are known risk factors for the disease; however, research is currently investigating hormonal (such as androgens) and circadian disruptions as influences on prostate tumorigenesis (Zhu *et al.*, 2003). These known risk factors leave few avenues for research directed towards a more effective treatment or preventive measure. One hormonally-linked factor thought to increase prostate cancer risk is obesity. In recent decades, obesity has reached epidemic proportions in well-developed countries (Ribiero *et al.*, 2005) and is considered to be a major health issue negatively affecting the course of, or increasing the risk of, several diseases, including hypertension, heart disease, diabetes, and dyslipidemia (Goren *et al.*, 2008), as well as cancer. It has been reported that obese men at least 50% more likely to develop cancer of any type. Also, the mean mortality rate from prostate cancer increases from approximately 67 per 100,000 individuals with normal body mass index (BMI) to just over 87 per 100,000 in obese individuals (BMI > 35) (Calle *et al.*, 2003). The National Health and Nutrition Examination Survey from 1999-2000 revealed that approximately 30% of men in the United States are obese, defined as a BMI greater than or equal to 30 kg/m^2. This figure has tripled in recent decades as 10% of the men in the US were classified as obese in the 1960s (Flegal *et al.*, 2002).

Obesity can negatively affect prostate cancer cells in many different ways, such as speeding up the progression of the cancer in men diagnosed, or leading to a more dangerous stage of cancer, resulting in a prostatectomy at a much younger age (Onuma *et al.*, 2003). It is thought that obesity may not be a direct risk factor for prostate cancer, but rather obesity is thought to enhance the lethality of the disease (Frankenberry *et al.*, 2004). However, there is evidence to show that men living in Westernized cultures where high fat diets are prevalent are far more likely to develop prostate cancer. Yu *et al*, (1991) determined there to be a 26-fold higher incidence of prostate cancer in the United States than in China and concluded that this may be due to diets higher in fat. Figure 1 highlights the similarity in the increasing prevalence of obesity and prostate cancer (Ribeiro *et al.*, 2006). These corresponding trends further support that idea that obesity may be a risk factor for prostate cancer.

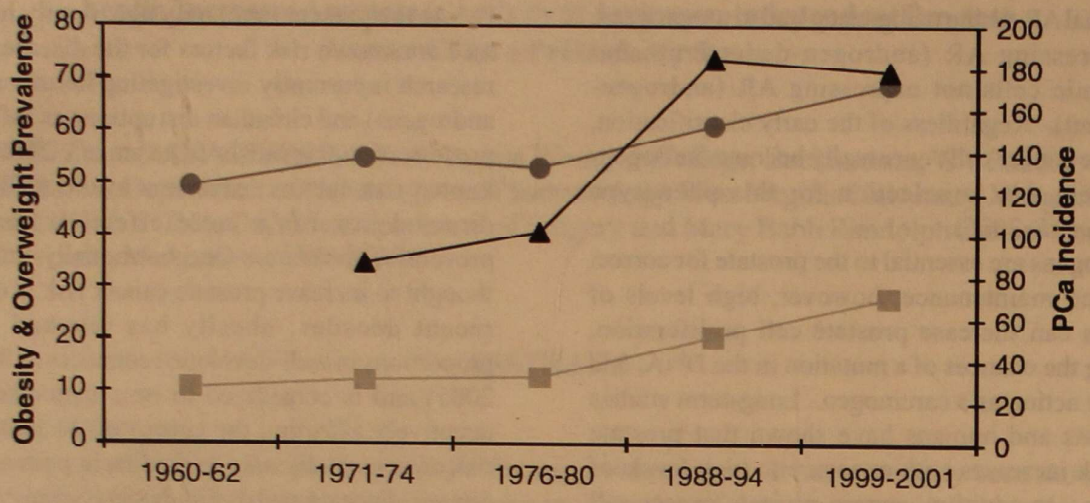

Figure 1. The trends in the prevalence of overweight and obese individuals, as well as prostate cancer, have increased since 1960. Obesity and overweight prevalence was measured as percent of population. Prostate cancer (PCa) incidence measured as number of cases per 100,000 individuals. Gray circles denote overweight; gray squares denote obesity; black triangles denote prostate cancer (Ribiero *et al.*, 2006).

The secretions of adipocytes (fat cells) have been proposed to be the link between prostate cancer and obesity (Ribiero *et al.*, 2006). The hormones and other major cytokines produced and secreted by adipocytes control body weight homeostasis by balancing the amount of energy that a person takes in and the amount of energy that is expended by that person. Leptin is one such protein hormone. It is encoded in the obesity gene (*ob*) and mutations of this gene can lead to type II diabetes mellitus, as well as profound obesity (Onuma *et al.*, 2003) Leptin exists at a higher concentration in the blood of obese individuals, and the hormone has been shown to act as a growth factor on many organs, both normal and obese, the prostate included (Somasundar *et al.*, 2003). Research has revealed that leptin may also affect prostate carcinomas, increasing proliferation of androgen-independent prostate cancer cells by inhibiting apoptosis (Somasundar *et al.*, 2004a).

The proposed normal role of leptin *in vivo* is to regulate body weight by decreasing appetite and increasing metabolism when bound to receptors on the hypothalamus. A strong positive correlation exists between leptin in circulation and both total body fat and adipocytes' volume. Given this, obese individuals possess exaggerated levels of plasma leptin. A lean person has approximately 4 ng/mL of leptin circulating in his blood, whereas an obese individual has greater than 100 ng/mL (Somasundar *et al.*, 2003). The proposed physiological role of leptin as an appetite regulator contrasts with its aberrant expression in obese individuals. Research has shown that even in obese individuals, plasma leptin levels drop during fasting (Wolf *et al.*, 2002) and increase after feeding (Dieudonne *et al.*, 2002), leading to the conclusion that leptin is an energy-balance signaling hormone (Ahima *et al.*, 1996 and Flier *et al.*, 1998).

In addition to increased adipose tissue, insulin and age may influence concentrations of plasma leptin. A study has demonstrated that leptin levels fluctuate along with insulin levels, increasing following meals and decreasing when insulin levels drop (Mueller *et al.*, 1998). Ahren *et al.*, (1997) report elevated plasma leptin levels in aged mice. The same result was demonstrated in humans (Li *et al.*, 1997). Other metabolic factors such as corticosterone and glucocorticoid, along with reproductive changes, such as puberty, may also have an effect on plasma leptin levels (Somasundar *et al.*, 2003). Regardless of these additional factors, plasma leptin levels are greater in obese individuals.

Given that obesity is a risk factor for cancer, possibly including that of the prostate, and that plasma leptin is elevated in obese individuals, it is logical to hypothesize a connection between leptin and cancer. The aforementioned study by Somasundar *et al.* (2003) revealed the likely effects of leptin on prostate carcinomas (increased cell proliferation through

inhibited apoptosis). Since a mechanism by which leptin directly affects the prostate cancer cells has not been proposed and supported, it is logical to assume that there may be a missing link between leptin and its subsequent effects on prostate cancer cells.

Aromatase may be this molecular link that bridges obesity and prostate cancer. Aromatase is an enzyme that is involved in the production of estrogen that acts by catalyzing the conversion of testosterone to estradiol (Bray, 2002). Research has found that aromatase expression in breast tissue is greatest in the vicinity of tumors, and aromatase inhibitors have been developed and used to successfully treat breast cancer (Brueggemeier *et al.*, 2005). Catalano *et al.* (2003) found that leptin stimulates increased aromatase mRNA expression, content, and enzymatic activity in breast cancer cells. It is plausible that aromatase may have a similar effect on the prostate for two reasons. First, prostate cancer cells have been found to express aromatase activity, while benign cells have not (Ellem *et al.*, 2004). Secondly, prostate cancer cells express at least one type of estrogen receptor on the cell surface. Estrogen receptor-β (ERβ) is expressed in the stroma and the epithelial cells of the prostate (Taylor and Al-Azzawi, 2000), leading to the idea that estrogens have a direct effect on prostate cells. In addition, the International Agency for Research on Cancer categorizes estrogens as carcinogenic (Liehr, 2000). Together, this evidence supports the idea that increased aromatase activity in the prostate, and thereby increased local estrogen levels, may influence prostate cancer cell activity.

Information regarding the effects of high leptin levels on aromatase activity is lacking. The aforementioned evidence suggests that increased aromatase activity may be implicated as a cause of prostate cancer cell proliferation, and inhibition of this enzyme may slow the progression of the disease. Therefore, we hypothesize that aromatase activity will increase with increasing concentrations of the adipokine leptin in androgen-dependent and -independent prostate carcinomas.

METHODS AND MATERIALS

CELL CULTURE

The androgen-dependent cell line LNCaP, and the androgen-independent cell lines DU145 and PC3 were obtained from the American Type Culture Collection (Gibco, Rockville, MD) and maintained at 37°C in a 5% CO_2 atmosphere. DU145 and LNCaP cells were maintained in RPMI-1640 media (Seromed Biochrom KG, Berlin, Germany) and PC3 cells were maintained in Hamm's F12K media (Mediatech, Inc., Herdon, VA). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mmol/L of glutamine (Seromed Biochrom, KG), and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin sulfate; Seromed Biochrom, KG).

