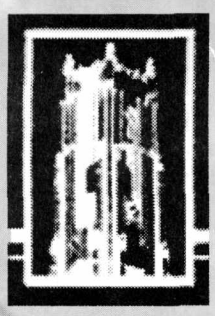

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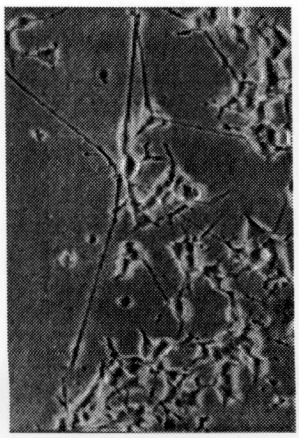

These pheochromocytoma (PC12) cellswere grown in the presence of NGF (100 ng/ml) for 96 hrs. The PC12 cell line is a new addition to Dr. Jay Blundon's Neuroscience program and is being used to study neuronal differentiation.

Picture provided by Brian Wamhoff and Dr. J. Blundon.

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PREFACE

The Rhodes College Science Journal (RSJ) is a student-edited, annual publication which recognizes the scientific achievements of Rhodes students. Founded thirteen years ago as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.

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Courtship Behavior and Effect of Substrate on Effectiveness of Courtship in *Schizocosa saltatrix* (Hentz) (Araneae: Lycosidae)

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ABSTRACT

The courtship behaviors of a species of wolf spider, *Schizocosa saltatrix*, were described fully for the first time. Collection attempts in a deciduous woods near Memphis, TN yielded no members of this species, while a pine-deciduous mix forest yielded many individuals. Females were placed into containers with one of three substrates: control, deciduous leaf litter, and pine-deciduous leaf litter, and the effectiveness of male courtship was examined. The pine substrate was seen to be the most conducive to successful courtship (i.e. more copulations occurred here than in any other treatment), while the deciduous treatment resulted in no copulations at all. The control substrate was found to be an intermediate.

INTRODUCTION

Biologists have long been fascinated by the topic of spider courtship behaviors (see references in Robinson 1982, as well as Davis 1989, Hebets in press; Kronestedt in press, Rovner 1968, 1975, 1989, 1991; Stratton & Uetz 1983, 1986; Uetz & Stratton 1982). As predators, spiders have been known to exhibit instances of sexual cannibalism (Foelix 1982, personal observation). Therefore, there is strong selection for effective signals in courtship. Many sensory systems are used in detection of these behaviors. Acoustic (Rovner 1975, Barth 1982), vibratory (Kronestedt, in press; Uetz & Stratton 1982, Barth 1982), pheromonal (Rovner 1991; Foelix 1982), and visual (Rovner 1968, 1989; Robinson 1982; Lizotte & Rovner 1988) signals are all important in conveying species-specific information in courtship. To date, behaviors in all species studies have been species-specific and may function in reproductive isolation (Uetz & Stratton 1983, Stratton 1991, Stratton, personal communication). While several studies of courtship in Schizocosa have been completed, courtship behaviors for many species remain unknown.

Auditory and vibratory signals are probably the most important class of signals for wolf spiders. These animals are capable of producing courtship sounds audible to humans; *Lycosa gulosa* produces a sound that a human can hear from up to 6 meters away (Rovner 1975). Many sound-producing wolf spiders employ a stridulatory organ located in the tibio-tarsal joint in the palps of mature males (Rovner 1975). Spiders are able to sense airborne vibratory signals through numerous tiny hairs that extend perpendicularly from their exoskeleton, called trichobothria, as well as through a claw slit sense organ and a tarsal slit sense organ (Barth 1982). Solid-borne vibrations are sensed via a metatarsal lyriform organ, and the abundant slit sensilla probably play a role in this, too (Barth 1982).

Although wolf spider vision is second only to that of the saltacids (Robinson 1982), vision is a less effective means of communication than are auditory/vibratory signals. Lycosid vision is designed to perceive movement, not form (Lizotte & Rovner 1988, Rovner 1968, 1989). Therefore, any visual component of courtship must be prominent and clear. Lycosid courtship sequences that have an obvious visual component include those of *S. ocreata,* a brush-legged spider, which rhythmically taps its brushed front pair of legs (Stratton & Uetz 1983) and *S. retrorsa*, which performs a "push-up" behavior in its courtship (Hebets, in press). Species with less ornamentation, such as *S. saltatrix*, have less frequent and obvious movements, such as leg waving (Stratton & Uetz 1983). This is a general pattern in the lycosids: those with a morphology

that easily distinguishes males from females with some physical elaboration usually have a more visual courtship than species in which the sexes are more monomorphic (Stratton, personal communication).

Schizocosa saltatrix, first described by Hentz in 1844, is a medium-sized, dark red-brown lycosid with a wide pale median band and non-bristled legs I (Dondale & Redner 1978). Closely related to *S. ocreata* and *S. rovneri*, this species is monomorphic, distributed throughout the entire United States, and is believed to inhabit hardwood forests (Dondale & Redner 1978). Adults usually become mature in the early spring (Stratton & Miller 1994). Rovner found that this spider possesses one of the largest stridulatory organs relative to its body size of the known sound-producing lycosids (Rovner 1975). Thus, this species is capable of producing relatively loud stridulations. While portions of the courtship of *S. saltatrix* have been described (Rovner 1974 in Dondale & Redner 1978, Rovner 1975), it has not been described fully in any one work.

My original intent in this work was to describe the courtship behaviors of this spring-maturing species. In a study by Stratton and Miller, *S. saltatrix* were collected in early March in a variety of forest compositions, from a pine forest to a pine-deciduous mix to a deciduous forest (Stratton & Miller 1994). However, 12 person hours spent in three habitats of Meeman-Shelby State Forest (MSSF), a deciduous forest 15 miles north of Memphis, Tennessee, on March 15 yielded no members of this species. From the information on its habitat and distribution, I assumed that I would be able to collect these spiders from MSSF. The day after this unsuccessful collection attempt, approximately 50 *S. saltatrix* were collected from the "Lonesome 80", a pine-deciduous mix near Oxford, Mississippi. Several questions thus arose from this finding: (1) Do the spiders have a preference for the pine-deciduous habitat? That is, will they preferentially court and copulate there? (2) Is there something about the composition of these habitats makes it more suitable for courtship and copulation? In addition to describing the courtship behaviors for *S. saltatrix*, I have investigated the effectiveness of courtship in differing habitats using leaf litter collected from these two locations.

METHODS

Spiders were collected on March 16, 1996 from a pine-deciduous woods in Lafayette Co, MS, 8 miles southeast of Oxford by G. Stratton and P. Miller. The spiders were transported to the laboratory and were housed individually in 8 cm. x 4 cm. plastic cages that contained a stick to provide a climbing surface. Water was provided via a cotton wick dipped into a reservoir. A controlled environment was maintained, as the spiders were kept on a light/day cycle of 12/12 and temperature was held constant at 70 degrees Fahrenheit. The spiders were fed several small crickets two to three times per week. 10 pairings were recorded at Rhodes College (RC) from March 26 to April 2. Substrate preference experiments were conducted on April 11 using 15 females and 8 males. 8 pairings were recorded on April 17 at the University of Mississippi (UM). All recordings began at approximately 9:00 a.m. and proceeded for several hours. The UM recordings continued into the late afternoon. A summary of the individuals I used, molting dates, and receptiveness to courtship (i.e. whether the individual copulated) during different trials is given in Appendices 1 and 2.

Courtship and copulatory behaviors. Females to be tested were placed in separate containers on a liner of filter paper 12 hours before pairing was to begin. All subjects were fed several small crickets 24 hours prior to testing to minimize hunger. Testing arenas were constructed from a 9.5 cm. diameter petri dish platform and acetate walls. For the trials that took place at UM, cage liners were cut to fit the arena, with an oblong portion cut to extend from the arena floor through a slit in the arena wall. A stereo needle was placed on this portion to collect sounds produced by the spiders. The stereo needle transducer was attached to an EG&G PARC, Model 113, pre-amp (Gain set at 5K, low roll off set at .3Hz, high roll off at 10kHz) and overlaid onto the videotape (as

described in Hebets in press). No specialized audio equipment was used to record sound at the trials that took place at RC. Recordings at UM were done with a Panasonic HD-5000 video camera with either a 105 mm macro (1:1, f/2.8) lens for close-up recording of the male or a 10.5-125 mm zoom (1:16, 12x) for sequences involving both the male and female. Video equipment used at RC included a Panasonic Color TV Camera WV-3320 and control unit, a TV Camera Zoom Lens V6 X 16, 16-100mm 1:1.19. Behavioral sequences were recorded by a Betamax Video Cassette Recorder SL-HFR 70 on a Betamax video tape. Lighting at UM was provided by a standard light bulb suspended one foot above the recording arena, and RC recordings were done under standard fluorescent room lights. Temperature at UM was 24 degrees Celsius, and at RC was 21 degrees Celsius.

Females and their cage liners were placed into the arenas and allowed to acclimate for several minutes. The recorder was turned on and males were gently introduced through a hollow glass tubing. If no courtship was seen from the male within 10 minutes, the male was scored as a negative and removed. If the male began to display evidence of courtship (stridulation, chemoexploration, or abdominal bouncing), behavior was recorded until either copulation took place or 20 minutes passed with no receptivity response from the female. Behavioral descriptions were made from the recorded sequences.

Effects of substrate on courtship and copulation. All subjects were fed 24 hours prior to testing. 16 hours before testing, 15 females were placed into shoebox-sized containers, each of which had a base of paper towels. One of three substrates was added to each of the boxes before introduction of the female: (1) a control substrate with no addition, (2) deciduous leaves collected from Meeman-Shelby State Forest or (3) pine needles and deciduous leaves collected from a habitat similar to the habitat of the "Lonesome 80." 5 mature males that had molted within the previous two weeks were selected for testing. I also selected several "back-up males" in case one of the originally selected males copulated or were eaten by the females. These males were to be rotated among the habitat types to measure their latency to courtship on each of the different substrates. To prevent any unforeseen effects of a particular rotation on the males (for example: pine to control to deciduous), each was given a different rotation.

After introducing the male, I observed each trial for 20 minutes. The first three trials were observed individually so that I would be guaranteed to observe all behaviors occurring on each of the substrates. I recorded the time of the initiation of each behavior and its duration. After these trials, three boxes were observed simultaneously using a scan-sampling approach. I scanned each of the boxes every 30 seconds and recorded the behaviors that both the male and female were showing. Behaviors recorded include: chemoexploring, stridulation/abdomen bounce, exploring, walking, resting, leg raising, leg rubbing, hiding, female orientation, mounting, copulation, stalking and attacking.

In my analysis of the data, I measured latency from introduction of the male to the performance of the first recorded courtship behavior and from this first behavior to each subsequent behavior.

RESULTS

Courtship and copulatory behaviors. Out of a total of 33 trials (10 at RC, 15 experimental, 8 at UM), 7 courtships ending in copulations occurred (0 at RC, 5 experimental, 2 at UM). The experimental courtships were not recorded on videotape, but scan sample records were kept, allowing me to view all components of courtship. Male behavioral characteristics seen in courtship include chemoexploration, stridulation/abdomen bouncing, exploring, leg raising, and leg rubbing, mounting, and copulating. Female receptive (or "courting") behaviors include orientation towards the male. Non-courting behaviors that may be exhibited by either sex during pairing include

resting, walking, stalking, and attacking. A summary of the correlation of each behavior to the occurrence of subsequent copulation is shown in Table 1.

Table 1: The correlation of each behavior to the subsequent copulation of pairs

Behavior	# Showing behavior	# Showing behavior + copulation	# Showing behavior without copulation
Chemoexplore	7	3	4
Stridulation	13	5	8
Exploration	13	5	8
Leg raise	8	4	4
Leg rub	7	5	2
Female orient	4	4	0

Male behaviors: Chemoexploration occurs when males rub the dorsal surface of their palps against the substrate. This behavior, usually one of the first behaviors seen in courtship, only lasts a few seconds and may be repeated several times during courtship. It is presumed that the male is detecting pheromone left by the female during this behavior (Tietjen 1977 in Stratton & Uetz 1986).

