The Rhodes Journal of Biological Sciences

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Statement of Purpose

The Rhodes Journal of Biological Sciences is a student-edited, annual publication which recognizes the scientific achievements of Rhodes students. Founded fourteen 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.

Acknowledgements
Dr. John Olsen
Dr. David Kesler
Rhodes Biology Department Faculty

Editorial Note

Previously entitled *The Rhodes College Science Journal,* this journal formerly accepted reports from all branches of the natural sciences. The contents of the journal now reflect contributions by the faculty and students of the Biology Department. To eliminate confusion, the journal has been renamed *The Rhodes Journal of Biological Sciences*

Cover

An electron micrograph of a mouse retina by Edson Erkulwater '98 performed in Dr. T. Hill's electron microscopy class. **ROS**- Rod outer segment

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Research Information in Biology

Dr. Gary Lindquester: My latest project involves the development of a reverse transcriptase polymerase chain reaction (RT-PCR) assay for the detection of active equine herpesvirus 2 (EHV2) infection in horses and its use in correlating active infection and disease for this agent of unknown etiology. EHV2 has been isolated from up to 90% of apparently healthy horses and has been implicated in immunosuppression in foals, upper respiratory tract disease, conjunctivitis, general malaise, and poor performance. As a gammaherpesvirus latent in B lymphocytes, it is hypothesized that EHV2 is a significant factor in a number of unexplained illnesses, either upon primary infection or reactivation of latent virus. Because of the difficulty in identifying reactivation by antibody titers, RT-PCR detection of a gene expressed late in active infection will be used. The PCR assay will also distinguish and categorize EHV2 strains based on their extensive heterogeneity by use of restriction site differences within the amplified fragment. Reagents have been designed and tested for these experiments while on sabbatical leave. Upon optimization of the assay with in vitro reagents a larger scale epidemiological study assessing active EHV2 infection in clinically relevant horse populations may begin.

Dr. Alan Jaslow: My research interests are in the areas of vertebrate functional morphology and animal behavior. Past student projects include studies of mammal hair ultrastructure, mimicry, acoustic behavior, size and shape relationships in vertebrae, and scaling relationships in leg bones of ungulate mammals and ratite birds. I am also interested in other topics relating to amphibians, reptiles, and tarantulas.

Dr. Terry Hill: My research deals with the mechanisms of growth of microorganisms, especially of fungi and fungus-like protists. I've been investigating the mechanisms of secretion of enzymes involved in nutrition and morphogenesis- in the latter case, those that restructure the cell wall, permitting growth. The methods employed are those of traditional enzymology and protein chemistry (spectrophotometric enzyme assays, ultracentrifugation, gel exclusion and ion exchange chromotography, and electrophoresis), and more recently I've begun to employ immunochemical methods- i.e., those involving antibodies to locate and characterize the proteins of interest. For about the past two years, I've been collaborating with Dr. Loprete in Chemistry on some of these projects, and we've shared direction of some students working on the characterization of cellulase enzymes in the protist *Achlya ambisexualis*. Students have been involved in all aspects of this work, from enzyme isolation to raising monoclonal antibodies. One student co-authored poster has been presented at a regional meeting of the A.C.S., and one student co-authored paper has appeared in an international journal (Hill and Pott, 1997).

While it would be helpful for students to have had experience with specialized research techniques to participate in some aspects of the work, most of the techniques can be learned "on the job." Neither advanced class standing nor specific course work is required. Students with less than ideal grades are also welcome to apply.

In all cases, a sincere interest in pursuing a career in biological research will be considered a strong "plus."

Dr. Carolyn Jaslow: My primary research interests are in the areas of biomechanics, functional morphology, and the development of bone and other mineralized tissues. Biomechanics is the application of physical and engineering laws to study biological materials; for instance, can we predict the design of a tooth or a leg bone based on the forces and physical demands that the structure will experience? Biomechanics is a part of the study of functional morphology, which seeks to correlate an organism's form (morphology) with its functional or ecological demands. I am willing to take on students for projects in these areas depending on their previous course

experience and interests. I am also interested in working with students who want to use their electron microscopy experience to develop a research project.

Dr. David Kesler: I will support a wide range of research dealing with freshwater ecology. Examples of student research are the role of predation on larval dragonfly community structure, limnological characteristics of an area lake, and sediment load carried by the Wolf River. Students have generated a field guide of aquatic macroinvertebrates and sampled a local lake for freshwater mussels.

I am of the philosophy that "finding the problem" is important. If a student is interested in a realistic problem, I'll support it.

My current research deals with freshwater mussels. These threatened species have dwindled due to degradation of water quality, habitat loss, and invasion of exotic species. I am interested, among other things, in the rate at which these organisms grow and for how long they live. I have measured internal rings as indicators of age and growth rate, and I have also marked, measured, and annually sampled mussels. These two estimates of growth rate do not agree, and I am in the process of figuring out why they do not.

Dr. John Olsen: My research interests are threefold. I have an interest in the ultrastructure of plants, involving scanning electron microscope investigation. This has generated a number of student projects since the SEM is a relatively easy instrument to learn to operate and it generates very interesting images. Recent projects have examined differences in textile fibers. Next, I am interested in chromosomes as systematic tools. Our current level of technology in the department makes it possible to produce high quality images of mitotic and meiotic chromosomes and to compare organisms for chromosome morphology and number. A number of students have undertaken research projects that generate karyotypic data on a wide variety of organisms. Finally, I am interested in plant pigments, primarily that group of compounds that are called secondary compounds. Using the HPLC, it is possible to separate complex mixtures of these compounds for identification and comparisons between taxa.

Dr. Bob Jones: My research is on the genetics of esterase enzymes in the Cotton Boll Weevil. Weevils from the Gast Boll Weevil Rearing Laboratory and weevils collected from cotton fields in Shelby County are examined for the frequency of various esterases. Wild weevils are crossed with laboratory ones to culture genes for other studies.