AROMATASE ASSAY

PC3, DU145, and LNCaP cells were plated in duplicate in 6-well plates at $1x10^6$ cells per well and allowed to attach overnight. Cells were serum-starved for 22 hours to rid the medium of any growth hormones present in the serum, and also to synchronize the cells to the same phase of cell division. The cells were then treated for 24 hours with human recombinant leptin (R&D Systems, Minneapolis, MN) diluted in serum-free medium at both a normal physiological concentration (4 ng/mL) and a concentration typically found in morbidly obese individuals (100 ng/mL). Cells were then rinsed in 1X phosphate buffered saline and treated for 24 hours with androst-4-ene-4-3,17-dione (androstenedione), the substrate for aromatase. One milliliter of medium was collected and filtered through a charcoal/dextran pellet twice for 30 minutes each to remove excess substrate. Aliquots (300 mL) were added to excess scintillation fluid for counting. Aromatase activity was measured by the release of tritiated water from the radiolabled androstenedione precursor. Aromatase activity was expressed as concentration of substrate produced in picamoles (pmol) per mg of total protein.

PROTEIN ASSAY

A total protein assay was carried out to provide a basis for comparing aromatase activity of different cells. The cells were lysed in 300 uL cold RIPA buffer (25mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and scraped gently to lift from the plate. Cells were centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatant was stored on ice. A BCA protein assay was performed per instructions to determine the total protein of the cells.

STATISTICAL ANALYSIS

Data were analyzed in JMP© software using a Student's t-Test for all cell lines. The p-value was set at 0.05. Means are reported with standard errors.

RESULTS

We examined the ability of leptin to stimulate an increase in aromatase activity in the androgen-dependent LNCaP prostate adenocarcinomas and in the androgen-independent DU145 and PC3 prostate adenocarcinomas. Aromatase activity was detected in the LNCaP cell line and increased from 1.887E-3 ± 7.773E-05* for the control to 4.735E-3 ± 5.46E-4 for the 4 ng/mL leptin treatment group. The aromatase activity was 3.044E-3 ± 3.06E-4 for the 100 ng/mL treatment group. There was no statistical difference between either of the treatment groups and the control (Figure 2).

Aromatase activity was detected in the DU145 cell line and was significantly increased from 2.179E-3 ± 1.77E-4 to 5.006E-3 ± 4.66E-4 for the 100 ng/mL treatment group ($p<0.05$) (Figure 3). Aromatase activity was detected in the 4 ng/mL treatment group at 3.778E-3 ± 6.71E-4, but this was not significantly different from either the control or the 100 ng/mL treatment group.

Aromatase activity was also detected in the PC3 cell line at and was significantly from 7.945E-3 ± 2.22E-4 for the control to 9.053 ± 1.18E-4 for the 4 ng/mL treatment group and to 9.891E-3 ± 4.53E-4 for the 100 ng/mL treatment group (Figure 4). There was no significant difference between the 4ng/mL and the 100 ng/mL treatment groups.

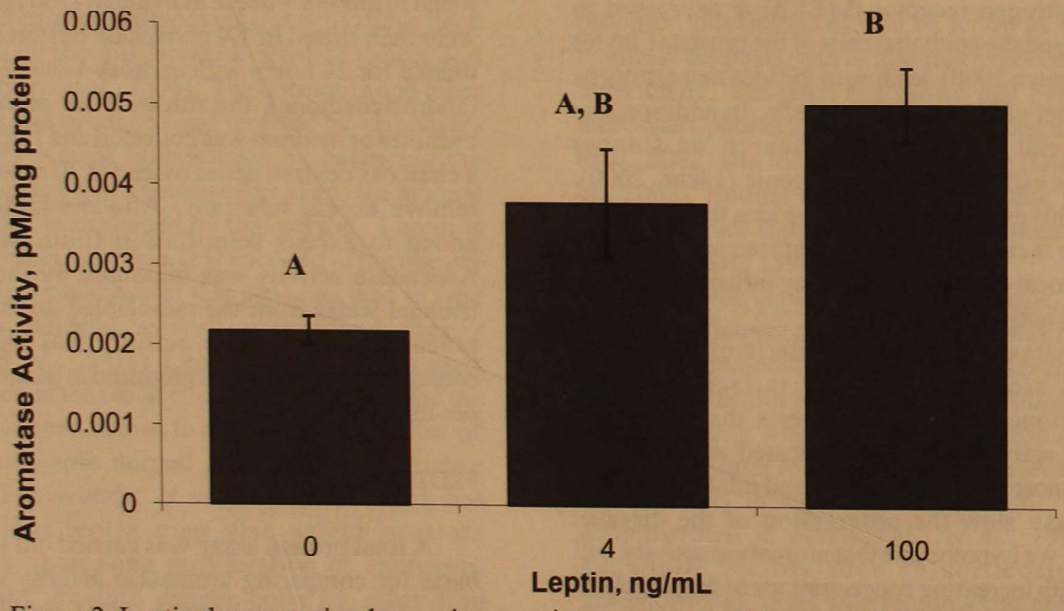

Figure 2. Leptin does not stimulate an increase in aromatase activity in androgen-dependent LNCaP cells. Groups not connected by the same letter are significantly different ($p<0.05$). Error bars represent the standard error of the mean.

*All values reported as mean ± SEM in picomoles of substrate cleaved per milligram of total protein.

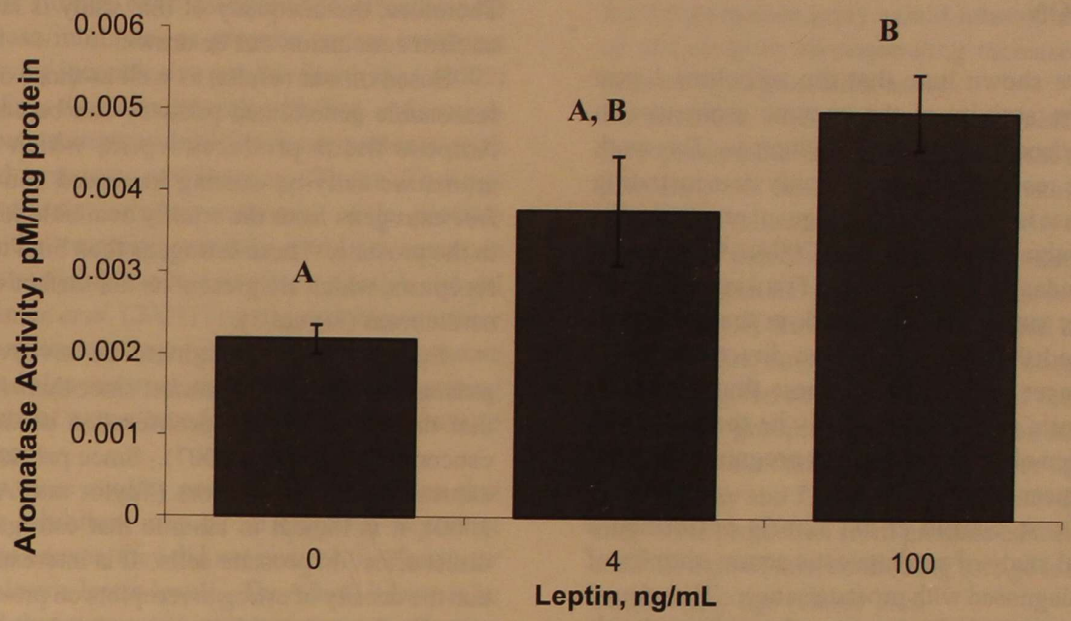

Figure 3. Leptin stimulates an increase in aromatase activity in androgen-independent DU145 cells. Groups not connected by the same letter are significantly different ($p=<0.05$). Error bars represent the standard error of the mean.