Stridulatory pulsing is the most frequently-occurring behavior during courtship. During stridulation, the abdomen appeared to pulse downward from a pivot at the pedicel. The frequency of stridulatory pulsing was approximately 0.74 pulses/sec soon after courtship began, and increased to approximately 1.45 pulses/sec in the few minutes before copulation took place. The duration of stridulation varied, with bouts of stridulation lasting from several seconds to several minutes. These bouts were interspersed with resting periods also of several seconds to several minutes.

In exploring, a courting male moves about its container, sometimes stridulating, and may orient towards the female. In other cases, the male walks about the container while not stridulating.

A leg raise occurs infrequently and with no apparent pattern, but was seen in many cases of courtship. This behavior entails raising the patellar joint of the leg I of one side to a point, which usually results in the femur being almost perpendicular to the substrate. The distal portion of the leg is sometimes raised as well, though at less than a 90° angle, and sometimes the tarsal claw continues to rest on the substrate.

Only one male that was unsuccessful at stimulating a female to receptivity showed the leg rub. All other demonstrations of this behavior were seen in successful males (Table 1). This is a very quick behavior, lasting only a few seconds. Legs I and II are held off the substrate and rubbed against each other very rapidly in an almost bicycling motion. Several times, males performed this behavior first on one side, then on the other immediately after. Other males only leg rubbed on one side.

Mounting occurs just before copulation begins. The male, when he is within one-half inch of the female, approaches her vigorously stridulating, and climbs on top of her, his ventrum to her dorsum. His anterior end is facing her posterior end and she begins to rotate her abdomen through approximately 90° in a position in which the males palps can reach her epigynum.

Copulation begins when the male first inserts his palp into the female's epigynum and expands the hematodocha. The male always inserts the closest palp into the female, his right palp into her right side, and his left palp into her left side. Several insertions, but only one expansion per insertion, occur on one side before the female rotates her abdomen to the other side. Copulation lasted at least two and one half hours for the two accurately recorded trials.

Female behaviors: Orientation signifies the behaviors that a female demonstrates when she is receptive to mating. She makes small moves lasting several seconds, often turning less than 90° in the process. At the conclusion of each bout of orientation, the body is held low to the ground and the front two pairs of legs are extended out in front of the body, also low to the ground.

Non-courting behaviors: Walking is the act of either a female or a male moving about the container or arena, with no apparent notice of the other member of the pair. Walking is not mistaken for exploring, as the pace is much quicker and no stridulation occurs in male walking. Female walking usually involves rapid movement, sometimes in an attempt to climb the walls of the arena. Commonly a walking spider will encounter the other member of the pair and a scuffle will ensue, as if the walker had no cues that the other spider was present.

Resting signifies a lack of movement from either spider, while not visible was recorded when the spider could not be located in the container (only in experimental tests).

Stalking occurred when one spider (usually the female) began slowly and seemingly deliberately approaching the male with her legs I raised. In one instance, the female was momentarily mistaken for the male as her abdomen was pulsating during her approach, a behavior that was remarkable similar to the male's abdominal movements during stridulation. Attacking occurred when the stalking spider jumped for the other and a fight ensued. Attacking resulted in death only two times out of numerous attacks.

Effects of substrate on courtship and copulation. Two out of five trials on the control substrate ended in a copulation. No copulations were seen on the deciduous substrate, and three out of four trials on the pine substrate ended in copulation. Table 2 shows a summary of the averages of latencies on each substrate. The latency from introduction of the male to the first behavior was greatest for the non-copulating male on pine and shortest for the non-copulating males on the control substrate (Figure 1). Standard deviation data indicates that none of these differences are significant. The greatest range of deviation is found for the non-copulating males on deciduous. This results from one male with a latency of more than 600 seconds longer than the other three males, all who exhibited their first behaviors at the same time. If this piece of data is excluded, then it is clear that there is little difference between the males on control and the males on deciduous. It is also clear that the males on pine had a longer average latency to the first behavior. The longest first latency was seen in the non-copulating male on pine (220 sec.). For the males that copulated, a trend can be seen in latency to first behavior performance. Males on control began courting at an average of 40 seconds, whereas males on pine began courtship at an average of 101 seconds.

Table 2: Average latencies from introduction of male Schizocosa saltatrix to first behavior and from first behavior to each subsequent behavior. No data is shown for those trials in which a behavior was not seen.

Behavior	Control Copulators	Control Non-Copulators	Deciduous Non-Copulators	Pine Copulators	Pine Non-Copulators
1st Behavior	40	27.67	169.75	101.67	220
Chemoexploring	0	23.50	90	254.5	
Stridulation	65	59.33	71.5	199.33	90
Exploring	200	537	55.75	52.33	60
Leg raising	215	105.67	210	29	
Leg rubbing	120	600	249	574.33	
Orientation	210			557.33	
Mounting	520			795	

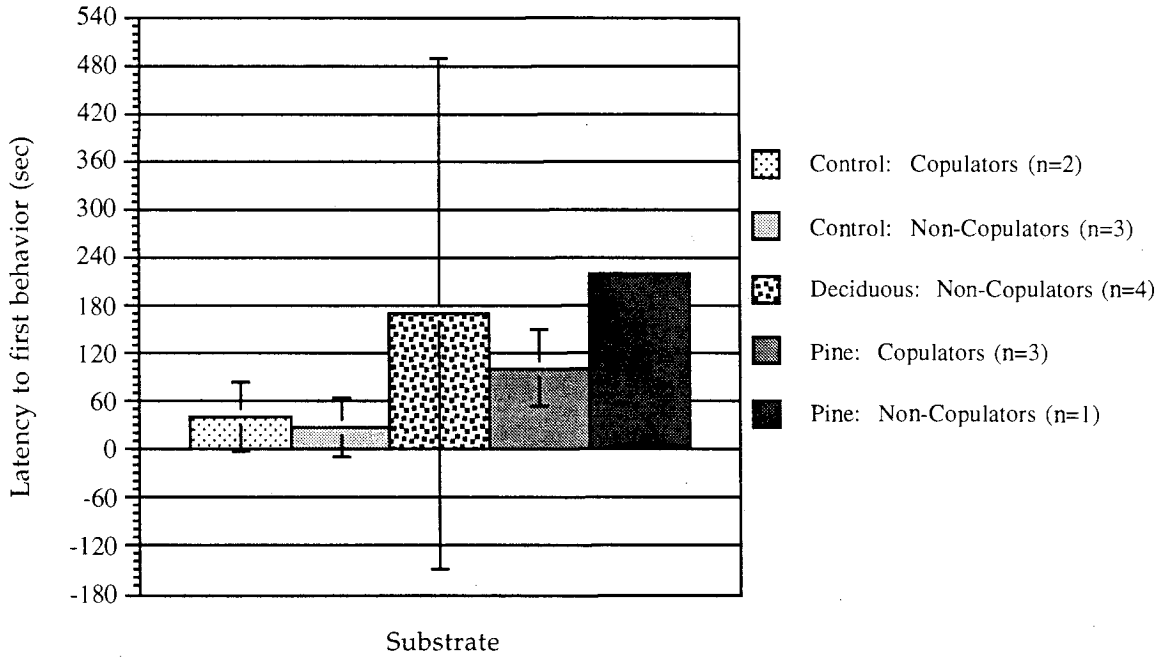

Figure 1: The average latency from introduction of the male to performance of the first courtship behavior by *Schizocosa saltatrix* on control, deciduous, and pine substrates.

Latency from the first behavior to subsequent behaviors for copulating males on control and pine were not significantly different for stridulatory pulsing, leg raising, female orientation, and mounting (Figure 2). However, a difference can be seen in the latency to chemoexploration, exploration, and leg rubbing. Only one male on control male was recorded to perform chemoexploration, so this data is most likely not significant. Exploration, however, was seen in both males on control and was much higher (285 sec.) than for males on pine (22.33 sec.). The leg rubbing behavior also had very different latencies for the two treatments. This behavior was seen much earlier in males on control (120 sec.) than in males on pine (574.33 sec.).

Difference between latency to performance of each later behavior by non-copulating males on control and on deciduous were not significantly different (Figure 3). A trend, however, does seem to be apparent. For most behaviors (excluding exploration and leg rubbing), the males on control averaged a slightly shorter latency than did the males on deciduous. Both exploration and leg rubbing however, were begun earlier on deciduous than on control. One non-copulating male on control exhibited this behavior much later (1050 sec) than did the other two of his condition. Excluding this piece of data would lead to an average latency of 280.5 sec. and a standard deviation of approximately 27.5. This change would make the difference in exploration latencies significantly different. Secondly, the leg rubbing behavior was seen in only one non-copulating male and this occurred on two different substrates, control and deciduous. It is interesting to note that the same individual performed this behavior 351 seconds earlier on deciduous than on control. Another point to note is that this male was rejected by females a total of five times throughout the length of all pairings.

DISCUSSION

Courtship and copulatory behaviors. Because stridulation comprises the majority of the courtship of *S. saltatrix*, this species' pattern is very auditory/vibratory. Visual signals make up only a small portion of the courtship sequence and one of these, the leg rub, may in fact produce sound out of the range of human hearing. Also, although the pulsing accompaniment of stridulation is very obvious to a human observer, this movement may only be an equal and opposite reaction to the flexion of the palps and may communicate no signals to the female (Stratton, personal communication; Rovner 1975). The female's orientation to the male, because it involves several small advances and turns, is both an auditory and a visual signal, though it is difficult to say which is more important. Her movements are crucial to the male's locating her, as a still, silent object is much more difficult for spiders to locate than a loudly moving one (Rovner 1975).

Effects of substrate on courtship and copulation. A trend is apparent in *S. saltatrix* preference of copulatory substrate. Pine was preferred over either a control substrate or deciduous litter. Behaviors on this substrate usually had a longer latency than on control or deciduous, but were more successful in the end. Perhaps female pheromone was more difficult to locate in this treatment, but was substantial enough to stimulate the male to exhibit the full range of courtship.

Although behaviors occurred with a shorter latency period on the control substrate, copulations were less frequent. I would hypothesize that the female's pheromones are much more easily detected on the control and therefore, the male would become stimulated more rapidly. The latencies to exploring for all males on control versus males on either pine or deciduous demonstrate this. Males on control had a much longer latency to exploring than did males on either of the other two treatments. I would suggest that the leaf litter treatments afford the male a "hint" of the female's pheromone, and thus stimulate him to begin searching for higher concentrations of it, and consequently for the female. The pheromone on control is probably abundant enough to stimulate the full range of courtship behaviors. However, females in this treatment seemed more likely to hide underneath the paper towels than did females in either of the other two treatments. Males may be courting more rapidly, but the females may be less receptive to it.