Dr. Bruce Abedon: My research interests are diverse, ranging from the developmental biology of maize to disease resistance in *Brassica rapa.* Maize has a juvenile (seedling) and adult vegetative phase of development on the main stem that are anatomically and physiologically distinct. I am interested in how the rate of transition between vegetative phases affects different aspects of agronomic performance such as yield, disease resistance, and stalk strength. In addition to understanding this relationship, per se, my goal is to identify and isolate specific developmental rate-determining genes to facilitate improvement of maize agronomic performance. This research will be conducted primarily during summers at the Agricenter International, although I have several projects that can be done during the spring semester in my laboratory. In addition to the maize research, I am interested in studying the relationship between levels of resistance to several races (variants) of white rust (Albugo candida), a fungal pathogen, in B. rapa, and how resistance varies at different times in development. This research will be conducted in my laboratory during spring and fall semester. I welcome students to participate in all aspects of my research. If any students are interested in conducting research with me, or would like me to advise them on a project of their own making, they should stop by my office so we can discuss specific projects in greater detail.

Dr. Charles Stinemetz: The research conducted in my laboratory attempts to understand the response of plants to specific environmental factors. I am particularly interested in studies dealing with the response of plant roots to gravity stimulation (gravitropism) and water gradients (hydrotropism). The investigations I am interested in mentoring can be at either the whole organ or molecular level. I am also interested in developing a research programs which utilizes Wisconsin Fast Plants, Arabidopsis, or grapevines.

Dr. Jay Blundon: I am interested in the cellular mechanisms that allow neurons to communicate with other cells. Neurons release neurotransmitters or hormones, and these chemical messages serve as signals that communicate with other neurons, muscle cells, glands or organs. While neuroscientists have known for years that calcium ions play a major role in activating neurons to release neurotransmitters, we are only just beginning to understand many of the complex details of this process.

In the brain, signaling within neurons ultimately results in the collective activities (such as thinking, reasoning, planning, reacting, feeling, remembering) that we attribute to the mind. One such process within our brain gives us the ability to form memories of past experiences. Neuroscientists now believe that our ability to produce short term memories (memories that are only remembered for at most a day or two) is due to the capability of neurons in the brain to temporarily boost their communication with other neurons. I am currently researching the mechanisms that provide neurons with the capacity for short term memory. Neurons within the mammalian brain aren't the only neurons that show this capacity for changes in signaling ability. My research for the past several years, beginning with his postdoctoral research at the University of Texas at Austin, has centered around neurons found in crayfish. Neurons within crayfish limbs actually show patterns of activity similar to mammalian brain neurons, although crayfish neurons are much larger and easier to manipulate and study. Most recently, however, I am expanding my research on neuronal communication by studying cellular processes of mammalian brain cells grown in culture.

Though I welcome any students who are interested in contributing to the above mentioned research, he also supervises students in a wide variety of other physiological and neurobiological experiments. Typically, students will have taken Bio 340 Animal Physiology and/or Bio 370 Neuroscience prior to taking Bio 451/452 Independent Research or Bio 495/496 Honors Research.

ON THE PROCESS OF DIGESTION‡

BY: Tom Logue

‡ *The following was written for Dr. C . Jalow's Core Zoology Lab in response to the question "Describe three specific things that can happen to food from the moment it crosses your lips to its arrival in the stomach."*

There once was a large chunk of food
Which was put in the mouth and chewed.
As teeth tore and crushed,
The food became mushed,
And food preparation continued.

Bolus:
1 c. salivary amylase (for proper starch breakdown)
3 tsp. water
5 mucins, finely chopped
a pinch of HCO_3 (varies for pH preference)
1 set salivary glands
32 teeth
food
**Mix first four ingredients; load mixture into salivary glands. Check settings on teeth and salivary glands: for thick bolus, use low settings; for thinner bolus, set appliances to 'high.' Insert food into mouth. Release saliva mixture from glands and use teeth to mix saliva and food. When bolus forms, swallow. Serves 1.

A Marxist Interpretation of the Swallowing Process
The food in this case very clearly plays the role of the proletariat, abused in hideous ways and to horrifying extremes in an attempt to serve the mighty capitalist body. The soft palate, in blocking passage of the food and liquids into the nose, is analogous to agreements between capitalists and workers, which serve to prevent an uprising by keeping the workers in their place. (This place, it may well be notes is down -- to their dorm). Just as contractions of the tongue muscles force softened balls of food into the pharynx, market contractions gradually worsen the position of the proletariat by forcing wages down and allowing capitalists to exploit workers more effectively.

"Bottleneck" *also by Tom Logue*

Little boys in shorts
squatting in mud and ditches
eating some tadpoles.

Nine of ten tadpoles
suffering in the boys' hands
possess the Green Gene.

The remaining 'tads'
black, slimy, and endearing,
are mostly blue-gened.

Soon there will be frogs,
but where there would have been green
there will now be blue.

The population
has experienced a great change:
Thanks a lot, Jimmy.

THE COURTSHIP BEHAVIOR OF THE WOLF SPIDER *Schizocosa avida* (Walckenaer) (Araneae; Lycosidae)

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Faculty Supervisor: Gail Stratton, Ph.D.[2]

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ABSTRACT: Wolf spider courtship behavior generally consists of visual, acoustic, and chemical signals. In this study, video-recordings and sound recordings were used to record, analyze, and describe the courtship behavior of the wolf spider *Schizocosa avida.* Out of the 26 pairings of *S. avida* observed, 12 males showed courtship behavior and 3 pairs copulated. All of the females who copulated had matured in the laboratory and thus were known to be virgins. The courtship of *S. avida* was compared to the courtship behavior of 15 other wolf spider species. The courtship behavior of *S. avida* was most similar to *S. retrorsa* and *S. mccooki*, two other species who perform palpal drumming. The courtship behavior of the male *S. avida* was found to have unique elements, confirming that courtship behavior is species specific. Some prominent behaviors observed during a male courtship sequence include Extend and Tap (legs 1), Extend and Vibrate (legs 1), Step Vibrate (legs 1), and Rapid Palpal Drumming. The Rapid Palpal Drumming produced audible sounds and is the most distinctive behavior of *S. avida* courtship, however its role in courtship is unclear and requires additional study.