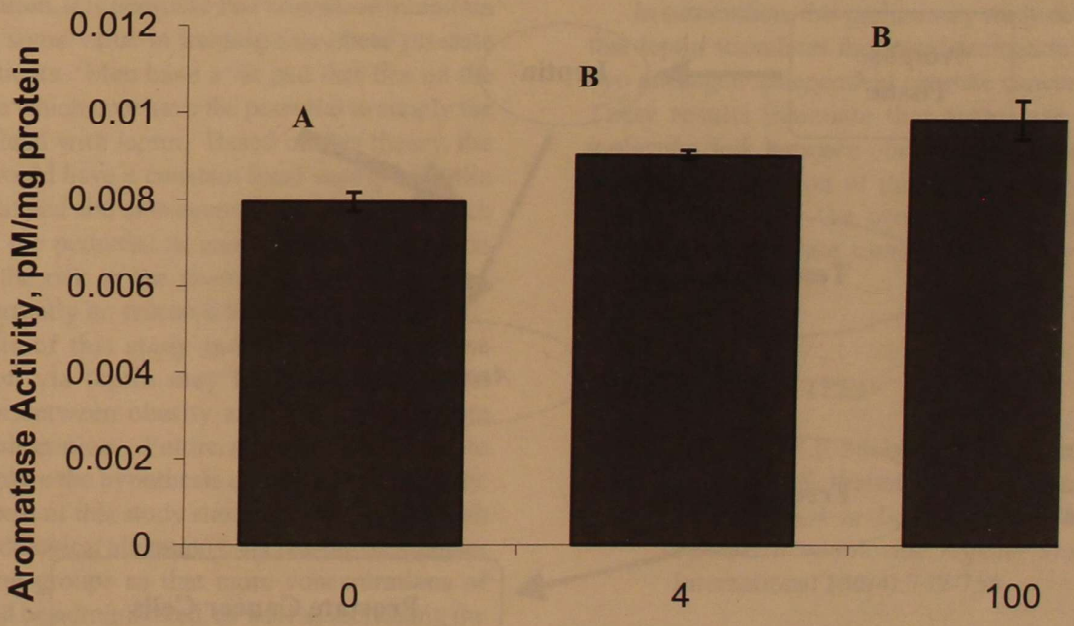

Figure 4. Leptin stimulates an increase aromatase activity in androgen-independent PC3 prostate cancer cells. Groups not connected by the same letter are significantly different ($p<0.05$). Error bars represent the standard error of the mean.

DISCUSSION

We have shown here that the adipokine leptin increases the activity of the enzyme aromatase in androgen-independent prostate carcinomas. This work supports the results of an earlier study demonstrating that aromatase is expressed in malignant prostate cells, but not in benign cells (Ellem *et al.*, 2004). Also, studies by Somasundar *et al.* (2004a) and Frankenberry *et al.* (2004) offer support for this work in that they both demonstrated that leptin can have direct effects on prostate cancer cells. Together, these findings imply that that leptin and aromatase may be the molecular links between obesity and the poor prognosis of obese prostate cancer patients.

In contrast, Stattin *et al.*, (2003) performed a longitudinal study of prediagnostic serum samples of men later diagnosed with prostate cancer. They found no significant association between plasma leptin levels and prostate cancer risk; however, they could not conclude that elevated plasma leptin levels correlate with increased disease progression. In addition, one aspect of this study compromises its legitimacy. The mean time reported between blood collection and prostate cancer diagnosis was 17 years. This seems to be an ample window of time for the development of obesity and onset of prostate cancer due to this obesity. Therefore, the adequacy of this study is lacking and no firm conclusion can be drawn.

Based on our results, as well as those of others, a reasonable generalized pathway can be constructed. Adipose tissue produces leptin, which increases aromatase activity, causing increased conversion of free estrogens from the readily available testosterone in the prostate. These estrogens then bind to estrogen receptors, which are present on the surface of prostate carcinomas (Figure 5).

Estrogens are thought to be involved in the pathogenesis of prostate cancer since there is evidence that they stimulate the proliferation of the prostate cancer cells (Carruba, 2007). Since prostate cells do express estrogen receptors (Taylor and Al-Azzawi, 2000), it is logical to assume that estrogens have a direct effect on prostate cells. It is interesting to note that the density of estrogen receptors on prostate cancer cells has been found to increase following androgen deprivation therapy (ADT) (Kruithof-Dekker *et al.*, 1996). By starving prostate cells of androgens, the substrate for aromatase has thereby been removed and no estrogen is formed through this process. Thus, the cells are deprived of estrogen and it is possible that the up-regulation of estrogen receptor expression is a cellular response in an attempt to bind more of the hormone.

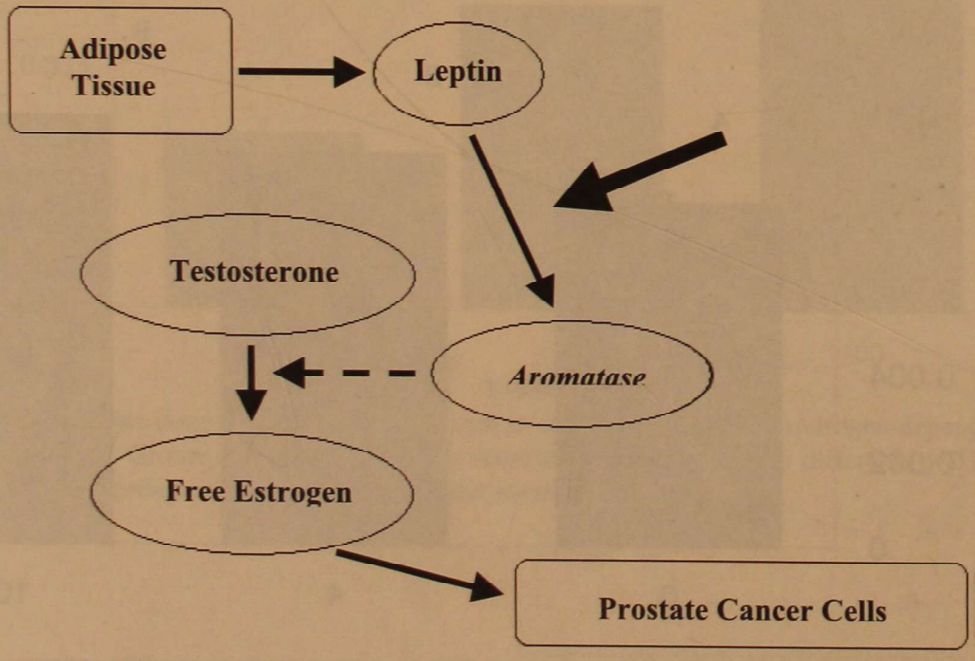

Figure 5. A proposed general pathway though which adipose tissue effects prostate cancer cells. Adipose tissue secretes leptin, which stimulates the activity of the enzyme aromatase, leading to enhanced conversion of testosterone to estrogen, which then acts on the prostate cancer cells. The bold arrow specifies the mechanism step that is the subject of this study.

Recent studies have assessed the effectiveness of aromatase inhibitors as prostate cancer treatment with relatively inconclusive results. Smith *et al.* (2002) report findings from clinical trials with first- and second-generation aromatase inhibitors on patients with advanced prostate carcinomas. These findings demonstrate relatively low responses of the prostate tumors to the treatment, yet they could not conclude that aromatase inhibitors had no effect. Another clinical trial by Santen *et al.* (2001) investigated the aromatase inhibitor anastrozole on patients with advanced prostate cancers and also reported inconclusive results, stating that no patient experienced an objective response to the treatment.

While these studies provide little evidence that aromatase inhibitors may be an effective treatment for prostate cancer, they did not control for obesity or elevated plasma leptin levels. Therefore, it cannot be concluded that aromatase inhibitors are not effective prostate cancer treatments for obese individuals. Our work focuses on increased aromatase activity in response to leptin, which is found in higher concentrations in obese individuals. It is possible that aromatase inhibitors may be effective in the treatment of prostate cancers in obese men, as inhibition of the enzyme aromatase could offset the effects of high plasma leptin levels and slow the progression of the disease.

In addition, it is plausible that aromatase inhibition may have some value in treating non-obese prostate cancer patients. Men have a fat pad that lies on the pubic bone which may have the potential to supply the prostate gland with leptin. Based on this theory, the prostate would have a constant local supply of leptin from the fat pad and androgens from the testes which may have the potential to increase the proliferation and even the risk of the prostate cancer. However, there is currently no research to support this theory.

The results of this study indicate that aromatase stimulation via leptin may be implicated in the correlation between obesity and increased prostate cancer proliferation. Future research is merited to further explore the hypothesis examined in this study. First, a repeat of this study should be carried out with two methodological alterations. Increasing the number of treatment groups so that more concentrations of leptin could be administered, as well as increasing the number of replicates in each treatment group, may improve the consistency of the data and therefore yield more statistically significant results. In addition, a Western blot separation of the cell lysates collected for the aromatase assay would substantiate the results of this study by demonstrating increased aromatase activity through another assay.

Furthermore, studies on aromatase inhibitors should incorporate both laboratory and clinical components. The chemical structure of the aromatase inhibitors should be evaluated via structure-activity relationships in order to determine if minor molecular alterations, such as functional group substitutions, could increase the potency of the inhibitors. Additional clinical trials are called for, but should control for obesity and age. Controlling for obesity would reveal the efficacy of the current group of aromatase inhibitors in treating obese prostate cancer patients. As for age, a review by O'Malley and Taneja (2006) concluded that obesity may offer protection from prostate cancer in younger men. In light of this, controlling for age is necessary to remove any age related bias.

Finally, the concentrations of leptin necessary to induce proliferative effects of prostate cancer cells should be established. The pubic fat pad that lies on the pubic bone may supply the prostate with a local supply of leptin; however, it is unknown if this concentration would be sufficient to affect the prostate in either a normal or diseased state. Determining if the secretion of leptin from the pubic fat pad is sufficient may reveal whether or not the effectiveness of aromatase inhibition is strictly limited to obese patients.