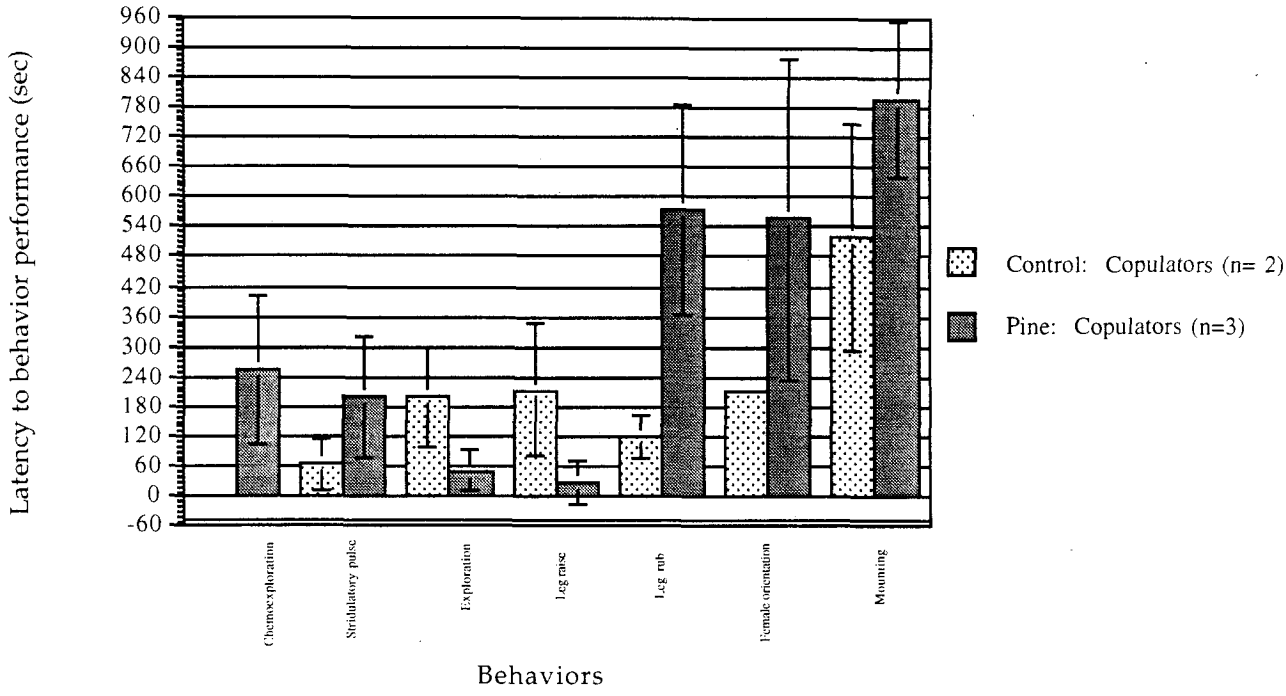

Figure 2: Average latency from performance of first behavior to performance of each later behavior by successfully copulating *Schizocosa saltatrix* spiders on a control and pine substrate. One standard deviation is shown.

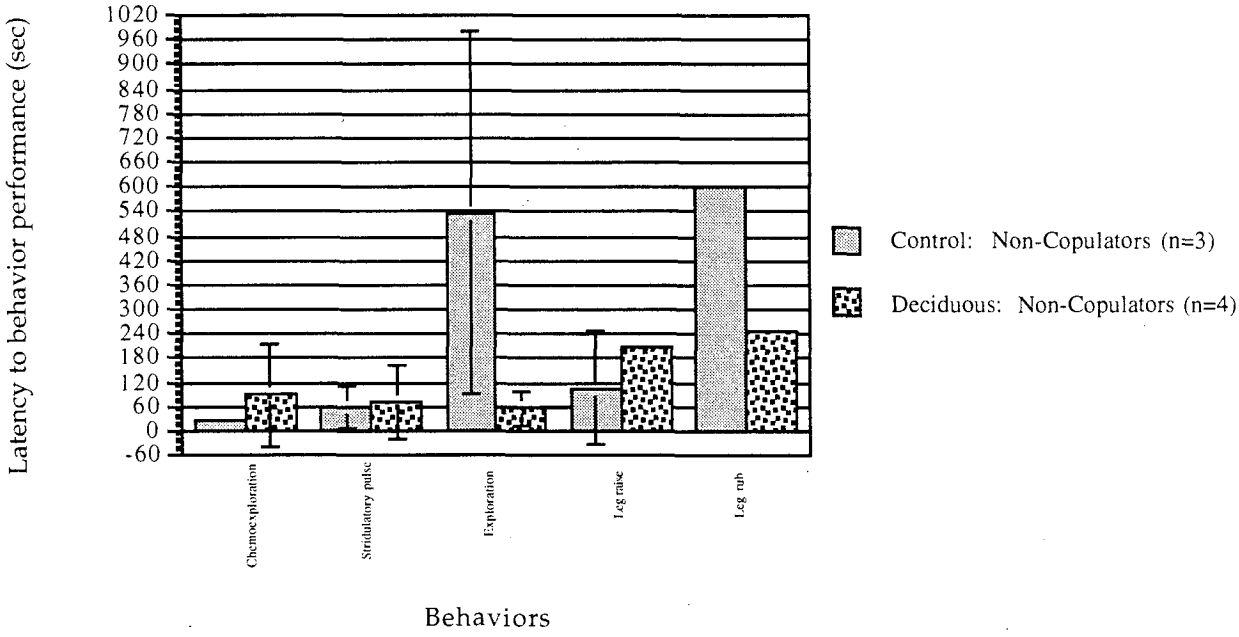

Figure 3: Average latency from performance of first behavior to performance of each later behavior by non-copulating *Schizocosa saltatrix* spiders on a control and deciduous substrate. One standard deviation is shown.

The deciduous treatment was remarkable in that no copulations took place at all. Males did court, but were more likely to hide or rest for long periods of time (4 out of 5 were hidden for 10 minutes or more). Females seemed to be much more active in this treatment than in any other (3 out of 5 vigorously moved for five minutes or more). Therefore, there does seem to be something about this substrate that discourages courtship and copulation.

Although it is difficult to determine the ultimate explanation for this substrate preference, several suggestions come to mind. First, the distribution of *S. saltatrix* may have been an historical accident, with no spiders dispersing to the deciduous forest of Meeman-Shelby State Forest. The spiders that did disperse to the Lonesome 80 site could have evolved their own local preference for substrate. Since it has been shown that *S. saltatrix* can be found in a variety of habitats (Stratton & Miller 1994), an interesting investigation would be to examine *S. saltatrix* pairs from other localities and determine if they have a preference for their natal habitat.

Another suggestion is that the deciduous litter, while it may provide more air pockets to amplify the stridulatory sound (personal observation), may not be conducive to an equal distribution of female pheromone, thus leading to a less vigorous courtship by the male. If pheromones are difficult to locate, so too would be potential mates, as this is one of the primary means of locating them. Also, as these spiders are sit-and-wait predators, the many hiding/hunting places that deciduous litter affords may be too much to pass up. An analysis of the benefits of predatory behavior as compared to courtship behavior would test this hypothesis.

Other possible explanations involve the habitat particular to Meeman-Shelby State Forest. A *S. saltatrix* predator may be present that is not found at the Lonesome 80. Also, exposure on the loess bluffs of Meeman-Shelby State Forest may contribute to lower humidity, a condition which can cause death of these spiders (Stratton, personal communication). Other features of this area that can be tested are: spring rainfall, soil type (is there some characteristic of the soil that is detrimental to the spiders?), prey availability, and competition from other predators.

One notable weakness with my experimental design and results is that of a very small sample size. The conclusions that I have reached would be much stronger with evidence gathered from more trials on each substrate. Since I also found great variability in the responses from males on the different substrates, I would recommend to future researchers that they examine the variability of behaviors on different substrates. Does one substrate stimulate a more consistent response than others? I would also recommend that future researchers examine the movements of solitary females on each of these different substrates to determine if one habitat stimulates different behaviors more than others. For example, I have suggested that the deciduous habitat stimulates hiding behavior due to its many "pockets." This hypothesis could be tested by examining the frequency of exploration versus resting or hiding under leaf cover.

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Appendix 1: Maturation dates and results of different trials for male *Schizocosa saltatrix* individuals

Individual Sex and #	Molt date	RC copulate?	Experimental copulate? (4/11/96)	UM copulate? (4/17/96)	Notes
1	3/24	4/2 no	yes	no	
2	3/24	4/2 no			eaten by fem.
3	3/24	4/2 no		yes	
6	3/24	3/29 no			eaten by fem.
7	3/28		yes		
8	3/28			yes	
9	3/24	3/29 no	yes		
10	3/24	3/29 no	no, no, no	no	rejected 5 times
11	3/31		yes	no	
12	3/24	3/26 no			
13	3/28		yes		
14	3/21	3/26 no			
15	3/28			yes	
17	3/24	3/26 no			
18	3/26		no		
19	3/31			no	
23	3/26	4/2 no			

Appendix 2: Maturation dates and results of different trials for female *Schizocosa saltatrix* individuals

Individual #	Molt date	RC copulate?	Experimental copulate? (4/11/96)	UM copulate? (4/17/96)	Notes
25	3/26	4/2 no			ate male
26	4/7			no	
28	3/31		yes		
29	4/3			yes	
30	3/31		no	no	
31	3/31		no	no	
33	3/25	3/29 no	yes	no	
34	4/3			no	
35	3/31		no		
36	3/31		no		
37	3/24	3/29 no	no		
38	3/28		no	yes	
39	3/27	4/2 no	no		
41	3/24	3/26 no	yes		4/18 egg sac
42	3/24	3/26 no	no		
43	3/24	3/26 no	yes		
44	3/24	3/29 no			ate male
46	3/24	4/2 no	no		
47	3/27	4/2 no	yes		

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The Effects of Forskolin, Dexamethasone, and Nerve Growth Factor on PC12 Cell Growth, Morphology, and Acetylcholinesterase Activity

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ABSTRACT

By studying the effects of different neurobiological agents on undifferentiated rat pheochromocytoma (PC12) cells, we can learn more about the process of neural cell development. In this experiment, we investigated the effects of forskolin (FSK), dexamethasone (DEX), and nerve growth factor (NGF) on undifferentiated PC12 cells raised in cell culture. The characteristics of the cells that we monitored were percent confluency (a measure of growth rate), neurite number and length, and acetylcholinesterase (AChE) activity.

Cells were grown in five different chemical environments: control (low serum growth medium), FSK, DEX, NGF, and DEX+NGF. Measurements of growth and morphology were recorded every 24 hours for 6 days. Cells were then frozen on day 6 and assayed for AChE a week later. Cells placed in low serum growth medium continued to divide and failed to show any evidence of differentiation. Forskolin, which increases intracellular cAMP, induced the extension of short, stout neurites from the cells but failed to induce AChE activity. Dexamethasone caused PC12 cells to differentiate into chromaffin-like cells. NGF caused the cells to sprout long thin neuritic extensions and also induced the expression of acetylcholinesterase enzymes. Cells treated first with DEX and later with NGF began to sprout neurites following NGF exposure, but did not test positive for AChE after 3 days of NGF exposure.

Overall, we observed that the fate of PC12 cells was not fixed, but instead was changeable and dependent upon the chemical environment. In developing organisms, this local environment allows neural cells to differentiate, migrate, and form proper synaptic connections. Without the necessary chemical cues, organization of a functional nervous system would be disrupted.

INTRODUCTION

During development, the vertebrate embryo undergoes a series of changes that transform a single celled zygote into a complex yet predictable multi-cellular, multi-system organism. Throughout this process, the nervous system, with its extensive network of neurons and glial cells, arguably undergoes the most complex developmental processes with the organism. First, neurons differentiate from ectoderm. The cells then migrate and extend axons in response to trophic substances and other guidance cues. Finally, many axons undergo a process of competition for limited trophic substances, resulting in survival of some neurons and elimination of others (Kandel *et al.*, 1991).

In recent years the rat pheochromocytoma adrenal cell line (PC12) has become the model system for investigating neuronal differentiation and development. This is due in part to their rapid induction, by such agents as nerve growth factor (NGF) and glucocorticoids, to differentiate into either sympathetic neurons or adrenal chromaffin cells, respectively (Levitan and Kaczmarek, 1991). Moreover, these cells possess a low mortality rate, and undergo rapid proliferation (Rieger *et al.*, 1980).

In this paper, we chose to reproduce and extend the research of several classic PC12 differentiation experiments. We proposed to compare the effects of several neuroactive substances on PC12 morphology and biochemistry. Most importantly, we were interested in determining any interrelatedness among the mechanisms by which these substances affect cellular differentiation. Specifically, we chose to measure changes in cell confluency, neurite formation and length, and acetylcholinesterase (AChE) activity. We accomplished this by incubating cultures of PC12 cells for several days in solutions of control growth medium, FSK (an agonist of adenylate cyclase), DEX, and NGF. We also performed a modification of an experiment by Pollock *et al.* (1990) in which PC12 cells were treated simultaneously with DEX and NGF. They found that DEX blocked NGF's ability to induce cellular expression of sodium channels without hindering neurite growth. We treated cells with DEX for three days before adding NGF, this time to determine if the same results are reached after DEX-induced differentiation. For all cells, morphological changes were measured by visual inspection. The presence of AChE activity was measured by a colorimetric assay using spectrophotometers.