INTRODUCTION

Courtship behavior must be conspicuous enough to attract the female's attention, but such behaviors may also attract the unwanted attention of predators (Platnick 1971). In spiders, courtship behaviors place the male in danger of predation from natural enemies and from the female spider. In many spider species, the female is larger than the male and could attack the male while he is in close proximity (Robinson 1982). Thus, one of the functions of courtship behavior in spiders is to suppress the female's predatory instincts. A second function is to stimulate both the female and the male for copulation. A third function is to facilitate species recognition between the two spiders (Robinson 1982, Platnick 1971). Spider courtship behavior is generally thought to be species specific and may act as a reproductive isolation mechanism between some species pairs. However, a systematic analysis of behavior for a large number of species of spiders has not been performed.

Wolf spiders make up the family Lycosidae. Most of these spiders do not build webs to capture prey. Instead, a wolf spider hunts for prey on the ground, preying on insects smaller than itself. Wolf spiders have very large eyes and the second best eyesight of all spiders. Wolf spider courtship behavior consists of visual, acoustic, and chemical signals. Visual signals may consist of enhanced colorations or structures on the male in combination with distinct movements performed by the male. Acoustic signals can be produced in a variety of ways and may allow courtship to continue into the night or in a dark environment when visual cues are ineffective (Platnick 1971). Chemicals cues include female pheromones that induce courtship behavior in males (Rovner 1968). Many males show a "chemoexploration" behavior which is the rubbing of the dorsal side of the palps on the substrate, presumably detecting chemical cues left by a female.

Schizocosa is a genus of wolf spider that is found throughout the Southeastern U.S. *Schizocosa avida* (Walckenaer) is a medium to large wolf spider found in meadows and grasslands of the Eastern U. S. *S. avida* is identifiable by a dark heart mark on the dorsal side of the abdomen and a light colored shield on the ventral side of the abdomen. Males and females have identical coloration and markings. In 1978, Dondale and Redner briefly mentioned that male *S. avida*

spiders drum their palps during courtship, but a detailed description of courtship has not been made for this species until now. This study examines the courtship of *S. avida* males in order to determine what behaviors are used in courtship and which behaviors distinguish *S. avida* from other species of wolf spiders.

METHODS

S. avida spiders were collected from Marshall and Lafayette counties, Mississippi, during the summer of 1996. Spiders were found in grassy areas and on the edges of woods. Spiders were housed individually in 8cm X 4cm plastic containers at the University of Mississippi under a 14L/10D lighting schedule. The spiders were fed 2-4 crickets once or two times per week and had continuous access to water.

Behavioral studies were carried out at the University of Mississippi using a video camera to record visual behavior and a stereo needle to record sound. The arena used for behavioral trials consisted of a clear petri dish encircled by a wall of clear acetate paper and lined with the filter paper cageliner of the female being observed. It has been shown that male spiders will perform courtship behavior when exposed to the female's pheromones on a used cageliner (Rovner 1968). The stereo needle recorded the sounds inside the trial arena by touching a portion of the liner that extended through a slit in the acetate paper. Spiders were fed one to two days before each pairing.

For each trial, the female was placed first behind a barrier in the arena. The male was introduced into the arena five minutes later, after the female had settled. Video-recording of behavior started when the male was placed in the arena. After observing the two spiders, the barrier surrounding the female was lifted if she showed no predatory behaviors in response to the male. Once the barrier was lifted, the two spiders were allowed to interact until 1) antagonistic behavior was shown by one of the spiders (chasing/alarming each other), 2) male courtship behavior was shown for an extended period of time (10-20 minutes) during which copulation did not occur, or 3) copulation was initiated by the spiders.

Only mature spiders were used in the behavioral trials. Male-female pairings were matched randomly based on the spiders available. The videotapes of *S. avida* were viewed numerous times and prominent behaviors were described. In addition, courtship videotapes of 15 other wolf spider species were analyzed for prominent behaviors for use as a comparison for *S. avida* (see Table 1 for list of species). These courtship videotapes were made during previous studies of wolf spiders and stored in a library of spider videotapes at the University of Mississippi.

Table 1: Fifteen wolf spider species whose courtship behavior was analyzed for comparison with *S. avida* courtship behavior. Two species of Schizocosa have yet to be formally described but are believed to be distinct species.

Schizocosa crassipes	(Walckenaer)
Schizocosa duplex	Chamberlin
Schizocosa floridana	Bryant
Schizocosa mccooki	(Montgomery)
Schizocosa ocreata	(Hentz)
Schizocosa nr. ocreata	
Schizocosa retrorsa	(Banks)
Schizocosa rovneri	Uetz & Dondale
Schizocosa saltatrix	(Hentz)
Schizocosa nr. saltatrix	
Schizocosa stridulans	Stratton
Schizocosa uetzi	Stratton

Gladicosa bellamyi	(Gertsch & Wallace)
Lycosa annexa	Chamberlin & Ivie
Trochosa avara	Keyserling

RESULTS

Thirteen female and ten male *S. avida* were used to videotape 26 sequences of male/female interaction. Of those 26 sequences, 12 pairs showed behavior subsequently defined as courtship, and 3 pairs showed courtship followed by copulation. Eleven pairs did not show courtship. All three females who copulated were known to be virgins (Table 2).

Table 2: Pairings and outcomes of *S. avida* courtship behavior trials. Out of 26 total pairing, 11 males did not show courtship behavior, 12 males showed courtship behavior, and 3 pairs copulated. An "*" indicates a female known to be a virgin.