In summation, this preliminary study demonstrates that leptin stimulates increased aromatase activity in two androgen-independent prostate cancer cell lines. These results insinuate that aromatase may be a molecular link between obesity and prostate cancer. Successful inhibition of this enzyme may have the potential to slow the progression of androgen-independent prostate tumors, especially in obese patients.

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APPENDIX

LSMeans Differences Student's t - LNCAP

α=
0.050 t=
3.18245
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	0	4	100
0	0 0 0 0	-0.0012 0.00051 -0.0029 0.00039	-0.0006 0.00051 -0.0022 0.00106
4	0.00125 0.00051 -0.0004 0.00289	0 0 0 0	0.00067 0.00051 -0.001 0.0023
100	0.00058 0.00051 -0.0011 0.00222	-0.0007 0.00051 -0.0023 0.00097	0 0 0 0

Level		Least Sq Mean
4	A	0.00218729
100	A	0.00152200
0	A	0.00093860

Levels not connected by same letter are significantly different.

LSMeans Differences Student's t – DU145

α=
0.050 t=
3.18245
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	0	4	100
0	0 0 0 0	-0.0016 0.00063 -0.0036 0.00037	-0.0028 0.00063 -0.0048 -0.0008
4	0.00162 0.00063 -0.0004 0.00361	0 0 0 0	-0.0012 0.00063 -0.0032 0.00078
100	0.00283 0.00063 0.00084 0.00482	0.00121 0.00063 -0.0008 0.0032	0 0 0 0

Level			Least Sq Mean
100	A		0.00500627
4	A	B	0.00379883
0		B	0.00217875

Levels not connected by same letter are significantly different.

LSMeans Differences Student's t – PC3

α=
0.050 t=
3.18245
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	0	4	100
0	0 0 0 0	-0.0017 0.00042 -0.0031 -0.0004	-0.0029 0.00042 -0.0043 -0.0016
4	0.00173 0.00042 0.00038 0.00307	0 0 0 0	-0.0012 0.00042 -0.0026 0.00013
100	0.00295 0.00042 0.0016 0.00429	0.00122 0.00042 -0.0001 0.00257	0 0 0 0

Level			Least Sq Mean
100	A		0.01039053
4	A		0.00917083
0		B	0.00744473

Levels not connected by same letter are significantly different.

A Review of Nesting Ecology and Larval Period for *Desmognathus ochrophaeus* (Amphibia: Caudata) with Specific Comments for Populations in West Virginia

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ABSTRACT

Seventeen nests of the Allegheny Mountain dusky salamander, *Desmognathus ochrophaeus,* were discovered in shallow depressions within the stream bank in Camp Hollow at Fernow Experimental Forest in Tucker County, West Virginia. Nests were typically found in a substrate of soil and pebbles associated with water. At this stream, egg deposition occurs from late July to mid-August for *D. ochrophaeus*. The mean clutch size was 12.0 (± 4.5), which is consistent with clutch sizes from *D. ochrophaeus* populations in Virginia and North Carolina, but this is smaller than what has been reported for some populations in Ohio. Mean snout-vent length (SVL) of larvae was 8.7 mm. However, this SVL was smaller than hatchlings reported in other parts of the range. The larval period in northern West Virginia was brief, lasting one to three weeks, and the larvae transformed in or near the nest at or near their hatchling SVL.

INTRODUCTION

The Allegheny Mountain dusky salamander, *Desmognathus ochrophaeus*, is part of the *D. ochrophaeus* species complex. This species complex has experienced much taxonomic controversy and instability in the past due to the presence of complex geographic patterns of genetic diversity and the presence of extreme morphological conservatism. For example, Tilley and Mahoney (1996) split populations of the *D. ochrophaeus* species complex into four distinct species based on allozyme variation. Currently, these species include *D. ochrophaeus* in eastern Tennessee and southwestern Virginia to New York and southern Quebec; *D. orestes* in southwestern Virginia, northeastern Tennessee, and northwestern North Carolina; *D. carolinensis* in east-central Tennessee and west-central North Carolina; and *D. ocoee* in southeastern Tennessee, southwestern North Carolina, northern Georgia, and northeastern South Carolina. Due to the recent description of new species from what was once thought to be a single species, *D. ochrophaeus*, and the presence of widespread geographic variation for many traits (e.g., color, pattern, size at sexual maturity, time of reproduction; Petranka 1998), the literature on these species contains numerous ambiguities and points of confusion and controversy (Tilley 1973).

The reproductive ecology of *D. ochrophaeus* has been shown to vary among local populations (Bishop and Chrisp 1933; Tilley 1974; Keen and Orr 1980; Bruce 1990). For instance, Keen and Orr (1980) reported a larval period of eight months for Ohio populations, and Bishop and Chrisp (1933) reported that the larval period lasted from a few days to a few weeks for populations in Pennsylvania. The nesting ecology and reproductive habits of *D. ochrophaeus* have been documented throughout its range, except in West Virginia. The present study aims to describe the nesting behavior and reproductive ecology of *D. ochrophaeus* in northern West Virginia. Specifically, the purpose of this study is: (1) to describe ecological characteristics, including substrate type, temperature preference, and moisture content, of nesting sites; (2) to determine the length of the larval period and the size of hatchlings; and (3) to compare findings of the reproductive habits of *D. ochrophaeus* in West Virginia with published data from across the known geographic range, and make comparisons with the additional members of this species complex.

MATERIALS AND METHODS

This study was conducted in Camp Hollow at Fernow Experimental Forest, which is owned and managed by the United States Forest Service (USFS), Tucker County, in northern West Virginia. The study was confined to the stream bank of Camp Hollow, a slow-flowing, first-order stream located in a mountainous, mixed mesophytic forest at a maximum elevation of 868m.

We searched for nesting sites for *D. ochrophaeus* by hand from June through August 1993 by removing the upper layers of soil and rocks in stream banks and searching beneath rocks and in rock crevices. When a nest was discovered, eggs were counted and substrate temperature recorded. Substrate samples were taken from four different nesting areas. In the laboratory, each sample was divided into four subsamples for repeatability. Each subsample was weighed to the nearest 0.01 g and then dried at 21°C for 24 h. The subsamples were then reweighed to the nearest 0.01 g and the moisture content of each subsample was calculated.

Snout-vent length (SVL), the length of the salamander from the tip of the snout to the posterior portion of the cloaca, was taken for larvae and transformed juveniles. It was noted if the larva possessed a remnant yolk plug. These data were used to determine size at hatching and transformation for *D. ochrophaeus* at this study site. In addition, notes were made on the status of the gills, noting whether they were reduced or completely absorbed.

RESULTS

Seventeen nests of *D. ochrophaeus* were found in interstitial spaces within the stream bank. Nesting cavities were shallow, concave depressions, with only enough space for the eggs and female. In all cases, the female was found guarding the nest. Eggs were arranged in grape-like clusters, each containing individual eggs attached to the ceiling of the cavity by a short, gelatinous stalk. All nests were found in a substrate of soil and small gravel with seeping water. On average, the stream bank substrate temperature was 16°C, and the average substrate moisture content across the 17 nesting sites was 29%.

The first nest was found on 25 July 1993, and we judged this nest to be only a few days old since egg division was not yet evident by visual inspection (Tilley 1972). Based on the early developmental stage of the eggs in this nest and later developmental stages of eggs in nests found after 25 July 1993, we determined that egg deposition in northern West Virginia for *D. ochrophaeus* occurs from late July to mid-August. We calculated the mean (± SD) clutch size in northern West Virginia to be 12.0 ± 4.5 (range 3 – 19; n = 17). Seventeen larvae were found on 10 October 1993 and all had abdomens filled with yolk plugs and exhibited no signs of intestinal or liver development, indicating that eggs had recently hatched (Montague 1987). Ten of the 17 hatchlings showed signs of gill reduction or complete loss of gills, indicating a short larval period of a few weeks. Mean SVL of these larvae was 8.7 mm (range 8 – 9.5 mm; n = 17), which was smaller than those of hatchlings reported in other parts of the range of *D. ochrophaeus* (Bishop and Chrisp 1933; Tilley 1974; Keen and Orr 1980; Bruce 1990).

Additional evidence for a short larval period in northern West Virginia was found on 25 February 1994, when two recently transformed juveniles were captured in the stream bank and two others were found in the stream. These transformed juveniles showed signs of yolk plug remnants that could be seen through the abdominal wall. The large intestine and liver were forming; however, yellow yolk still remained visible indicating that these were recent hatchlings. Based on the reduction or loss of gills of the larvae found on 10 October 1993 and juveniles found on 25 February 1994, we determined that the larval period in northern West Virginia is often as brief as one week, but may last up to three weeks. Larvae transform in or near the nest at the SVL near their hatching size.