Our data show a steady increase in confluency for all treatments, with the rate of growth being greatest for cells raised in growth medium alone. We observed the highest number of neurites and the longest neurites in cells exposed to NGF. FSK induced neurites that were shorter, stouter, and fewer in number than NGF induced neurites. Cells grown in DEX+NGF displayed neurites comparable in number but shorter than those of NGF-treated cells. AChE activity increased significantly only for NGF exposed cells.

In light of these observations, we conclude that all drug treatments used in this study reduced cell proliferation and initiated cell differentiation. We also conclude that cAMP, activated by FSK, is likely to be involved in the differentiation of PC12 cells exposed to NGF, but cAMP does not fully mimic the effects of NGF. Moreover, we propose that NGF is able to restore neurite expression even after three days exposure to DEX alone. The role of DEX in inhibiting NGF induced expression of AChE warrants further investigation.

METHODS

Rat pheochromocytoma (PC12 H-line) cells were obtained from Dr. David Fischer at the University of Tennessee at Memphis. These cells were allowed to proliferate in complete growth medium (RPMI 1640 supplemented with 5 % fetal bovine serum, 10 % heat inactivated donor horse serum, 25 μg/ml streptomycin, 25 units/ml penicillin), 2 g/L sodium bicarbonate, and 20 mM HEPES and were maintained according to previously outlined culture maintenance procedures (Greene and Tischler, 1976).

These cells were then given to us in a T-25 flask and further handling of cells took place according to a strict aseptic technique (Wamhoff and Blundon, 1996). Because the cells had attached themselves to the side of the flask, it was necessary to resuspend the cells. First, the cells were examined under an inverted microscope to gather a general idea of size and shape under normal growth conditions. Then 2 ml of 0.05% trypsin and 0.02% EDTA were added to the flask and the mixture was swirled for approximately 2 minutes. Once a majority of the cells were detached, 5 ml of RPMI low serum medium (RPMI LS, an incomplete growth medium containing RPMI 1640, the above antibiotics and buffers, and 2% donor horse serum) was added to stop the cleaving action of the trypsin (Wamhoff and Blundon, 1996). The suspension was triturated to break up remaining clumps of cells and the mixture was pipetted into a 15 ml culture tube and centrifuged at 1000 rpm for 5 minutes. The resulting supernatant was then removed and fresh RMPI LS was added to the remaining pellet. A final thorough trituration was performed.

In order to count the cells, 200 μl of the suspension was transferred to a 1.5 ml Eppendorf tube and vortexed for 1 minute. Next, 0.2 ml of 0.08% trypan blue was added to the tube and

allowed to sit for 3 minutes to determine the percent of dead cells in the volume. Ten µl of the stained suspension was removed and the cells were counted using a hemacytometer. The number of cells per ml suspension was then calculated (Wamhoff and Blundon, 1996).

Next, the cells were subcultured to 3 collagen coated 4-well plates at a density of 2 - 3 X 10^4 cells/ml. One ml of the cell suspension was added to each well and the 3 plates were then incubated at 37°C at 8% CO_2. The cells were allowed to reattach for 6-8 hours. The cells were then exposed to their respective drugs after removing 500 µl of the existing medium. On day 1, plate A wells 1 and 2 were not altered and served as control. Wells 3 and 4 were given 10 µM of forskolin (FSK). All wells on plate B were given 20 µM of dexamethasone (DEX). All wells on plate C were administered 100 ng/ml of nerve growth factor (NGF). All of the wells were then filled with 500 µl of fresh RPMI LS medium and placed back into the incubator. On day 4, the cells were fed by removing 500 µl of medium from each well and replenishing it with new medium. Wells receiving NGF and FSK were given second doses of the drugs, while wells 3 and 4 of plate B, which initially received DEX, were administered 100 ng/ml of NGF. Observations of cell culture confluency, number of neurites per cell, and neurite length for each well were monitored at 24 hour intervals for 6 days. On day 6, samples of all cells were photographed.

The cells were then harvested for an AChE assay. All of the medium from each well was removed and the wells were rinsed with Dulbecco's phosphate buffered saline (DPBS) and then triturated with 750 µl of DPBS for resuspension. The cells from each well were then transferred to microfuge tubes and centrifuged at 1000 rpm for 5 minutes. The DPBS was then carefully removed using a suction needle attached to a vacuum and the remaining pellet was resuspended with 400 µl of solubilization buffer. Each tube was then assayed according to previously recorded procedures for the level of AChE activity present (Ellman *et al.*, 1961).

RESULTS

The mean confluency of PC12 cells increased in RPMI LS medium as well as the four experimental solutions. The greatest increase in percent confluency was seen in the control and NGF samples (Fig. 1). In these two samples, confluency increased to 57 % for the NGF treated cells and to 77 % for the RPMI LS cells by day 6. Also by day 6, DEX+NGF treated cells increased to 45 % confluency, while FSK and DEX treated cells increased to 32 % and 22 % confluency, respectively.

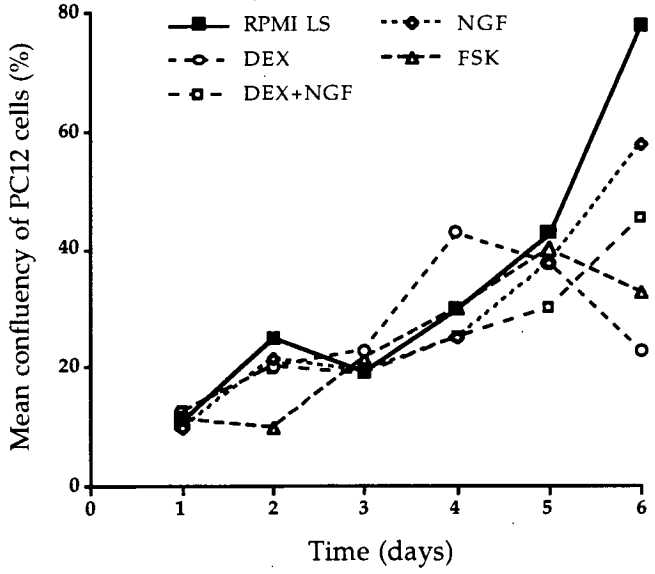

Figure 1. PC12 cell confluency was compared among five different treatments daily for 6 days. RPMI low serum growth medium (control) cell confluency was visibly greater than exposures to NGF, DEX+NGF, FSK, or DEX.

PC12 cells cultured in RPMI LS media showed no growth of neurites during the six day observation period (Fig. 2). DEX+NGF and NGF exposed cells expressed the highest number of mean neurites per cell (5-5.5/cell), while the neurites formed in FSK and DEX treated cells were substantially fewer.

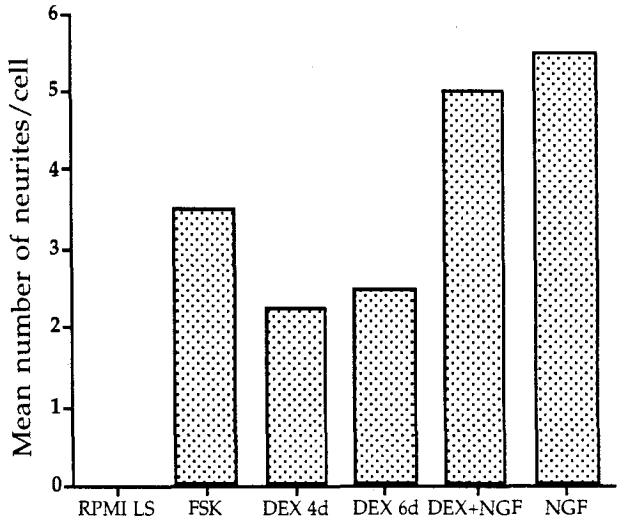

Figure 2. Mean number of neurites per cell for various pharmacological treatments of PC12 cells after an exposure of six days. NGF and DEX+NGF exposed PC12 cells show a greater number of neurite extensions relative to all other cells. Note the absence of neurites in PC12 cells cultured in control RPMI LS medium.

Of the PC12 cells that grew neurites during the experiment, those exposed to NGF had the longest mean neurite length (50 μm, Fig. 3). Cells exposed to all other drugs grew neurites of approximately the same length (mean length ranged from 24 to 32 μm). Although cells exposed to DEX and then to NGF had approximately the same number of neurites as NGF exposed cells (Fig. 2), the former cells had only been exposed to NGF for three days, and neurites were notably shorter than neurites from cells exposed to NGF for six days.

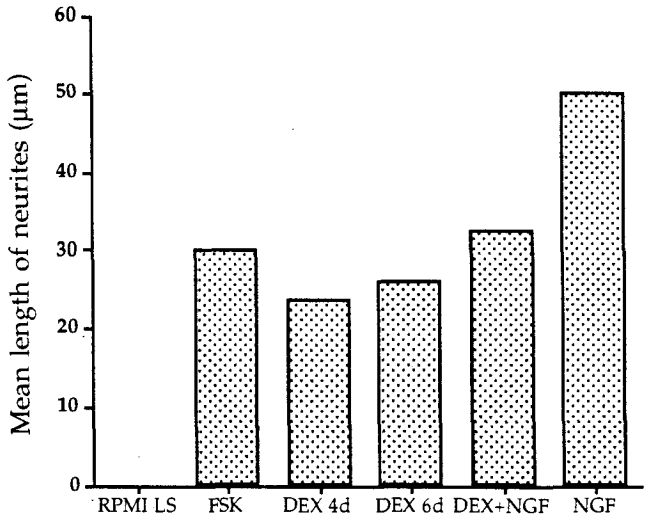

Figure 3. A comparison of the mean length of neurites (in μm) for various pharmacological treatments of PC12 cells after an exposure of six days. NGF exposed PC12 cells had substantially longer neurite extensions relative to all other cells.

The relative activity of AChE was noted after six days of treatment exposure (Fig. 4). The NGF-treated cells were the only cells that had AChE activity significantly greater than the spectrophotometer cuvettes containing no cells (Kruskal-Wallis multiple comparison test, $P<0.001$; Sokal and Rohlf, 1981).

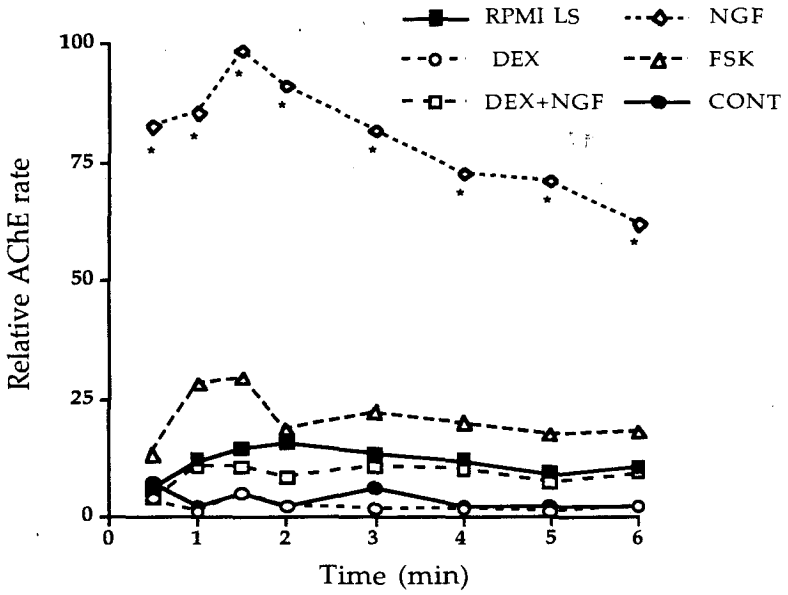

Figure 4. A comparison of relative AChE activity for various pharmacological treatments of PC12 cells after an exposure of six days. A Kruskal-Wallis multiple comparison test revealed that the NGF cells were the only cells to have a significant increase in AChE activity relative to the control blank cuvette (* $P < 0.001$).