Date	Male	Female	Outcome
May 28, 1996	231	234	No Courtship
May 28, 1996	233	234	No Courtship
May 29, 1996	231	234	No Courtship
May 29, 1996	230	234	No Courtship
May 29, 1996	232	234	No Courtship
June 26, 1996	238	222	No Courtship
June 26, 1996	232	222	No Courtship
June 26, 1996	237	222	Courtship
June 26, 1996	233	240	Courtship
June 26, 1996	230	240	Courtship
June 27, 1996	238	222	No Courtship
June 27, 1996	232	222	No Courtship
June 27, 1996	237	222	Courtship
June 27, 1996	230	222	Courtship
July 5, 1996	237	240	Courtship
July 5, 1996	214	222	Courtship
July 10, 1996	232	221	No Courtship
July 10, 1996	230	*244	**Copulation**
July 10, 1996	231	272	Courtship
July 10, 1996	239	276	Courtship
July 10, 1996	214	274	Courtship
July 10, 1996	238	275	No Courtship
August 14, 1996	246	249	Courtship
August 14, 1996	239	245	Courtship
August 14, 1996	230	*223	**Copulation**
August 14, 1996	248	*247	**Copulation**

In a trial that consisted of courtship but not copulation, generally, the male would begin rubbing his palps on the substrate as he walked (**Chemoexploration**) when first placed on the female cageliner in the trial arena. Then male would begin displaying a variety of behaviors involving leg tapping and palpal drumming (see Table 3 for definitions of behavior seen in *S. avida* and other species). As he moved about the arena, he would hold out his forelegs and tap the substrate (**Extend and Tap**) or hold out his forelegs and slightly vibrate them up and down (**Extend and Vibrate of Leg 1**). He would also vibrate his forelegs as he walked (**Step Vibrate**), and tap his palps very quickly on the substrate producing a rattling or percussive sound (**Rapid Palpal Drumming**). During this time, the female either remained motionless or waved legs 1 & 2 in an arching manner (**Double Arch**). Once the barrier between the male and female

was lifted, the male would continue leg taps, vibrations, and palpal drumming as he walked. If he came into contact with the female he would dart away and drum his palps much louder than before, while positioned such that his forelegs were raised with the tips pointed outward (**Front Leg Brace**). During this time, the female would actively walk around the cage, remain motionless, or walk slowly with her body held low to the ground with legs outstretched (**Slow Walk**). At the end of the slow walk, with her body low to the ground, she would perform double arches. Eventually, the female would resume actively walking around the arena.

Table 3: Ethogram of Behaviors defined in *S. avida* and fifteen other species of wolf spider. Behaviors seen in *S. avida* are bolded.

Chemoexploration. The dorsal side of the palps touches or rubs along the substrate.
Double arch. Legs 1 and 2 lifted into an arch position, extended and lowered. Can occur quickly or slow & deliberately.
Extend and vibrate of leg 1. Leg(s) 1 is extended parallel to substrate and vibrates up and down.
Step vibrate. Leg(s) 1 vibrates slightly while walking.
Extend and tap. Leg(s) 1 is extended and the tip of the leg touches the substrate.
Rapid palpal drumming. Palps move up and down very quickly, touching the substrate; much faster than palpal drumming.
Front leg brace. Leg(s) 1 is lifted with the angle of the femur pointed backwards and the rest of the leg pointed forward.
Slow walk. The body is held low to the ground with legs outstretched while walking.
Rearing. Legs 1 are waved high above and in front of body.
Leg rubbing. Two adjacent legs are rubbed together very quickly.
Single arch. Same as double arch, but with only leg 1.
Double leg raise. Legs 1 and 2 are lifted into an arch and lowered similar to a double arch, but has more height and is more linear.
Single leg raise. Same as a Double leg raise, but with only leg 1.
Extend of leg 1. Leg(s) 1 is extended parallel to the substrate.
Leg-palp tap (= "extended leg tap" of Hebets). Leg 1 and/or leg 2 is extended and quickly tapped on the substrate simultaneously with a palpal tap.
Minute leg tap. Leg(s) 1 is barely lifted from the substrate and returned to the same position with a slight tapping sound.
Short leg tap. Leg(s) 1 is lifted from the substrate and returned to the same position with a tapping sound. This tap is higher than the minute leg tap, but not over the body like the high leg tap.
High leg tap. Leg(s) 1 is lifted from the substrate over the body and returned to the same position with a tapping sound.
Vibrating Wave. Leg(s) 1 or legs 1&2 is waved up and down from nearly vertical above the body to nearly horizontal to the substrate. The leg is vibrating as it moves up and down.
Paired Arch Legs I are lifted above the body in an arch, extended and lowered.
Pushup. While palpal drumming, body is raised slightly resulting in a slightly louder drumming sound.
Abdomen bob with Stridulation. The abdomen is moved up and down but clearly does not touch the substrate, accompanied by simultaneous palpal stridulation.
Abdomen bob without Stridulation. The abdomen is moved up and down but clearly does not touch the substrate, not accompanied by simultaneous palpal stridulation.
Abdomen bounce. The abdomen is moved up and down and touches or nearly touches the substrate.
Cheliceral bounce. The body is lowered quickly with the chelicerae impacting the substrate.
Palpal stridulation. The tips of the palps stay stationary on the substrate as the limb portion moves back and forth.
Rhythmic stridulation. Stridulation occurring in distinct units (pulses), usually 1-2 pulses per second or 1 pulse every 2 seconds. The noise is on-off, on-off, etc. With only one sound being repeated.
Extended stridulation. Stridulation occurring uninterrupted for 1- 2 seconds, includes body movement that appear to make the sounds louder or exaggerated. The noise is on-on-on with only one constant sound.
Extended pulse stridulation. Stridulation consisting of distinct units of sound occurring for at least 1 second or

more. At least two discernible sounds can be heard. Noise 1 is on-on-on, sound 2 on-off-on-off, etc. Noise 2 overlays Noise 1.
Palpal drumming. Palps move up and down quickly, touching the substrate.