DISCUSSION

The reproductive ecology, including nesting habits, length of larval period, and size at metamorphosis and hatching, of *D. ochrophaeus* varies across the known geographic range and among the species within the complex. For instance, *D. ochrophaeus* exhibits a stronger tendency to build underground nests than do other, more southern, members of the *D. ochrophaeus* complex (Keen and Orr 1980; Petranka 1998). In northern West Virginia, we found that *D. ochrophaeus* nests within the interstitial spaces of a stream bank. In contrast, in northern Pennsylvania, Bishop and Chrisp (1933) discovered nests of *D. ochrophaeus* in shallow excavations underneath logs and rocks in moist areas. In Ohio (Orr 1989) and southwestern Virginia (Wood and Wood 1955), nests of *D. ochrophaeus* were found within the banks of seepages. For *D.*

orestes, Organ (1961) reported that nests in Virginia were underground and on the surface under cover objects such as rocks, leaf litter, or moss mats in or adjacent to a small stream or seep (Petranka 1998); *D. ocoee* and *D. carolinensis* exhibit similar nesting habits in North Carolina and Tennessee (Martof and Rose 1963; Tilley 1973; Forester 1979; Bruce 1990).

Dates of oviposition in the literature for species within the *D. ochrophaeus* complex begin as early as March, peak in mid-May, and continue into September (Bishop and Chrisp 1933; Fitzpatrick 1973; Huheey and Brandon 1973; Tilley 1973; Keen and Orr 1980; Orr 1989). We determined that oviposition occurs in late July and extends into August in northern West Virginia. This is comparable to other northern *D. ochrophaeus* populations. Bishop and Chrisp (1933) determined that egg deposition occurs in August in Pennsylvania and the same was true for populations in northwestern Ohio (Keen and Orr 1980). For each of the species in the *D. ochrophaeus* complex, females reproduce annually (Organ 1961; Petranka 1998); however, there have been reports of biennial reproductive cycles (Organ 1961; Tilley and Tinkle 1968). For West Virginia, data on the frequency of reproduction are unknown, and this is an area in need of future research (Green and Pauley 1987). However, we predict that *D. ochrophaeus* reproduces annually in northern West Virginia, as they do in Pennsylvania (Bishop and Chrisp 1933). It is unknown if they reproduce annually or biennially in Ohio (Orr 1989).

Generally, eggs of *D. ochrophaeus* are deposited in small grape-like clusters in concave depressions (Petranka 1998) with each egg individually attached to the overhead substrate with a gelatinous stalk. Additional eggs may be laid singly (Petranka 1998) or in small numbers next to the main grape-like cluster (Bishop 1941). Organ (1961), for *D. orestes*, and Bishop and Chrisp (1933), for *D. ochrophaeus*, described egg masses as resembling a cluster of grapes that may be twisted. We found eggs with tending females in shallow, concave nesting cavities in northern West Virginia. At these nesting sites, eggs were arranged in grape-like clusters attached to the cavity's ceiling with a single gelatinous stalk. The mean number of eggs reported per clutch for *D. orestes* is 10 in Virginia (Organ 1961); for *D. carolinensis*, 12 in rock-face and 17.5 in woodland populations in North Carolina (Tilley 1974); and for *D. ochrophaeus* 16 in Pennsylvania (Hall 1977), 19 in Ohio (Pfingston 1966), and 12 (this study) to 19 or more in West Virginia (Green and Pauley 1987). Eggs for *D. carolinensis* hatch from summer to early spring in North Carolina or in September, depending on habitat type (Tilley 1973; 1974); for *D. orestes* from August to October in Virginia (Organ 1961); and for *D. ochrophaeus* from September to March in Pennsylvania (Bishop and Chrisp 1933), September and October in Ohio (Keen and Orr 1980; Orr 1989); and October to March in New York (Bishop 1941). In northern West Virginia, we suggest that *D. ochrophaeus* eggs hatch between October and late February.

Previous research has shown that the larval period for species within the *D. ochrophaeus* complex may last from two to three weeks to as long as eight months (Bishop and Chrisp 1933; Keen and Orr 1980). Eaton (1956) found that the larval period terminates shortly after hatching; whereas, Dunn (1917) found that the larval period apparently occurs while in the egg. The length of the larval period for *D. orestes* is 11 months in Virginia (Organ 1961); nine to 10 months in North Carolina for *D. ocoee* (Bruce 1989); two to three months in North Carolina for *D. carolinensis* (Tilley 1973); and for *D. ochrophaeus,* one to eight months in Ohio (Keen and Orr 1980), few days to a few weeks in Pennsylvania (Bishop and Chrisp 1933), and one to three weeks in West Virginia (this study). Factors, such as seasonal drying patterns of aquatic habitats and time of oviposition, may directly affect the length of the larval period (e.g., eggs laid in spring typically transform in early autumn; whereas, eggs deposited in summer may overwinter; Petranka 1998). Transformed larvae have been collected from March through October across the geographic ranges of the species within this complex (Petranka 1998). Seasonal timing of oviposition is poorly documented throughout portions of the geographic range of *D. ochrophaeus* (Petranka 1998) and more research is necessary to improve the understanding and knowledge of the reproductive habits of species within this complex.

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Status of the Federally Threatened Cheat Mountain Salamander, *Plethodon nettingi* (Amphibia: Caudata): Sixty years later

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ABSTRACT

In 1948, Maurice Brooks described the habitat, seasonal and daily activities, reproduction, and sympatric species of the Cheat Mountain Salamander (*Plethodon nettingi*) at four localities. In 1976, I began studying this species and during the ensuing 32 years have examined over 1300 sites. In this report, I compare my data for these four topics with data presented by Brooks 60 years ago.

INTRODUCTION

Plethodon nettingi (Cheat Mountain Salamander) is endemic to five counties in the high Allegheny Mountains of eastern West Virginia (Pauley 2007). Graham Netting and Leonard Llewellyn first observed *P. nettingi* on Barton Knob (Randolph County) in 1935 (Brooks 1948). In 1938, N. Bayard Green described it as a new species based on specimens collected at Barton Knob. I reported previously (Pauley 1991; 2005) that *P. nettingi* was first found on White Top (Randolph County), but after searching early documents by N. Bayard Green (1938) and Maurice Brooks (1948), I discovered that it was first found on Barton Knob, not White Top.

Brooks collected *P. nettingi* on more than 50 occasions at four localities between 1939 and 1947. He reported his findings over these nine years in 1948. I have conducted surveys for *P. nettingi* since 1976 (32 years), and during this time I have examined over 1300 sites and have found them in approximately 135 locations. I conducted areal extent studies in 18 locations and found that several sites were part of the same population. It now appears there are approximately 80 disjunct populations within the total range of *P. nettingi*.

In this manuscript, I compare the current *P. nettingi* range, habitat, seasonal and daily activities, reproduction, and sympatric species with what Brooks (1948) reported 60 years ago.

METHODS

Brooks (1948) and I used similar survey methods. Day surveys involved overturning rocks, logs, and searching leaf litter, while night surveys entailed searching the forest floor for salamanders with the aid of flashlights. We both identified salamanders to species, described the range and habitat characteristics, recorded nest information, and noted associated salamander species.

RESULTS AND DISCUSSION

RANGE

Brooks (1948) described the known range of *P. nettingi* as "...the high, southern portion of the Cheat Mountains, in Randolph and Pocahontas counties, West Virginia." He continued with a detailed description of the range by stating, "It has been found from a point along the headwaters of Condon Run, near Bickle's Knob, Randolph County, on the north, to the southern end of the Cheat Range at Thorny Flat, Pocahontas County, on the south. This is an air-line distance of almost exactly 45 miles. Its known altitudinal range is between 3557 feet [1084 m] at Cheat Bridge, and 4842 feet [1476 m] atop Bald Knob, Pocahontas County, the highest point in the Cheat system."

Currently, the total range of *P. nettingi* extends north to south for approximately 92km (57.2 miles) from Blackwater River Canyon (Tucker County) to Thorny Flat on Cheat Mountain (Pocahontas County) (Pauley 2007). The west to east range varies in distance from less than 3.2 km (2.0 miles) at the southern tip of the range to approximately 31km (19.3 miles) near the northern end (Pauley 2007). The altitudinal distribution ranges between 2000 ft (610 m) in the Blackwater Canyon to 4,862 ft (1482 m) at Spruce Knob (Pauley, unpublished data). The known range today is greater than what Brooks described, presumably because I have searched many more areas than Brooks.

Brooks reported *P. nettingi* at four localities, Gaudineer Knob, Barton Knob, Bickle Knob, and

Thorny Flat. After 60 years, *P. nettingi* still occur at Gaudineer Knob, Barton Knob, and Thorny Flat. I have searched Bickle Knob for *P. nettingi* without success but have found them on Stuart Knob, approximately 1.5 miles east of Bickle, and east to Condon Run. Brooks (1945; 1948) discusses the abundance of *P. nettingi* 1.5 miles east of Bickle Knob to the headwaters of Condon Run. I believe Brooks' Bickle Knob site is the same area where *P. nettingi* occurs today.

Timber on Barton Knob has been cut at least once since 1948, and this site was strip mined for coal on the west, north, and south sides. The population today is restricted to the north-facing slope (Pauley, unpublished data). Brooks (pers. comm.) found *P. nettingi* at Thorny Flat from West Virginia Route 66 upslope to the ridge. After numerous searches there, I have located them only on the ridge (Pauley 2007).