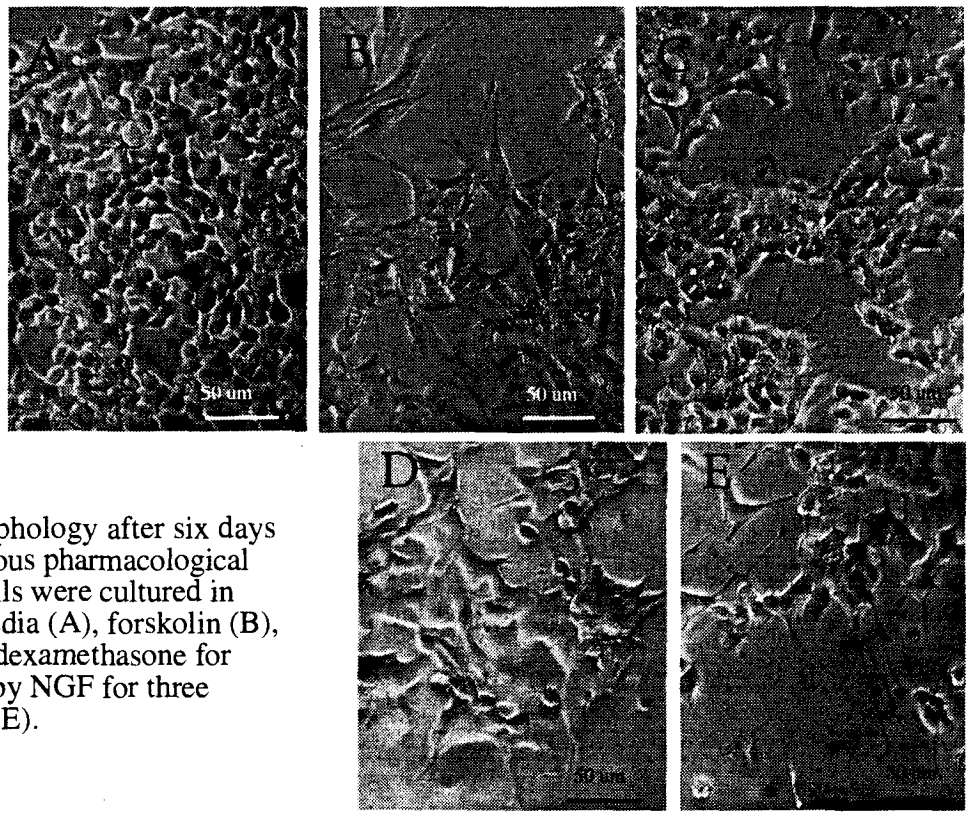

Figure 5. PC12 morphology after six days of cell culture in various pharmacological treatments. PC12 cells were cultured in control RPMI LS media (A), forskolin (B), dexamethasone (C), dexamethasone for three days followed by NGF for three days (D), and NGF (E).

Photographs taken at day 6 of the five different treatments of PC12 cells in this experiment show the variation of confluency and neurite length/abundance discussed previously (Fig. 5). Control cells clearly have no neurite growth and are quite dense (Fig. 5A) whereas the NGF cells have considerable neurite extensions (Fig. 5E). Intermediate degrees of neurite lengths and abundances per cell can be seen with the three other treatments (Fig. 5B-D).

DISCUSSION

Our results indicate that adding different drugs to the medium of PC12 cells has varying effects when compared with the PC12 cells grown in control RPMI LS growth medium. These drugs can be considered an artificial change to the cellular environment that mimics differences in normal cellular microenvironments *in vivo*. Our results are in agreement with previous research that has been done on the effects of these drugs on PC12 cells. Administration of these drugs in our experiment reduced cell division and led to differentiation into chromaffin-like cells (DEX) or sympathetic neuron-like cells (FSK, NGF, DEX+NGF) (Figs. 1-5).

We found that the number of neurites was higher in cells exposed to NGF, DEX followed by NGF combination, and FSK. The DEX and the control RPMI LS cultures showed the lower neurite outgrowth (Fig. 2). Addition of NGF to PC12 cells caused the cells to begin neurite outgrowth similar to neurites produced by sympathetic neurons in primary cell culture (Greene and Tischler, 1976; Caillaud *et al.*, 1995; Fig. 5E of this study). Direct addition of cAMP (Pollock *et al.*, 1990) or treatment with FSK, an activator of adenylate cyclase that raises intracellular cAMP levels (Fig. 5B), produced cellular extensions somewhat shorter and stouter than NGF induced neurites. Addition of DEX and NGF to PC12 cells has been found to cause neurite formation of a different kind than addition of NGF alone (Pollock *et al.*, 1990). Cells exposed to DEX for six days showed few neurites/cell, while cells exposed to DEX for three days and then NGF for three days expressed many more neurites/cell (Fig. 2). Neurites from DEX+NGF cells were shorter than those from NGF cells, perhaps because neurites from the former treatment had been NGF exposed for only three days instead of six days.

Although pre-exposure to DEX doesn't antagonize morphological formation of neurites, DEX exposure does alter neurite excitability. In experiments with simultaneous treatment of PC12 cells with DEX and NGF, Pollock *et al.* (1990) have shown that neurites that form in the presence of DEX fail to express sodium currents, whereas NGF induced neurites express sodium currents. Pollock *et al.* (1990) concluded that DEX actually inhibits the formation of sodium channels but does not antagonize NGF induced neurite outgrowth. This suggests that although some events influenced by NGF are overcome by DEX, some NGF induced events are not controllable by DEX. It appears that while sodium channel presence is antagonized by DEX, neurite morphological formation is by a distinct pathway that is not inhibited by the presence of the glucocorticoid.

Neurites expressed in PC12 cells exposed to FSK in our experiment are shorter and stouter than those seen in NGF exposed cells. It has been previously found that while FSK (through its product cAMP) induces some early events of neurite formation, functional neurites never form and are described as "spike like processes" (Pollock *et al.*, 1990). We suspect that cAMP is not the exclusive second messenger controlling the formation of excitable neurites with NGF exposure. While cAMP may begin to induce early neurite development, additional cues are necessary for functional neurite expression. It has been shown that PC12 cells grown in the presence of cAMP exhibited no increase in sodium channel formation and sodium current in the cells actually disappeared after the spike-like processes appeared (Pollock *et al.*, 1990). It is obvious that necessary steps in the production/and or membrane incorporation of sodium channel proteins are mediated by other factors related to NGF that have not yet been discovered. A potential candidate for such a step is activation of a c-fos gene. This gene is activated by NGF and has been thought to be a possible sodium channel gene (Pollock *et al.*, 1990). At least two other proteins may play an important role in proper neurite formation, the first of which is N-kinase (which is not cAMP activated). N-kinase has been shown to be active upon administration of NGF (Boniece and Wagner, 1995). Bar-Sagi and Feramisco (1985) have found another protein called *ras* that binds GTP and is homologous to other GTP-binding proteins. Intracellular *ras* activity may also be triggered by activation of NGF receptors. Injection of *ras* protein antibodies has been shown to block the effects of NGF on PC12 cells (Bar-Sagi and Feramisco, 1985).

Dexamethasone, a synthetic glucocorticoid, has been shown to promote the differentiation of PC12 cells and neural crest cells into adrenal chromaffin cells (Greene and Tischler, 1976; Doupe *et al.*, 1985). Chromaffin cells contain dense chromaffin granules but do not possess neurites. DEX exposed cells in our study reduced their rate of division and showed little expression of neurites or AChE compared to the cells exposed to NGF.

In order for neurons to be excited by neurotransmitter release, they must first synthesize and express ligand-gated receptors and the enzymatic machinery with which to degrade or remove synaptic neurotransmitter molecules. Upon addition of NGF, Rogers *et al.* (1992) have shown that there was no increase in RNA specific for acetylcholine receptor subunits, while NGF did result in an increase in the number of PC12 cells which expressed ACh-sensitive currents. They concluded that NGF regulation of ACh receptor formation does not involve receptor synthesis but may be linked to posttranscriptional assembly of receptor subunits. We now show that acetylcholinesterase (AChE) activity is present only in PC12 cells treated with NGF for 6 days (Fig. 4). Our results show no significant difference in AChE activity between all of our other treated and control cultures, even though the DEX+NGF cells show enhanced neurite outgrowth. It remains to be seen whether the DEX+NGF cells failed to express AChE activity because they had only been exposed to NGF for three days or because of a DEX-related inhibition of AChE synthesis and/or expression. Rieger *et al.* have shown that increases in AChE activity in NGF exposed PC12 cells was minimal after three days of exposure, but was much greater after five to eight days of exposure (1980).

While FSK exposed PC12 cells did produce neurite-like extensions, these cells failed to show AChE activity. Thus, while cAMP may be a component of neurite extension in PC12 differentiation, it may play a limited role in AChE expression. Indeed, in PC12 cells that were exposed to NGF but raised in suspension so that they could not form surface attachments, AChE expression proceeded in the absence of neurite extension (Rieger *et al.*, 1980). However, in their experiments, inhibition of neurite formation did cause one molecular form of AChE, 16 S AChE, to go unexpressed. From our results and those of Rieger *et al.*, (1980), it is evident that not all AChE activity is directly tied to neurite formation.

In summary, PC12 cells continue to divide most rapidly in the absence of external chemical cues. These chemical cues act in various ways to determine PC12 fate. NGF causes the cells to differentiate into a sympathetic neuron-like cells. FSK mediates some early changes similar to NGF by forming short, stout cellular extensions. However, FSK fails to initiate AChE activity by day 6, and therefore cAMP cannot be the only intracellular signal activated by NGF during differentiation of PC12 cells into sympathetic neurons. DEX causes PC12 cells to differentiate into chromaffin-like cells. NGF exposure for three days following DEX exposure cell causes neurite extension similar to NGF exposure alone. However, previous research indicates that the neurites of these cells may lack sodium channels and hence, may not be electrically excitable. AChE activity increases significantly only in the PC12 cells exposed to NGF for six days. Previous research shows that this increase is evident with or without neurite formation. However, one potentially important form of AChE (AChE 16 S) is evident only when NGF-induced neurite formation is allowed to occur.

Future student research in the Rhodes Neuroscience Program can now focus on the importance of cAMP and other second messengers during NGF-induced PC12 differentiation. Specifically, PC12 cells can be cultured with NGF in the presence of agents which interfere with activation of adenyl cyclase, cAMP dependent protein kinase, N-kinase, or even GTP binding proteins such as *ras*. Students can then assay the effects of these agents on neurite formation, AChE activity, and the expression of sodium channels or ACh receptors using patch clamp technology. Further, students can investigate the role of DEX in inhibition of NGF induced AChE activity. By measuring AChE activity after varying DEX and NGF incubation times, it should be quite possible to determine the critical period during which DEX inhibition occurs.

Finally, students at Rhodes are currently attempting to produce a monoclonal antibody that will bind to NGF that can be used to localize NGF receptors in PC12 cells. Development of this antibody will allow students to investigate effects of various neuroactive agents on NGF receptor density and distribution.

ACKNOWLEDGEMENTS

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Differential Activity of a 160Kda Membrane Protease in the Organs of Developing Chick Embryos

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INTRODUCTION

Proteases are thought to enable malignant tumor cells to invade adjacent tissues by disrupting the complex extracellular matrix assemblies composed of glycoproteins and proteoglycans (proteolysis) (reviewed in: Alexander and Werb, 1991; Liotta, et al., 1991; Mignatti and Rifkin, 1993; Stetler-Stevenson, et al., 1993). Proteolysis of extracellular matrix by invasive tumor cells occurs where plasma membrane protrusions extend from the cell surface and contact the matrix (Chen, et al., 1984; Kelly, et al., 1994) which implies that membrane associated proteases function on the surface of the protrusions to mediate the matrix degradation. These specialized membrane protrusions with abnormally high protease activity are termed "invadapodia" (Chen, 1989) and are the morphological feature that distinguish matrix-degrading cells from those that do not actively degrade the matrix.