In sequences that resulted in copulation, the male moved around the arena chemoexploring, tapping, and vibrating his legs as described above. The males in the three trials leading to copulation did not show any rapid palpal drumming. During these trials, the female would remain stationary rather than moving about the arena, and occasionally perform double arches. In two copulation trials, the male slowly approached the female while waving his forelegs above and in front of his body (**Rearing**). Once the male was within touching distance of the female, he held his forelegs in a front leg brace. He held this position until he mounted the female in a quick grasping motion.

The courtship behavior of *S. avida* shares some common characteristics with other wolf spiders. Chemoexploration is seen in many other species including *S. ocreata*, *S. rovneri* (Stratton & Uetz, 1986), *S. stridulans* (Stratton 1997), and *S. retrorsa* (Hebets 1996). However, rapid palpal drumming is a behavior unique to *S. avida*. A similar palpal drumming behavior is seen in *S. mccooki* and *S. retrorsa*, but *S. avida* drumming is much faster and is coupled with locomotion during courtship (Stratton & Lowrie 1984, Hebets 1996).

DISCUSSION

The courtship behavior of the male *S. avida* was found to have unique elements. This study confirms that courtship behavior is species specific. The most distinct behavior of *S. avida* is Rapid Palpal Drumming, where the male taps his palps quickly on the substrate when either approaching the female or retreating from the female. Drumming behavior is also seen in the wolf spiders *S. mccooki*, *S. retrorsa*, and *Hygrolycosa rubrofasciata* (see Figure 1). *S. avida* is distinguishable from *S. mccooki* and *S. retrorsa* because the *S. avida* male drums his palps much faster and is mobile throughout his courtship sequence (Stratton & Lowrie 1984). *S. avida* is distinguishable from *H. rubrofasciata* because the *S. avida* male drums his palps on the substrate rather than on his abdomen (Kronestedt 1996). In *H. rubrofasciata*. it was demonstrated that drumming activity correlates to viability (Kotiaho et al. 1996). This idea could also be tested in *S. avida*.

One of the perplexing features of the *S. avida* courtship behavior was that males who achieved copulation never performed Rapid Palpal Drumming. I originally thought that the Rapid Palpal Drumming was necessary for species recognition by the female and subsequent receptivity by the female. However, this is not the case since females copulated only with non-drummers. I then noticed that all three of the receptive females had just emerged from their final molt into adulthood and thus were virgins. I suspect that these females are much more receptive than non-virgin females. Perhaps they are more receptive because they have not yet mated and need to have one act of insemination before they can afford to show sexual selection of males, as was seen in the species *Lycosa tarentula fasciiventris* (Fernandez-Montraveta & Ortega 1990).

I hypothesize that copulating males did not show Rapid Palpal Drumming because this behavior serves to suppress the female's predatory instinct (Robinson 1982, Platnick 1971). The female may indicate her eventual receptivity to the male at the start of courtship. This signal may be the female's activity level. The receptive females remained stationary throughout the male's courtship rather than roaming around the arena like the non-receptive females. Norman Platnick reports that in species where non-receptivity is signaled by aggression "the female remaining motionless during certain periods my be just as specific a response and just as effective a releaser as an active display" (Platnick 1971). Therefore, if the *S. avida* male senses that the female is going to be receptive, the male does not need to suppress her predatory instincts by Rapid Palpal Drumming. This would also explain why whenever the female actively approached the male, the