HABITAT

Originally, *P. nettingi* habitat probably consisted of a red spruce (*Picea rubens*) forest, pure or mixed with yellow birch (*Betula alleghaniensis*), with a *Bazzania*-covered floor. After the original forest was cut and burned between 1890 and 1920 many red spruce forests were replaced with deciduous tree species and ground cover of mosses and a variety of herbaceous plants (Pauley 2008).

Brooks stated that throughout its known range in 1948, *P. nettingi* appeared "...to be limited to nearly pure stands or red spruce, or to forests in which red spruce is a prominent species." He found *P. nettingi* in higher numbers in younger timber than in mature stands. He also reported that boulders and small rocks, usually covered with moss, were typically at the surface or just below it.

After characterizing the habitat of the 80 known populations and over 1000 locations with appropriate elevation ranges where *P. nettingi* does not occur, I concluded that the typical habitat today is stands of conifers such as red spruce and occasionally eastern hemlock (*Tsuga canadensis*) or stands of mixed deciduous forests at elevations above 610m (2000 ft) in the northern part of the known range to above 1067m (3500 ft) in the southern part of the known range (Pauley 2005; 2007). The liverwort *Bazzania trilobata* is usually present on the forest floor. Brooks alluded to the presence of moss, which was presumably *Bazzania*.

SEASONAL AND DAILY ACTIVITIES

As expected, daily activity of *P. nettingi* has remained the same over these 60 years, i.e., they take refuge in decayed logs or under logs, rocks, and litter during the day and emerge at night to forage on the forest floor.

The earliest date I have found *P. nettingi* on the surface is March 24, 1979 (Stuart Knob) and latest October 18, 1987 (Spruce Knob). Brooks' earliest collection was April 10, 1945 (Gaudineer Knob) and latest was October 19, 1943 (Gaudineer Knob). Their presence on the surface is temperature and moisture dependent, thus dates of emergence and submergence depend on these environmental factors and can vary from year to year (Pauley 1978a; 1978b; Pauley 2005).

REPRODUCTION

Time of mating and egg deposition appear to have remained constant over the years. Mating probably occurs in late April or May but there may be an abbreviated mating period in late September and early October (Pauley, personal observation). Females lay eggs in well-decayed spruce logs or under rocks and logs (Brooks 1948; Green and Pauley 1987; Pauley personal observations).

Brooks (1948) reported 29 nests from May 28 to August 25. He found the number of eggs ranged from 4 to 17 (average = 9.5). I have observed 25 nests from May 15 to July 26. The number of eggs ranged from 5 to 11 (average = 8.5). I have found two nests with neonates and attending females in September indicating that eggs hatch in about four months. The discrepancy between the number of nests Brooks found (n=29) during five years and I found during 32 years (n=27) is most likely because I avoided tearing logs apart in order to preserve nesting habitat. All nests Brooks found were in well-decayed spruce logs, and of the 27 nests I observed, 24 were under rocks and three under logs. Decayed logs are important nesting sites that if destroyed could be detrimental to the status of some populations. Brooks and his colleagues were not aware of the potential fragile status of the salamander because the United States Fish and Wildlife Service did not list *P. nettingi* as a threatened species until 1989.

SYMPATRIC SPECIES

Brooks found *Desmognathus ochrophaeus* (Allegheny Mountain duskysalamander), *P. cinereus* (Eastern Red-backed Salamander), and *P. wehrlei* (Wehrle's Salamander) associated with *P. nettingi*. I

found these same species associated with *P. nettingi* in addition to *P. glutinosus* (Northern slimy salamander). My records show *P. cinereus* to be the most abundant sympatric species followed by *D. ochrophaeus*, *P. wehrlei*, and *P. glutinosus*. I found *P. cinereus* in 89% of the *P. nettingi* populations, *D. ochrophaeus* in 79%, *P. wehrlei* in 64%, and *P. glutinosus* in 56%. Brooks (1945) reported finding just two *P. glutinosus* at one (Bickle Knob) of the four *P. nettingi* sites, he studied. He collected *P. glutinosus* in deciduous forest at high elevations but not in the spruce forest. I observed them in over half of the known *P. nettingi* populations, but at fewer numbers than the other three species. Brooks (1945) commented on the unusual absence of *P. cinereus* in the Bickle Knob vicinity, but I found them to be common throughout the entire area where *P. nettingi* occurs. In addition to *P. nettingi*, Brooks also found *P. glutinosus*, *P. wehrlei*, and one *Eurycea longicauda* (Long-tailed salamander) at the Bickle Knob site. Except for *E. longicauda*, I found these same species at the Bickle Knob site.

Brooks found *D. ochrophaeus, P. cinereus*, and *P. wehrlei* on mountain ridges with remnant stands of spruce outside the known *P. nettingi* range at that time, and these species remain in those areas today. In addition, these three species are found on mountains within the *P. nettingi* range but where *P. nettingi* is absent (Pauley 2007).

CONCLUSIONS

Brooks found *P. nettingi* at four localities from Thorny Flat in Pocahontas County north to the vicinity of Bickle Knob in Randolph County, which he thought was the northern limit of their range. Thorny Flat remains the most southern point of the total range, but the northern end extends to the north side of Blackwater River Canyon in Tucker County (Pauley 2007).

Brooks found *P. nettingi* associated only with red spruce and yellow birch forests. My work reveals they are more common in red spruce and yellow birch forests, but I have located several populations in deciduous forests within the elevational range of *P. nettingi*. Deciduous forest sites that now support *P. nettingi* today were most likely red spruce forests before timbering and fire events between 1870 and 1920 (Pauley 2008).

Plethodon cinereus, D. ochrophaeus, and *P. wehrlei* continue to be common species associated with *P. nettingi*. My records show *P. glutinosus* to be much more commonly associated with *P. nettingi* than Brooks reported. The lack of *P. cinereus* in the Bickle Knob vicinity reported by Brooks is interesting, given that I observed them to be abundant in that area.

CONSERVATION CONCERNS FOR THE NEXT 60 YEARS

Plethodon nettingi is a federally threatened species, which provides it protection under the Endangered Species Act. Nevertheless, roads, ski slopes, utility rights-of-way, and hiking trails impact nearly every known population. These corridors potentially separate gene pools, which could result in subpopulations. The subsequent loss of gene flow in a population reduces genetic variability that could be costly to small, fragmented populations if diseases are introduced or should severe climatic conditions occur (Pauley 2005; Avise 2000; Lowe, et al. 2007). For these reasons, construction of structures and development of roads, hiking trails, utility rights-of-way, and ski slopes that fragment habitat should not be within 90 m (300 ft.) of known *P. nettingi* populations (Pauley 1991; Pauley and Watson 2003).

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Mercury-Resistant Bacteria in West Virginia Coal Waste

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ABSTRACT

Several species of bacteria were isolated from two environmental sources: the Kanawha River (near Montgomery, WV) and wastewater collected at an impoundment pond located within a coal surface mining facility (near Cabin Creek, WV). Undetectable levels of Hg were present in the Kanawha River water; however, an elemental analysis of the coal wastewater demonstrated the presence of Hg at an average of 11.5 ± 0.3 parts per billion (ppb) in the soluble fraction and at an average of 39 ± 3 ppb in the insoluble fine coal particles sedimented from this water. A total of three distinct bacterial species (*Citrobacter freundii, Enterobacter asburiae,* and *Klebsiella pneumoniae*) were identified using a standard series of morphological and biochemical tests. These isolates were subsequently cultured *in vitro* in the presence of Hg levels reaching 27,000 ppb. Ionic mercury (Hg^{2+}) in these cultures was shown to be reduced to elemental mercury (Hg^0). The presence of the mercury reductase gene (*merA*) was confirmed in the genomic DNA of the mercury-resistant bacteria by a polymerase chain reaction (PCR) assay designed to detect a portion of a highly conserved region of the *merA* gene. This report represents the first published study of mercury-resistant bacteria isolated from a coal impoundment pond.

INTRODUCTION

Both elemental (Hg^0) and ionic (Hg^{2+}) mercury are distributed widely in the environment: Hg^0 mainly in air, and Hg^{2+} predominantly in water and soil. Both occur as a result of natural processes, namely volcanic activity and the leaching of ores, but also as a result of human activities, including the burning of fossil fuels, the incineration of waste, and the use of mercury in industrial processes. Both forms are subject to conversion by biological processes into methylmercury and other organomercurial salts. The amount of Hg released into the biosphere by human activities has increased since the advent of the industrial age and today accounts for approximately 75% of the environmental input (Mason and Fitzgerald 1996). Globally, this figure may be as large as 70,000 tons per year (Robinson and Tuovinen 1984). A typical uncontaminated soil sample may contain between 20 and 150 parts per billion (ppb) Hg (Von Burg and Greenwood 1991). Appalachian coal typically contains between 150 and 240 ppb Hg. Coal from Northern Appalachia has the highest natural Hg content of all sources of coal in the USA (Telwart et al. 2005). In extreme cases, human activity has discharged enough Hg into the environment to cause large numbers of deaths and birth defects, most notably at Minamata Bay, Japan (Takeuchi et al. 1962).