Recently, a 160kDa membrane associated protease was identified, which may function on invadapodia during degradation of extracellular matrix. The protease is an integral membrane glycoprotein that degrades gelatin and is active at neutral pH (Kelly, et al., 1994). The 160kDa protease is apparently present on the plasma membranes of invasive transformed cells where it might facilitate invasion of extracellular matrix (Kelly, et al. 1994).

Extracellular matrix proteolysis is also important in normal biological processes such as: 1) migration of immune system cells out of blood vessels, through extracellular matrix and towards sites of inflammation; 2) the endothelial cell invasion of matrix that occurs during angiogenesis; and 3) the extracellular matrix remodeling that normally occurs during the process of organogenesis (Mignatti and Rifkin, 1993). This study explored the possibility that the 160kDa protease may have a function in the normal development of chicken embryos. It is reported here that the protease shows differential activity in the organs of 9-day old chicken embryos. The protease was found in certain organs and was absent in others, suggesting that it does have a role in organogenesis and other aspects of embryonic growth.

METHODS

Dissection. Fertile Peterson-Hubbard chicken eggs (Keith Smith Farms, Hot Springs, AR) were grown for 9 days with gentle rotation in a 39°C model 1202 incubator (G.Q.F. Manufacturing, Savannah, GA). The embryos were dissected and several tissues were removed: heart, brain, bone, liver, lung, kidney, gut, skeletal muscle, skin, and gizzard. The tissues were place in separate tubes and kept on ice until homogenization or stored for longer periods at -80°C.

Homogenization and phase partitioning. Tissues were weighed and homogenized with a Dounce homogenizer in a solution of 20mM Tris-HCl, 150 mM NaCl, pH 7.6 (Tris-buffered saline = TBS) and 5mM EDTA. After homogenization, 2.6% Triton X-114 was added to all samples, and an additional 1.3% Triton X-114 was added to liver and brain samples, to aid in extraction of membrane proteins. The total amount of buffer added to tissues was 20x the weight of the tissue. Samples were extracted with agitation for a minimum of 1 h at 4°C, and the insoluble precipitate was collected by centrifugation at 12,000 x g for 3 min. The insoluble pellets were frozen and the supernatant was transferred to new tubes. All samples were then phase separated by warming the

tubes in a 37°C water bath for 1-2 min and centrifuged at 12,000 x g for 2 min to acquire a detergent and aqueous phase (Bordier, 1981). After phase separation, TBS/5mM EDTA buffer was added to the detergent phases so their volumes would be equal to that of the aqueous phases.

Protein determination by BCA assay. A small fraction of each of the samples was removed and precipitated with trichloroacetic acid (TCA): 10µl of TCA was added to 100µl of sample and incubated on ice for 15 min. The TCA was used to precipitate protein out of the samples in order to resuspend it in a sodium dodecyl sulfate (SDS)-containing buffer. By this, the assay was not influenced by differences in buffer, such as variable Triton X-114 concentration, because a single buffer is used. The samples were centrifuged at 12,000 x g for 1 min in order to collect the precipitated protein, and the supernatant was discarded. TCA was removed from the pellets by washing with ethanol 2-3 times. All ethanol was removed and the pellet was resuspended in a solution of 100µl of 80mM Tris-HCl, pH 6.8, 2% SDS. If no visible pellet was found, 50µl of this same buffer was added in hopes of concentrating any protein to within the linear range of the assay. The protein pellets were put back into solution by vigorous vortexing, and any remaining insoluble material was precipitated by centrifugation. The protein in the supernatant was determined using the bicinchoninic acid (BCA) assay (Pierce). For this assay, 10µl of sample and 200µl of reagent were put into wells in a 96-microtiter plate and incubated at 37°C for 30 min. Absorbance was measured at dual wavelengths of 540nm and 490nm using a microtiter plate reader. The reading at 490nm is a measure of background absorbance and is subtracted from the 540nm reading that measures the color intensity produced by the BCA. The protein concentration was calculated so that an equal amount of protein from each of these samples could be used to isolate 160kDa protease activity by exposure to wheat germ agglutinin (WGA) agarose.

Protease activity determination and isolation of the 160kDa membrane associated protease & gelatin zymography. WGA beads (50µl) were incubated with 135µg of protein from detergent and aqueous phase extracts of the organs. The WGA beads were collected by centrifugation and resuspended in sample buffer (80mM Tris-HCl, 2%(w/v) SDS, 15%(v/v) glycerol, 0.01% Bromophenol blue) having the reducing reagent dithiothreitol (DTT) and 10% (w/v) n-actetyl glucosamine, which causes release of proteins bound to WGA agarose. The beads were sedimented by centrifugation and the released proteins in the supernatant were subjected to gelatin zymography by the method of Heussen and Dowdle (Heussen and Dowdle, 1980).

RESULTS

Table 1 shows that 19-39% of extracted protein is partitioned to the detergent phase (Table 1, %protein detergent). Gizzard, composed mostly of smooth muscle, had only 19% of protein in the detergent phase, compared to 39% detergent phase protein in bone and skin. Here, organs were extracted in 20 volumes of buffer based on the weight of tissue obtained; the volumes of aqueous and detergent phases for each sample were kept equal. Because the volumes were kept constant, the protein determinations obtained in mg/ml could be directly compared.

Table 2 shows that activity of the 160kDa protease is differential in organs of the 9-day old chicken embryo. The protease is found in brain, bone, lung, gut, skeletal muscle, skin and gizzard as judged by gelatin zymography (Table 2, detergent phase). Among the extracts that have detectable protease activity, there are differences in the level of activity as judged by the relative intensity of the negative-stained bands revealed in the gelatin zymogram (Table 2, detergent phase). The 160kDa protease is not found in the detergent phases of heart, liver, or kidney. Moreover, this membrane protease is not detected in the aqueous phase of any organ (Table 2, aqueous phase).

Table 1. Different organs from nine-day-old chicken embryos were extracted with Triton X-114 and then phase partitioned into detergent and aqueous phases. The protein in each fraction was determined (see Methods) and recorded in mg/ml (Detergent Phase and Aqueous Phase). These data were added to derive the total protein present (Combined Protein) and then the percentage of protein in the detergent (% Protein Combined) and aqueous phases (% Protein Aqueous) was determined.

Organ	Detergent Phase	Aqueous Phase	Combined Protein	%Protein Detergent	%Protein Aqueous
Heart	0.174	0.474	0.648	27	73
Brain	0.126	0.313	0.439	29	71
Bone	0.110	0.177	0.287	39	61
Liver	0.111	0.224	0.335	33	67
Lung	0.143	0.310	0.453	32	68
Kidney	0.196	0.650	0.846	23	77
Gut	0.140	0.287	0.427	33	67
Sk. Mus.	0.120	0.300	0.420	29	71
Skin	0.136	0.220	0.356	39	61
Gizzard	0.158	0.666	0.824	19	81

Table 2. Different organs from nine-day-old chicken embryos were dissected, extracted with Triton X-114, and phase partitioned as described (see Methods). For each protease activity determination, 189 ug of protein was incubated with 50 ul of WGA-agarose beads (see Methods). Proteins that failed to bind the WGA-agarose were washed away and the bound proteins were subjected to gelatin zymography. In this table, samples that had 160 kDa protease activity as judged by gelatin zymography are recorded as "+" while those lacking detectable activity are labelled as "-". In addition, the relative intensity of activity is indicated with "+++" exhibiting the strongest activity while the least intense 160 kDa protease activity is indicated by "+".

Organ	Detergent Phase	Aqueous Phase
Heart	-	-
Brain	++	-
Bone	+++	-
Liver	-	-
Lung	+	-
Kidney	-	-
Gut	+	-
Skeletal Muscle	+	-
Skin	++	-
Gizzard	+	-

DISCUSSION

Different organs of the 9-day old chicken embryo have from 19% to 39% of protein partitioning to the detergent phase. The 160kDa protease, partitions exclusively to the detergent phase and shows differential activity in the organs of 9-day old chicken embryos.

Proteins that partition to the detergent phase are generally considered to be those that are tightly associated with membranes (Bordier, 1981). The results reported here indicate that there is some variability between organs in the amount of protein that partitions to the detergent phase. The reasons for this variability are not completely clear. One possibility is that there is a higher volume of cell membranes in some tissues relative to others. This could occur by having an increased area of internal membranes such as endoplasmic reticulum and Golgi complex, by having an increased surface area of the plasma membrane caused for example, by microvilli, or by having an increase in both internal and external membranes. Another possibility is that the detergent phase proteins of some tissues may not be as easily extracted from the insoluble material as those of other tissues.

The fact that organs of the chicken embryo show varied levels of activity of the 160kDa protease suggests that the protease has a specialized role in embryogenesis and that it is not just a common "housekeeping" protease providing for metabolic homeostasis. If the 160kDa protease functioned to maintain cellular homeostasis then it would follow that all tissues should express the protease. However, because the protease was detected strictly by its activity, it is difficult to rule out its existence in the tissues that had no detectable activity in an unstable or inactive form.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kelly for his guidance and Dr. Terry Hill for technical assistance in publishing this paper.

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An Osteological Look at Ratite Wing Growth: Implications for Paedomorphism

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ABSTRACT

The wings of ratites and flightless birds are generally much smaller relative to body size than their flying bretheren. A study was done to investigate possible paedomorphic[1] mechanisms in ratite wing development and to look at growth rates of the wing bones versus leg and skull growth. It was found that within the Dromaiidae (emus) that the humerus grew at a faster rate than the femur, a finding consistent with birds of flight. The ulnae of these birds were found to grow at an even greater rate than the humerii, also a finding in earlier studies of flying birds (Carrier and Leon, 1990). Of special interest was the relation between wing development in the flightless birds and the birds of flight. This study focuses on the emu, with minor comparisons to ostriches (Struthionidae) and rheas (Rheidae).

INTRODUCTION

The flightless condition of the Ratites sets them apart from most of their avian relatives. It is also noticed that Ratites are much larger in size than most of their living relatives, but that wing size is much smaller in relation to body size. Past studies have concluded that these wings are neotonous (Raikow, 1985), and the current investigation hopes to look further into these paedomorphic characteristics and, more specifically, whether the forelimb bones of ratites grow more slowly than those in birds of flight or if the bones stop growth at an earlier stage than their flying relatives. Measurements were taken of several dimensions of the legs, wings, and skulls. Comparisons were made to rhea and ostrich specimens, and to a bird of flight, the California gull (*Larus californicus*). The data for *L. californicus* was taken from a 1990 study by Carrier and Leon in which the conflict between development and skeletal function was addressed by analyzing the ontogeny of the gull's skeletal strength.

We hope to describe the growth and osteological development of the emu wing in this study. Using comparative data we have analyzed the two major long bones of the wing, the humerus and the ulna, in relation to each other, and to the skull and a major long bone of the upper hindlimb, the femur.

MATERIALS AND METHODS

Skeletal preparations were done of several juvenile, fledgling, and adult emus; an 8-day-old ostrich; and a rhea of 4-5 years in age. The birds were skinned and de-fleshed by physical methods and then dried to jerky. The juveniles were further de-fleshed using a beetle colony and the larger specimens were prepared by boiling the remaining tissues off. After surfaces were cleaned and cleared sufficiently, measurements were made for length and mid-shaft diameter of the humerii, ulnae, and femurs of the specimens. Additional measurements were made of cranial parameters such as braincase length and width, as well as overall skull length. These data were converted to graphical form and analyzed using the literature at hand.

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[1] Paedomorphism is, for the purposes of this study, synonymous with neotony. It is the retention of a juvenile or larval characteristic in the adult form of an organism.

RESULTS

The analyses of the three long bones that were studied utilized the overall skull length of the birds as a parameter indicative of developmental growth.

In our analysis of the growth and osteological development of the wing of the emu, we have found several general trends that hold true for the emus and the California gull. The humerus bone of the upper forelimb was found to grow at a greater rate than the femur bone of the upper hindlimb (Figure 1). This is true for birds of flight and appears to be so for all birds, flying or not (Carrier and Leon, 1990). Also noteworthy is that the ulna (lower forelimb) of the emu grew at a proportionally faster rate than the humerus (Figure 2), a trend also seen in Carrier and Leon's study.