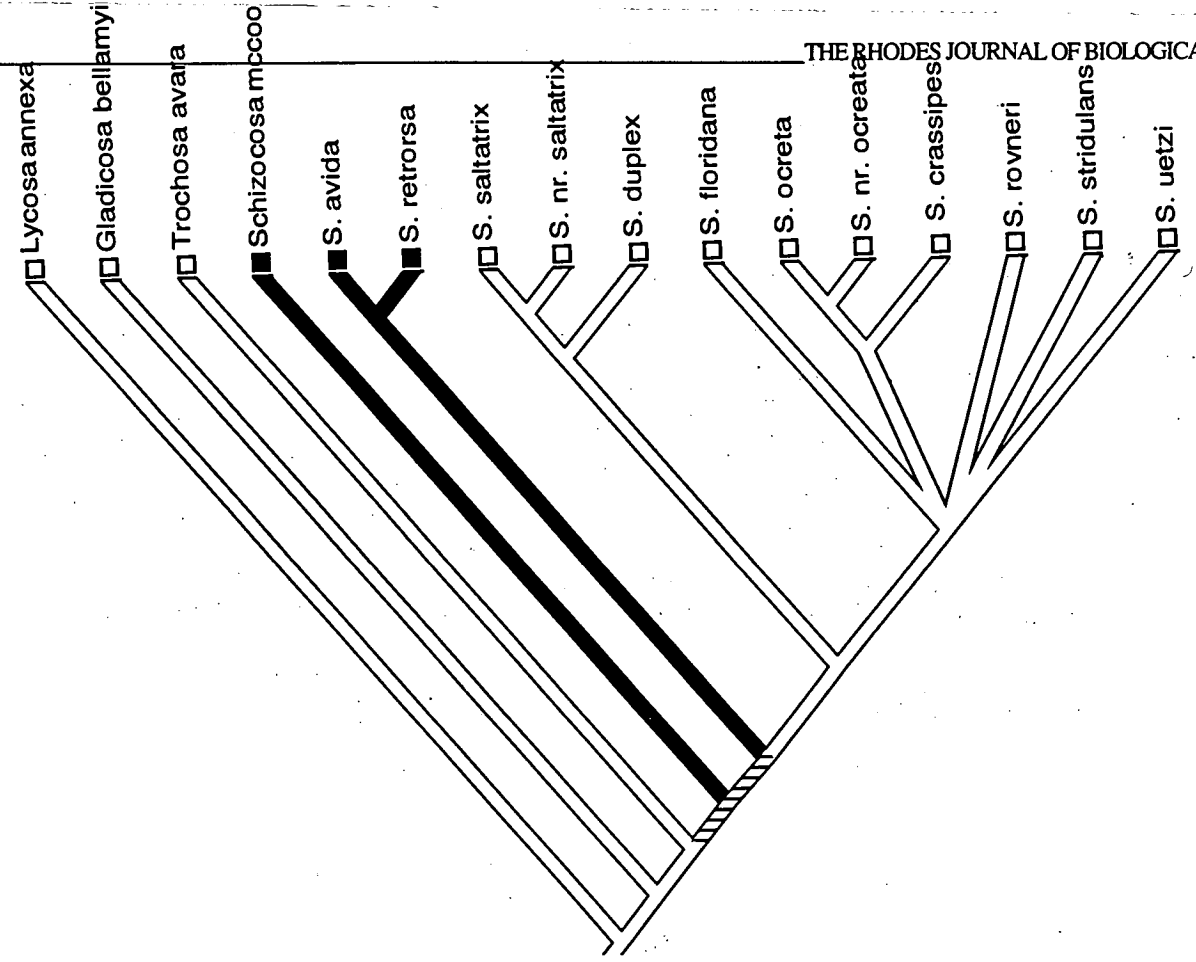

Figure 1: The distribution of Palpal Drumming (shaded portion) seen in the courtship behavior of 16 species of wolf spider overlaid onto a morphological phylogeny. Note: only *S. avida* displays Rapid Palpal Drumming, a more specific form of Palpal Drumming.

male responded by quickly retreating and drumming vigorously. By drumming in this situation, he may be trying to ward off a potential attack by the female by momentarily suppressing her predatory instincts. Rapid Palpal Drumming may be a risky behavior for the male because it might attract the attention of predators. Therefore, males may perform drumming when it is absolutely necessary but not when the female is perceived to be receptive.

There are still many questions about *S. avida* that have yet to be answered. Future studies could address the relationship between female virginity and receptivity, the role of Rapid Palpal Drumming in courtship, and the means by which the female signals her receptivity to the male.

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A HISTOCHEMICAL AND PHYSIOLOGICAL ANALYSIS OF PERFORMANCE IN THE PLANTARIS LONGUS MUSCLE OF THE FROG (*Rana pipiens*) AND THE TOAD (*Bufo terrestris*)

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ABSTRACT:Frogs typically move with powerful leaps for short periods of time, while toads display slower, lower levels of locomotion that can be sustained almost indefinitely. The objective of this experiment was to determine whether these differences in locomotory behavior of frogs and toads are reflected in the physiological properties of the plantaris longus (a.k.a. gastrocnemius) muscle, a primary muscle involved in movement. Frozen cross sections of frog (*Rana pipiens*) and toad (*Bufo terrestris*) skeletal muscles were stained for NADH dehydrogenase to investigate the primary pathway of ATP production in each fiber, anaerobic glycolysis or oxidative phosphorylation. Muscle fibers were also stained for myosin ATPase to determine whole muscle percent composition of slow twitch versus fast twitch fibers. Isolated whole muscle contractile performance was investigated by administering trains of stimuli and measuring rate of contraction fatigue at various points. Histological stains of the frog and toad gastrocnemius showed the frog gastrocnemius is composed of a significantly larger percentage area of fast twitch glycolytic fibers, while the toad gastrocnemius has a significantly larger percentage area composed of slow twitch oxidative fibers, in addition to a band of purely slow oxidative fibers comprising approximately 15-20% of the whole muscle. Fatigue tests showed that the frog gastrocnemius is capable of generating more force in the short term, while the toad gastrocnemius is able to sustain low levels of force over extended periods.

INTRODUCTION

Understanding the physiological basis for contractile properties of skeletal muscle is important in our understanding of the considerable diversity in behavioral responses and metabolic sources of energy that exists among amphibians during activity. Previous research has suggested that this range of diversity is well exemplified by *Bufo* (toads), and *Rana* (frogs) (Bennett 1974; Duellman and Trueb 1986).

The frog responds to threat or stimulation with rapid escape behavior, involving powerful leaps. However, stamina is low and exhaustion ensues in 2-5 minutes (Bennett 1974). In contrast, the toad seems incapable of exhibiting behavior beyond the scope of a moderate walk and can sustain low levels of activity almost indefinitely. Predation is avoided by the adoption of an inedible posture (the toad puffs itself up) or reliance on poisonous skin secretions rather than rapid escape behavior (Bennett 1974; Mendiola et al. 1991).

The objective of this research was to determine whether such different patterns of locomotory behavior are evident in the physiological properties of the frog and toad plantaris longus (a.k.a. gastrocnemius) muscle. Physiologists have known for years that vertebrate skeletal muscles are composed of different varieties of muscle fibers. These fibers vary in their source of energy (anaerobic glycolysis versus oxidative phosphorylation), their speed of contraction (fast

versus slow twitch), and rate of fatigue (fatigue prone versus fatigue resistant) (Eckert et al. 1988).

The gastrocnemius is a large, thick-bellied muscle that extends the foot. It originates by a slender dorsal tendon from the distal border of the aponeurosis covering the knee, and also by a short, cylindrical tendon formed by the union of two branches of the tendinous arc along the medial surface of the knee. The gastrocnemius inserts distally by a thick, flat tendon that spreads out on the plantar surface of the foot to form the aponeurosis plantaris, from which numerous tarsal and foot muscles originate. Contraction results in a straightening of the ankle joint, and hence it is a primary muscle used in locomotion (Duellman and Trueb 1986).