Most chemical forms of mercury (including Hg^{2+}) are highly toxic. This toxicity is due to the affinity of Hg for the sulfhydryl group present on the amino acid cysteine. By binding this sulfhydryl group, Hg is capable of rendering an enzyme non-functional. Methylmercury and related organic mercury

compounds present an even greater danger, in part because they accumulate in food chains. Despite this potent toxicity, however, some bacteria are capable not only of surviving, but of thriving in the presence of high levels of Hg. This remarkable capability has been studied extensively over the past thirty years (Barkay et al. 2003; Nascimento and Chartone-Souza 2003; Osborn et al.1997). It is achieved by the protein products of the *mer* operon. The seven Mer proteins encoded by this operon work collectively to confer bacterial resistance to multiple forms of Hg. In the case of Hg^{2+}, dedicated transport proteins, MerP and MerT, take up Hg^{2+} from the cell's exterior and deposit it in the cytoplasm, where a mercury reductase (MerA) carries out the critical step of reducing Hg^{2+} to the relatively inert elemental form, Hg^0. These transport and catalysis steps are accomplished with exquisite specificity for Hg^{2+}. Hg^0, due to its high volatility and low aqueous solubility, either partitions into the air (when diffusion allows), or otherwise coalesces into a liquid.

The *mer* operon is among the most diverse and ubiquitous in bacteria, having been identified in a broad range of species isolated from both environmental and clinical settings. *Mer* operons have been identified on plasmids (Clark et al. 1977; Griffin et al. 1987; Summers and Silver 1972), on transposons (Misra et al. 1984; Huang et al. 1999), and in genomic DNA (Inoue et al. 1991; Wang et al. 1987). As with resistance to antibiotics and other heavy metals, resistance to Hg can be disseminated through populations of bacteria by horizontal gene transfer (Bogdanova et al. 1998). For the current study, we investigated the impact of environmental Hg contamination on the presence and expression of bacterial genes that confer resistance to Hg. Specifically, we analyzed the Hg content of a waste sample collected from a West Virginia coal surface mining facility, isolated, identified, and cultured *in vitro* from this sample several native species of bacteria, examined the susceptibility of these cultures to high levels of Hg, and confirmed the presence of a mercury reductase gene in these bacteria.

MATERIALS AND METHODS

Samples were collected with sterile containers in 2004 from an impoundment pond at a coal surface mining facility in Cabin Creek, WV and from the Kanawha River near Montgomery, WV. Portions of four independent samples taken from each location were analyzed in triplicate for elemental content using a ThermoFisher Scientific S Series atomic absorption spectrometer equipped with a vapor generation system, furnace, or flame. Total mercury content (Hg^{2+}, Hg^{1+}, Hg^0, and organic Hg) was determined using cold vapor atomic absorption spectrometry. Procedures for each element analyzed were based on standard protocols provided by ThermoFisher (Waltham, MA). Reagents and standards for atomic absorption were obtained from Acros Organics (Geel, Belgium). Remaining portions of each environmental sample were used to inoculate liquid cultures. Five types of media were inoculated: nutrient broth (NB, Fluka Chemical, Milwaukee, WI), half-strength NB, Luria-Bertani broth (LB, Fisher Scientific, Pittsburgh, PA), tryptic soy broth (TSB, Sigma Aldrich, St. Louis, MO), and litmus milk (LM, Carolina Biological, Burlington, NC). Cultures were grown at either 25°C or 30°C for 24-48 hours with agitation. These cultures were subsequently streaked onto agar plates containing the same medium and grown at the same temperature for an additional 24-48 hours. At that point, individual colonies were isolated and expanded. Three such pure cultures were identified according to the taxonomic scheme of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1974). Each of these three isolates was then grown in a liquid medium supplemented with $HgCl_2$. Subcultures were initiated at regular intervals over a three month period. Mercury levels in these subcultures were increased incrementally up to a maximum of 27,000 ppb. Reduction of Hg^{2+} by these cultures was demonstrated by capturing volatile Hg^0 as it formed using $KMnO_4$ according to the method of Iwahori et al. (2000) followed by spectrometric analysis to determine the residual mercury content of the cultures.

Chromosomal DNA was prepared from bacterial cultures using a GenElute Bacterial Genomic DNA isolation kit (Sigma Aldrich) according to the manufacturer's protocol. DNA concentrations were determined using a DU 530 Spectrophotometer (Beckman Coulter, Fullerton, CA). PCR analyses were performed using Maxime PCR PreMix tubes (Intron Biotechnology, Seongnam, South Korea) containing 2.5 U of *i-Taq* DNA polymerase, 2.5 mM each dNTP, and 1x reaction buffer. To this premix was added 50 ng of template DNA, 200 ng of each forward and reverse primer, and distilled water to a total volume of 20 il. The forward (5'-TCCGCAAGTNGCVACBGTNGG-3') and reverse (5'-ACCATCGTCAGRTARGGRAAVA-3') primers used for this study are degenerate primers developed originally by Ní Chadhain et al. (2006) and

designed to amplify a 285 bp, highly conserved region of the *merA* gene. Both primers were synthesized by Integrated DNA Technologies (Coralville, IA). Abbreviations other than "A", "C", "G", or "T" represent the standard IUB designations for degenerate positions. The designation "V", for example, represents a combination of A, C, and G nucleotides. PCR was carried out in a Techgene thermalcycler (Techne, Minneapolis, MN) with the following amplification parameters: an initial denaturation at 94°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds with a final extension at 72° for 5 minutes.

PCR results were analyzed by electrophoresis on 2.0% agarose tris-borate-EDTA (TBE) gels alongside HyperLadder II size standards (Bioline, London, UK), stained with ethidium bromide (Sigma Aldrich), and documented using a FOTO/Analyst Investigator Electronic Documentation and Analysis System (Fotodyne, Hartland, WI).

RESULTS

We isolated on a variety of growth media a total of 26 bacterial clones: 10 from a sample of water taken from the Kanawha River and 16 from a sample of coal waste taken from an impoundment pond at a West Virginia surface mining facility. A preliminary analysis indicated some likely redundancy among these clones. Three unique clones we fully identified using standard morphological and biochemical assays (Buchanan and Gibbons 1974): *Citrobacter freundii* from the Kanawha River and *Enterobacter asburiae* and *Klebsiella pneumoniae* from the coal waste.

We analyzed heavy metal content, including that of Hg, in samples taken from the same two environmental sources using standard atomic absorption spectrometric techniques (Table 1). These analyses indicated that the Hg concentration was significantly higher in the impoundment wastewater than in the Kanawha River, West Virginia. The elements Mn, Ni, Fe, and Pb were detected in both the impoundment wastewater and the Kanawha River. The elements Cu, Zn, Se, and Sb were not detected in either source. Levels of Hg, Mn, Ni, Fe, and Pb were significantly higher in the fine coal particles than in either the impoundment wastewater or the Kanawha River.

Each of the three bacterial isolates we identified was grown under laboratory conditions. Subcultures containing incrementally higher levels of $HgCl_2$ were initiated at regular intervals over a three month period. All three species thrived in the presence of up to 27,000 ppb Hg. In contrast, 8,000 ppb Hg was sufficient to kill a laboratory strain of *Escherichia coli* (MM294). In the cultures of mercury-resistant bacteria, Hg^{+2} was reduced to Hg^0, as demonstrated by atomic absorption spectrometry using a gas generation system.

We subsequently extracted genomic DNA from each of these bacterial cultures, including *E. coli*, and analyzed this DNA for the presence of the *merA* reductase gene. This was accomplished using a PCR assay with primers designed to amplify a highly conserved, 285 bp region of the *merA* gene. The products of these PCR reactions were resolved by electrophoresis and are shown in Figure 1.

DISCUSSION

By using a variety of growth media, we were able to cultivate a sampling of bacterial species from two environmental sources: an impoundment pond at a coal surface mining facility in Cabin Creek, WV and the Kanawha River near Montgomery, WV. Due to the elevated levels of Hg detected in Appalachian coal, we considered the Cabin Creek impoundment pond a likely source of mercury-resistant bacteria. In contrast, we intended bacteria isolated from the Kanawha River to serve as negative controls, as we did not initially anticipate these bacteria to be mercury-resistant. It is likely that the cultivatable bacteria isolated from each of these two sites represent only a tiny fraction of the total microbial diversity present in the samples tested (Torsvik et al. 1990). Three bacterial isolates were selected for further study. The first, isolated from the Kanawha River, was identified as *Citrobacter freundii*, a member of the Enterobacteriaceae family of rod-shaped, gram-negative, facultatively anaerobic bacteria. The remaining two isolates were from the impoundment pond and identified as *Enterobacter asburiae* and *Klebsiella pneumoniae,* both of which are also members of the Enterobacteriaceae family. The presence of none of these species was surprising, as all three species are highly ubiquitous in water and soil.