Analysis revealed that the ostrich and rhea correlated to the emus in regards to femur growth, but not so much in ulna and humerus growth (Figure 3-5). The wing bones of the ostrich and rhea lie off the line of the curve fit of the emu bone growth, but this is only indicative of larger sized wings.

DISCUSSION

The wings of flightless birds, like the Ratites, have been studied in the past, but mostly as a description of fully developed specimens (Raikow, 1985). It is well known that the sternum of the flightless birds is reduced, the keel becomes shallow or is completely lost, the clavicles are reduced (and completely gone in ratites), and the angle between the scapula and the coracoid increases from acute to obtuse in relation to the birds of flight (Raikow, 1985).

Despite these differences outlined above, we found our data on growth of the wings of emus to agree with the general trends seen in birds, especially birds of flight. Carrier and Leon (1990) found that juvenile gulls had faster growth of the ulna in relation to the humerus, which was, in turn, faster in growth rate than the femur. However, structures can be the same across species, genus, and even family barriers, but the function of these developing structures is not necessarily similar (personal communication - Jaslow, A., 1996).

There are three major processes by which paedomorphosis is thought to occur. In the first, the rate of growth of the structure in question is significantly slower than the rate seen in "normal" growth. In the second method the structure grows at a normal rate, but stops growing before the usual time. Finally, a third possible paedomorphic process is that rate and time of growth are normal, but that the structure begins smaller. In this study we collected data that will be helpful in future, more thorough investigations into the issue of paedomorphic processes in Ratites. We also found that the second of the above methods is not the most probable option in emus.

The general trends for long bone growth in birds of flight also hold true for the emu. The growth of the humerus in relation to the femur is faster, as is the ulna in relation to the humerus. This seems to at least point toward the improbability of the second paedomorphic process described above (i.e. stopping growth early), since relation of the femur development to the overall development of the bird is consistent with data from flying birds. The growth of the wing bones of Ratites is also relatively consistent with the flying birds' data (when viewed in comparison to femoral growth). Whether or not the wings of ratites simply start off smaller or their rates of growth relative to rates seen in flying birds are slower remains to be seen.

Some of the problems we encountered in making a more direct comparison of our data to the findings of Carrier and Leon (1990) is that we did not use body mass as an indication of development, an assumption with much validity in juvenile semi-precocial birds. The source of

Relative Long Bone Growth Rates

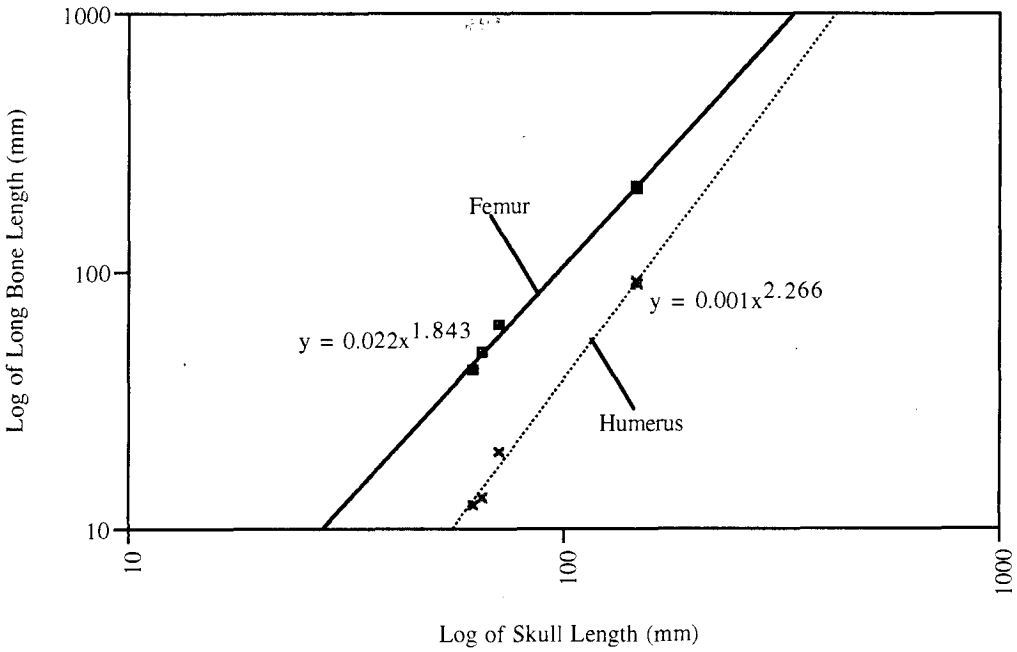

Figure 1. This figure shows a comparison of the relative growth rates (as logged) of the humerus and femur. Skull length is used as a value indicative of overall development.

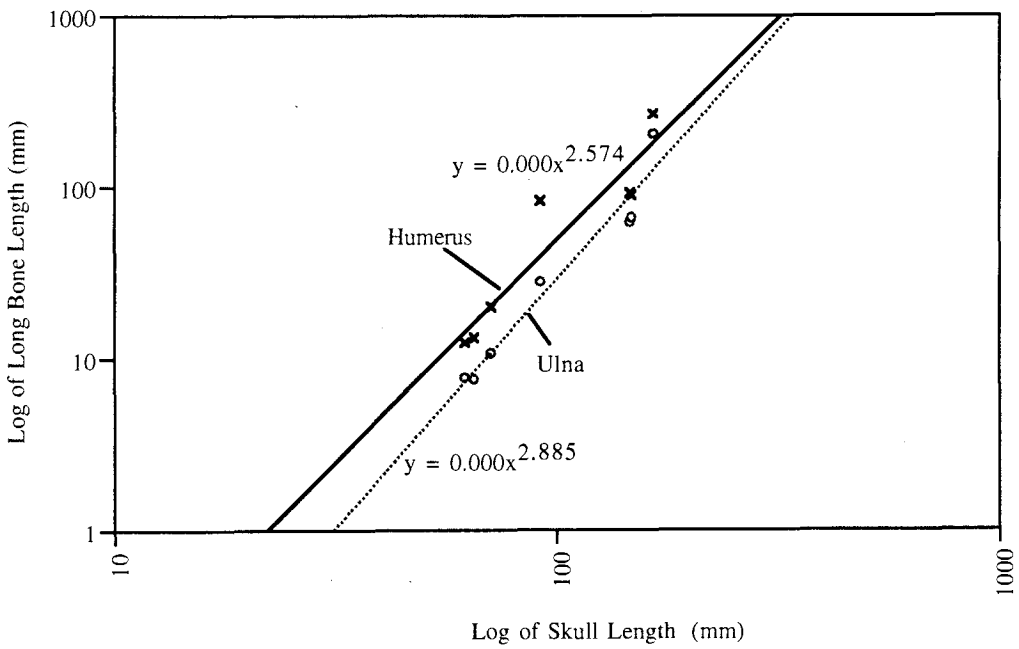

Figure 2. This figure shows a comparison of the relative growth rates (as logged) of the ulna and humerus. Skull length is used as a value indicative of overall development.

Comparison of Three Long Bones to Skull Length

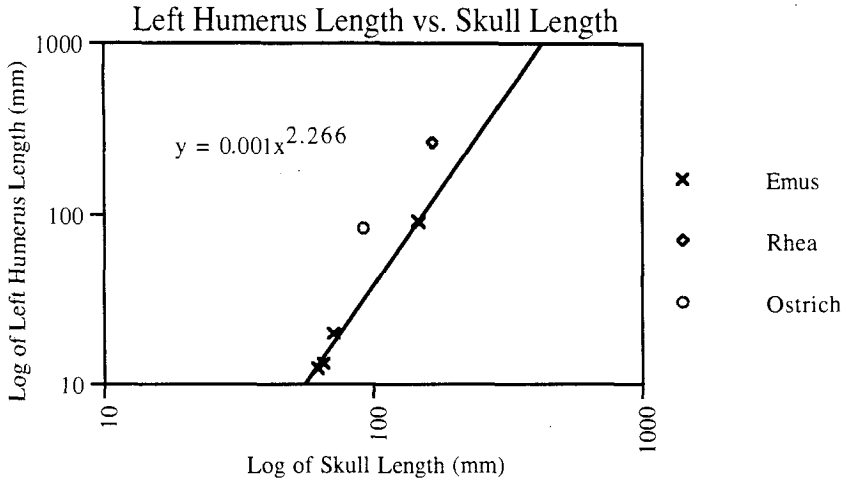

Figure 3. A comparison of emu humerus length in relation to overall skull length. Skull length is indicative of overall development. Note the position of the rhea and the ostrich.

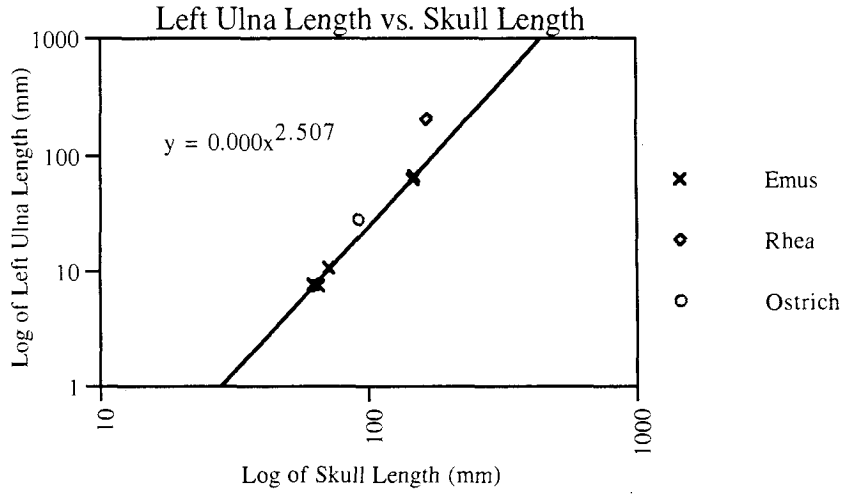

Figure 4. A comparison of emu ulna length in relation to overall skull length. Note the position of the rhea and the ostrich.

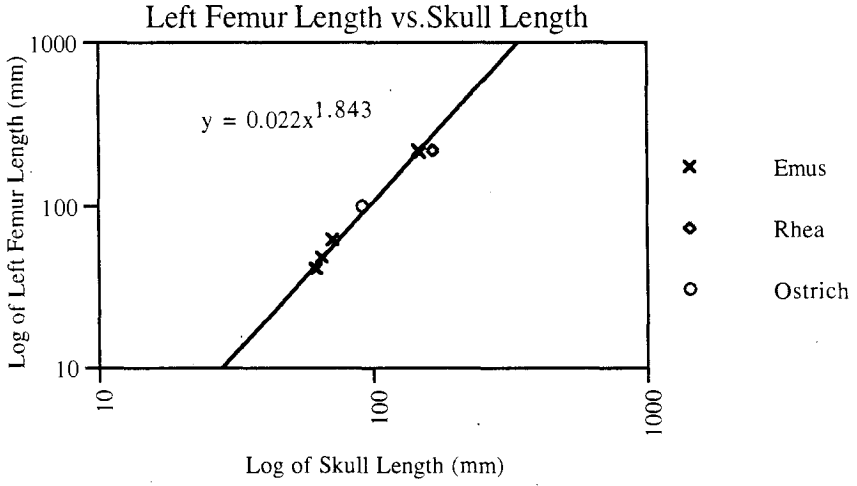

Figure 5. A comparison of emu femur length in relation to overall skull length. Note the position of the rhea and the ostrich.

our ratites was often through donation of autopsied specimens from a veterinarian. Not knowing how much mass had been removed during the autopsy procedure, we were forced to rely more on the skeletal features of these birds to indicate development. Our data for development is only one-dimensional and not easily related to Carrier and Leon's three-dimensional parameter. In the future we hope to find a more reliable indicator of development or further investigate the possibility of converting our data to a more comparable format.

Also in store for the future is possible comparison of our data to similar measurements to be taken from chicken specimens, a much more precocial bird than the California gull.