Frozen cross sections of the gastrocnemius muscle in *Rana pipiens* and *Bufo terrestris* were stained for NADH dehydrogenase to quantitatively determine the primary pathway of ATP production in each muscle fiber, anaerobic glycolysis or oxidative phosphorylation. Myosin ATPase stains were used to determine percent composition of slow twitch versus fast twitch muscle fibers. Whole muscle contractile performance was assessed by administering trains of stimuli until zero tension was reached, and then measuring fatigue rates. Results from the tests suggest that the frog gastrocnemius is better adapted to short bursts of high force responses because it contains a majority of large diameter fast twitch glycolytic fibers. The toad gastrocnemius is more suited to sustaining lower levels of tension over longer periods of time because it contains a much higher proportion of small diameter slow twitch oxidative muscle fibers.

METHODS

Twelve adult frog specimens and thirteen adult toad specimens were utilized in this experiment. Histological stains were done by first mounting the isolated gastrocnemius muscle on a cryostat chuck with dental wax and covering it with O.C.T. frozen tissue embedding medium. The muscle was then frozen by brief (10-12 s) immersion in isopentane cooled with liquid nitrogen. Cross-sectional slices were made midway along the muscle in a cryostat 16 μm thick at -30° C and immediately transferred to slides. The tissue was air-dried from 15-60 minutes prior to incubation. A fraction of these slides were stained for myosin ATPase at 4° C for 30 minutes (Padykula and Herman 1955). The remaining slides were placed in NADH-dehydrogenase incubation medium at room temperature in the dark for one to one and a half hours (Nachlas et al. 1958). Original staining procedures were slightly modified by Ogonowski and Lang (1979). A value for the percent of total muscle area that each fiber type composed was measured using computer imaging techniques in four randomly chosen sample areas for different tissue samples per muscle.

Physiological tension/fatigue studies involved attaching the isolated muscle to a force transducer and determining stimulation voltage and whole muscle length which produced the maximum tension response upon single 10 ms pulse stimulation. The muscle was then fatigued to zero tension by stimulating with trains consisting of five pulses (10ms duration pulses @200 Hz) delivered at 0.5 Hz. Rate of fatigue (measured as % decrease in tension per second) was then determined at tension values corresponding to 75%, 50%, and 25% of maximum muscle tension.

Data was analyzed using paired and independent T-tests (Systat).

RESULTS

Histochemical stains for the frog (Fig. 1) and the toad (Fig. 2) show that glycolytic fibers stain lighter than oxidative fibers for NADH-dehydrogenase due to the presence of fewer

mitochondria. Fast twitch fibers stain darker for myosin-ATPase activity than slow twitch fibers due to a greater density of the faster ATPase enzyme. The stains reveal that the gastrocnemius of *Rana pipiens* is composed throughout of a mix of fiber types; the gastrocnemius of *Bufo terrestris* also exhibits a mix of fiber types in approximately 85% of its area, but also displays a pure band of slow oxidative fibers composing $16 \pm 3.1\%$ of the total cross-sectional area.