Each of the three bacterial strains we isolated proved capable of thriving *in vitro* in the presence of 27,000 ppb Hg, despite the fact that the spectrometric analysis of water taken from the Kanawha River revealed no detectable Hg. In contrast, Hg was present at a level of 11.5 ± 0.3 ppb in wastewater collected from the containment pond, well below the level of 27,000 ppb that can be tolerated by these bacteria when

cultivated under laboratory conditions, yet still above the Maximum Contaminant Level of 2 ppb for drinking water as set by the Environmental Protection Agency (Table 1). Coal particles recovered from this pond were found to contain 39 ± 3 ppb Hg, below the average range of 150 to 240 ppb typical of Appalachian coal. Interestingly, a mixed culture of the three species we isolated plus additional unidentified species cultivated from coal waste was capable of surviving in growth media containing 50,000 ppb Hg. Spectrometric analyses indicated that the Hg^{2+} added to each of these cultures was being converted to Hg^0, suggesting the presence of the mercury reductase gene.

We therefore employed a sensitive PCR assay to confirm that these three bacterial isolates carry genes that confer resistance to Hg. Any such assay, however, is complicated by the broad diversity of *mer* operons. Consequently, we used for this assay primers designed to amplify a short but highly conserved region of the *merA* gene, the gene that encodes the inorganic mercury reductase (Ní Chadhain et al. 2006). The expected 285 bp PCR product was clearly observed when genomic DNA from our *E. asburiae* and *K. pneumoniae* isolates was amplified (Figure 1). A similarly sized, albeit much less intense, product was observed in the *C. freundii* reaction. Small amounts of larger amplicons were also present in the *C. freundii* reaction, but absent from the *E. asburiae* and *K. pneumoniae* reactions. Employing the same primer set, a similar array of large products was likewise observed by Ní Chadhain et al. (2006) in some species of bacteria isolated from mercury-contaminated soil. These larger products thus represent either nonspecific PCR amplicons or specific products of diverse *mer* operons (Ní Chadhain et al. 2006). We were unable to eliminate these extra products by altering PCR conditions. The expected 285 bp PCR product was absent from a laboratory strain of *E. coli*. Unlike the *E. asburiae, K. pneumoniae, and C. freundii* strains we identified, this *E. coli* strain was incapable of surviving high levels of Hg *in vitro*. Several alternately sized products were present in the *E. coli* reaction, however, including a particularly noteworthy product of approximately 700 bp. Again, attempts to eliminate these bands by altering PCR conditions failed. Importantly, however, our use of the forward *merA* primer in the absence of the reverse primer amplified the same 700 bp product, suggesting that this fragment is likely a nonspecific product unrelated to the *merA* gene (data not shown).

To the best of our knowledge, this report represents the first published study of mercury-resistant bacteria isolated from a coal impoundment pond. Our data suggest that native strains of mercury-resistant bacteria may be widespread in West Virginia. As demonstrated by the strain of *C. freundii* we isolated from the Kanawha River, their presence is not necessarily indicative of environmental Hg contamination, but rather of how ubiquitous the *mer* genes are among bacteria. Such bacteria are capable of thriving in the presence of high levels of Hg by employing the product of the *merA* gene, mercury reductase, to convert toxic ionic mercury (Hg^{2+}) to a less toxic volatile form (Hg^0). This raises the possibility of using such bacteria for the bioremediation of mercury-contaminated environments, assuming the volatile Hg^0 can be safely trapped (Chen and Wilson 1997; Von Canstein et al. 1999; Nascimento and Chartone-Souza 2003). Indeed, the lower than expected levels of Hg detected in the coal particles recovered from the same containment pond from which we isolated the mercury-resistant strains of *E. asburiae* and *K. pneumoniae* suggests a significant environmental role for such bacteria in volatilizing Hg^{2+}. Efficient bioremediation of Hg is likely to require a diverse range of microbial species (Von Canstein et al. 2002). The identification of additional species of mercury-resistant bacteria isolated from coal waste as well as detailed genetic and biochemical characterizations of such bacteria with respect to Hg metabolism will facilitate any attempts at bioremediation by allowing us to define a spectrum of genetic combinations optimal for the removal of Hg from coal waste.

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Table 1. Elemental Analysis of Metal Content in Coal Impoundment Wastewater, Kanawha River Water, and Fine Coal Particles.

Element	Impoundment Wastewater (ppb)	Kanawha River Water (ppb)	Fine Coal Particles (ppb)
Hg**	11.5 ± 0.3	0***	39 ± 3
Mn*	111.2 ± 0.2	77.5 ± 21.4	2216 ± 11
Ni*	229.7 ± 0.4	20.3 ± 2.3	4244 ± 246
Fe*	181 ± 51	131.0 ± 9.1	9759 ± 180
Pb*	28 ± 5	31 ± 14	5195 ± 1632

Table **1.** For the elemental analysis on a given element, the atomic absorption spectrometer was equipped with a * graphite furnace or a ** vapor generation system. ***Below detection limits. The metals *Zn, *Cu, and *Sb were also below detection limits. All values are means ± standard error of the mean, where in each case, n = 4. Each sample (n) was analyzed on the spectrometer in triplicate. The standard error was below detection limits for each element. The Scheffe-F-test was used to determine if the values were significantly different.

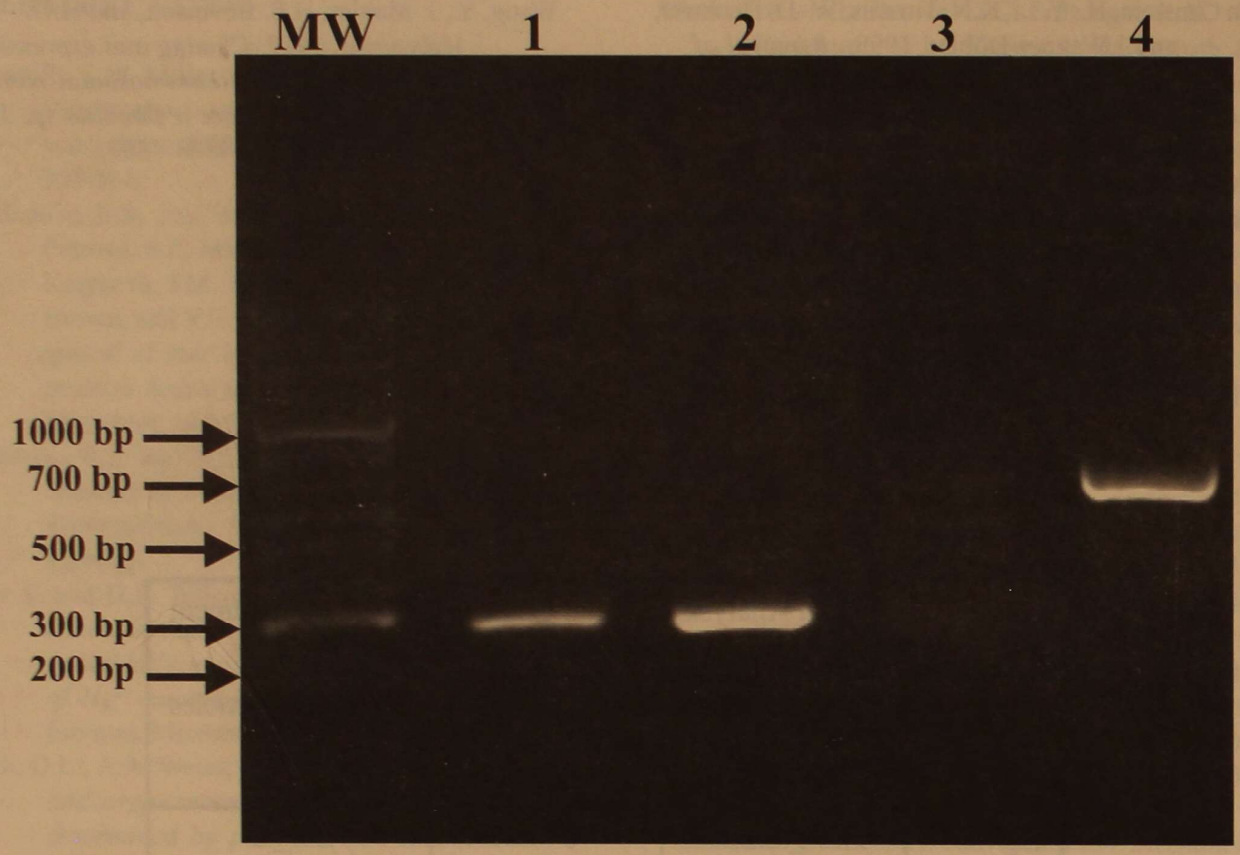

Figure 1. Results of PCR analysis of the *merA* gene in chromosomal DNA extracted from bacteria. MW: molecular weight marker with size standards indicated in base pairs (bp); lane 1: DNA from *Enterobacter asburiae* isolated from coal waste; lane 2: DNA from *Klebsiella pneumoniae* isolated from coal waste; lane 3: DNA from *Citrobacter freundii* isolated from the Kanawha River; lane 4: DNA from *Escherichia coli*, laboratory strain MM294. Primers used for the assay were expected to amplify a 285 bp product from a highly conserved region of the *merA* gene. PCR parameters are detailed in Materials and Methods.