This study of ratite osteological development can serve as a building block for future studies between flightless and flying birds, as well as relating wing growth between ratites. With at least one paedomorphic process removed from the picture future investigations will be able to more clearly address the issue of paedomorphosis in ratite wings. The emus were not found to deviate from the general trends seen in avian development despite their flightless condition, a situation long thought to be true, but not investigated until now.

ACKNOWLEDGEMENTS

I would like to thank Dr. Alan P. Jaslow for his help and guidance on this project, both in gathering data and analyzing the results. Also very worthy of acknowledgement are Ms. Debbie Smith and Mr. Chris Farris, who did earlier skeletal preparations of the ratites. Thank you all!

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The Effects of Malathion on Esterase Activity in the Adult Cotton Boll Weevil, *Anthonomus grandis* Boh

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ABSTRACT

The distribution of esterase activity within a population of boll weevils was first determined by an enzyme assay. The activity fell into three major groups corresponding to three different genotypes. The esterase enzyme was assayed in the presence of malathion, an organophosphate. The malathion acted to reduce esterase activity through direct reaction between the enzyme and the pesticide or through non-competitive inhibition.

INTRODUCTION

The cotton boll weevil (Anthonomus grandis, Boheman) is a small insect that is notorious for its destructive forces on cotton crops. In 1994, the National Cotton Council Bulletin reported that the cotton boll weevil has been responsible for more than $13 billion dollars in damages since its arrival in the United States. In 1958, the National Cotton Council adopted a resolution to eradicate the boll weevil by formulating the Boll Weevil Eradication Program (BWEP). Unfortunately, the eradication program has failed to eliminate the cotton boll weevil. Continued research at the federal boll weevil research laboratory in Starksville, MS, has focused on eradication through pesticidal programs. Other Key players involved in cotton boll weevil control have been the USDA and the National Cotton Council.

Many other insects, especially cockroaches, have displayed increased resistance to organophosphates; the heightened production of esterase activity is the culprit (Matsumura et al, 1968). Most insecticides are organophosphates. Mouches et al (1987) have reported that the overproduction of detoxifying esterases in organophosphate-resistant Culex mosquitoes is the mechanism for resistance. Two hypotheses exist regarding how esterases react with organophosphates in other insects. The esterase could be responsible for metabolizing the organophosphate, or the organophosphate could act as a non-competitive inhibitor to the esterase, rendering the esterase inactive. Resistance has not been reported in the cotton boll weevil; but there is a possibility that higher levels of insecticide application may serve as a selection pressure for resistance, as it has for other insects.

In our experiment, we explore individual variation in esterase activity of boll weevil homogenates. Est 4, a digestive lipase in the boll weevil gut, makes up more than 80% of the esterase concentration in a cotton boll weevil, and it is primarily responsible for the general esterase activity assayed (Jones et al, 1986). Evidence suggests that Est 4 has 3 genotypes and displays 2 major allozymes, 1 for high activity and 1 for low activity. The codominant genotype codes for equal production of both allozymes (Biggers et al, 1996).

Next, we create a protocol in which pooled samples of boll weevils were homogenated in order to determine Est 4 activity in the presence of various concentrations of Malathion. We realize that Malathion is not the organophosphate of choice for boll weevils, but we use its as a model because of its availability. The measured absorbance values show decreased esterase activity with increased malathion application.

METHODS

Animal care. A population of cotton boll weevils, Anthonomus grandis Boh, were supplied by the Robert T. Gast Rearing Laboratory at Mississippi State, MIssissippi, as young larvae in larval medium. Adult food supplements, larval medium, and holding conditions were consistent to Bancroft and Jones (1977), with some modifications.

Esterase assay. Individual boll weevils were weighed and placed in a 0.04 M $NaPO_4$ buffered solution. The volume of the buffer was determined by the following conversion: 1 mL buffer for 10 mg of boll weevil. The boll weevil was crushed, and its homogenate was centrifuged for 5 minutes at 13,000 rpm. When pool esterase samples were taken, 10 weevils were crushed simultaneously in 2 mL buffer.

The substrate, beta-naphthyl acetate (BNA), was prepared by adding 5.6 mg of BNA per 1 mL acetone. The substrate solution, made fresh for each trial, required 1 mL of BNA/acetone added to 99 mL of 0.04 M NaPO4 buffer. Each test tube was accurately administered 5 mL of this esterase assay substrate.

When individual weevil esterase activity was assayed, 200 uL of homogenate was added to the substrate. In the pooled samples, 50 uL of homogenate was added. The enzyme/substrate complex was incubated for 25 minutes at 37 C; the test tubes were removed, 1 mL of 2.5 mg Fast Garnet/mL of 3.4% Sodium Lauryl Sulfate was added, and each tube was vortexed for 2 seconds. The enzyme/substrate/dye complex was incubated for an additional 5 minutes. A Spectronic 21, set at 560 nm wavelength, recorded the absorbance of the enzyme/substrate/dye complex.

Esterase assay with malathion. Concentrations of 200 uL, 100 uL, 50 uL, 25 uL, and 12.5 uL of 50% malathion were brought up to 10 mL in distilled water. 200 uL of this malathion solution was added to the substrate directly after the 50 ul of enzyme was added.

The esterase/substrate/malathion incubated for 2.25 minutes. Then, we administered 1 mL of 2.5 mg Fast Garnet/mL of 3.4% Sodium Lauryl Sulfate that stabilized the reaction for 20 minutes. Absorbance was recorded at 560 nm wavelength, and a substrate/malathion solution was the control.

RESULTS

Distribution of individual esterase activity roughly fell into three main regions. Low-active esterase activity was observed between 0.2 and 0.3 absorbance. Much activity was seen in the second region between 0.4 and 0.5. Both low and high-active esterase activity was observed. Finally, between 0.6 and 0.7, a region of high esterase activity was observed (Figure 1).

A control reaction consisting of esterase and substrate was used to compare relative changes in esterase activity. As increased concentrations of Malathion were administeredto the esterase/substrate complex, the relative esterase activity decreased (Figure 2).

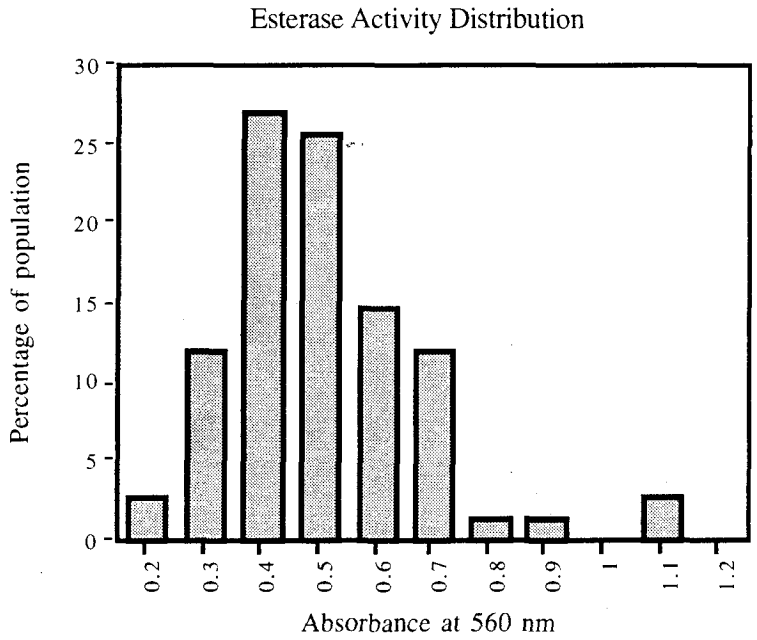

Figure 1. This bar graph illustrates the pattern of distribution of esterase activity. The y-axis gives the proportion within that absorbance range out of the entire population of weevils examined.

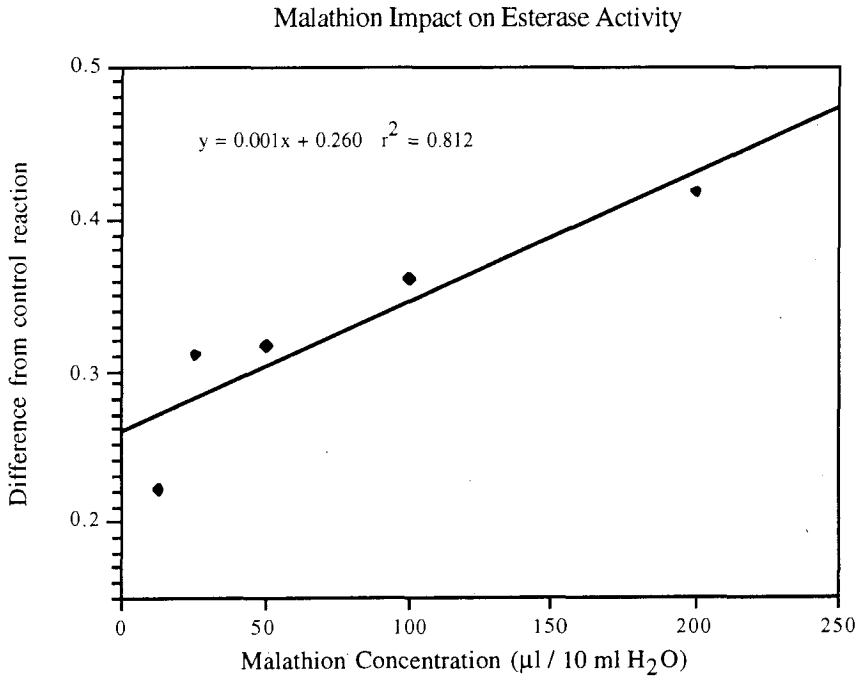

Figure 2. This graph represents the positive correlation between Malathion concentration and absorbance drop off from the control.

DISCUSSION

The collective data of individual esterase assays shows that 3 distinct regions of activity exist in the homogenate. Approximately 25% of the boll weevils display low esterase activity. Fifty percent show medium activity, and 25% show high esterase activity. Obviously, three genotypes are acting in esterase production. Data imply that Est 4 has 3 genotypes and display 2 major allozymes, 1 for high activity and 1 for slow activity. The codominant genotype codes for equal production of both allozymes (Biggers et al, 1996). Variation among individual boll weevils is clearly depicted.

The protocol that we designed to investigate the interaction of malathion and esterase was a difficult task. We created a protocol that could accurately and consistently allow for esterase, substrate, and Malathion to interact in a buffered solution.

Figure 2 shows that increased malathion concentration reduces the amount of enzyme and substrate complexing in the test tube. The mechanism for these results could be explained in two ways. As esterase concentration remains constant, the increased organophosphate may act as a competitive inhibitor with the beta-napthyl acetate. The constant esterase concentration could have more of its active sites bound by malathion; therefore, enzyme/substrate complexes would be less frequent. The second possible mechanism may involve the organophosphate non-competitively inhibiting the active site of the esterase. In that case, the active site would be permanently unreactive to substrate. If true, then increased organophosphate would inactivate more esterases; therefore, less enzyme/substrate complexes would be formed. The physiological mechanism for resistance for cockroaches is not entirely understood, but Donald G. Cochran (1989) suspects that multiple resistance mechanisms have evolved since organophosphates were introduced to the cockroach.

High esterase activity may be naturally selected in resisting organophosphates. Hence, increased malathion concentration, or doses, could actively eliminate boll weevils with lower esterase activity. This selection pressure would cause the resistant boll weevils to survive and pass their selected genes to their progeny. Further research needs to be done in analyzing and isolating the highly active allozyme. Data gathered from such exploration would be extremely valuable in understanding possible resistance in the boll weevil.

Future research on the cotton boll weevil should emphasize the individual correlation between esterase activity and organophosphate exposure. Such research could verify our claims that increased esterase activity is proportional to increased resistance of organophosphates at an individual level. Further, does malathion affect both allozymes equally, or does it preferentially affect one greater than the other? Understanding these implications could lead the researchers to explore possible cellular mechanisms to prevent the transcription or translation of those allozymes in the cotton boll weevil.

ACKNOWLEDGEMENTS

Thanks is expressed to Dr. Bobby Jones for his support, his friendship, and his enthusiasm.

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