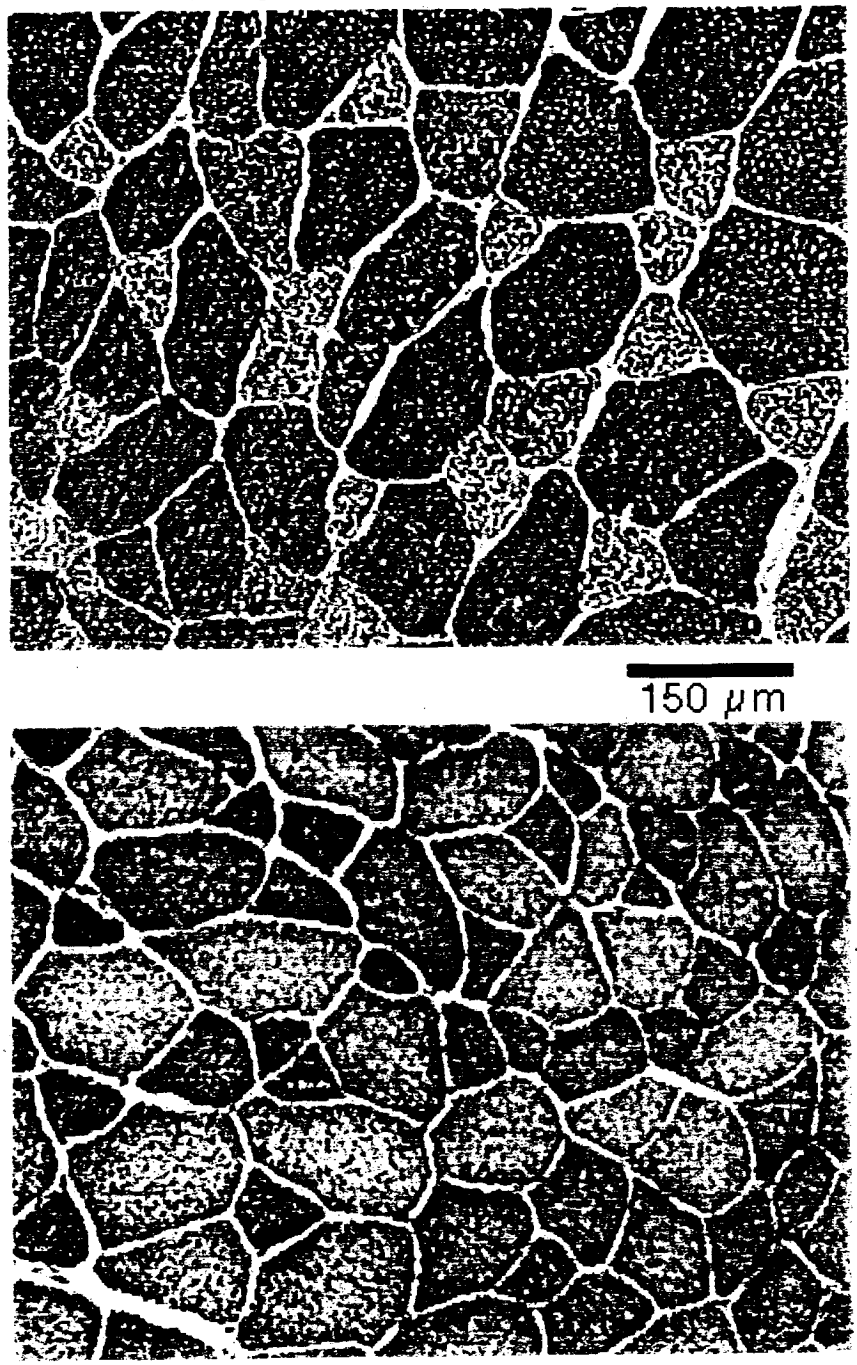

Figure 1: Histological stains in the frog (*Rana pipiens*) for myosin ATPase activity (top) and NADH dehydrogenase (bottom).

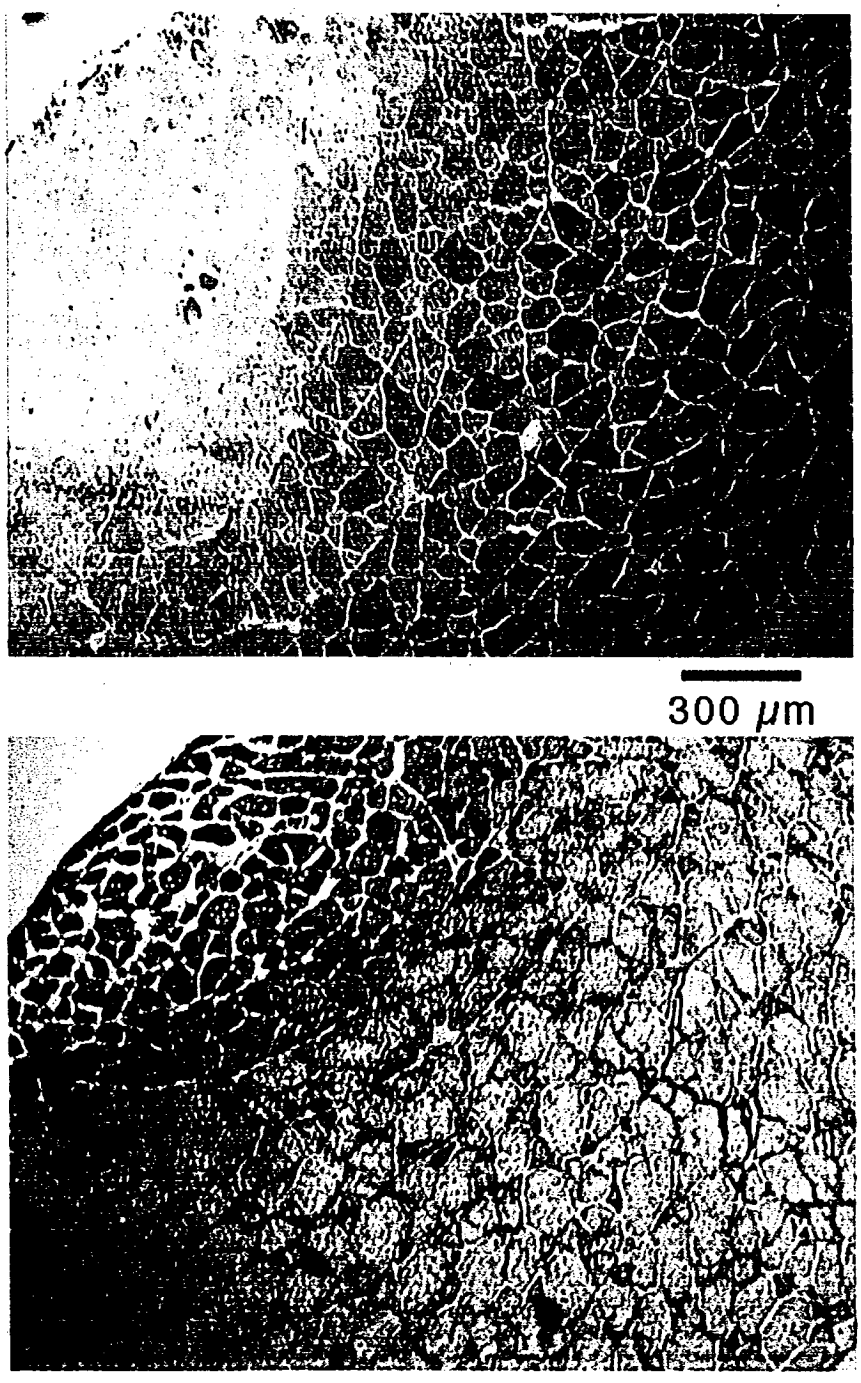

Figure 2: Histological stains in the toad (*Bufo terrestris*)for myosin ATPase activity (top) and NADH dehydrogenase (bottom).

A quantitative analysis of these stains reveals that glycolytic fibers compose 78 ± 6.7% of the total area in the frog gastrocnemius, and oxidative fibers compose 22 ± 6.7%. The toad gastrocnemius contains 48 ± 2.7% glycolytic fibers and 52 ± 2.7% oxidative fibers (Fig. 3). Frog fast twitch fibers compose 78 ± 5.0% of total muscle area, and slow twitch fibers compose 22 ± 5.0%. The toad gastrocnemius contains 47 ± 3.4% fast twitch fibers and 52 ± 3.4% slow twitch fibers (Fig. 3). Note that in the frog gastrocnemius, fast glycolytic fibers compose the majority of total muscle area. In the toad gastrocnemius, fast glycolytic and slow oxidative fibers compose approximately equal amounts of area.

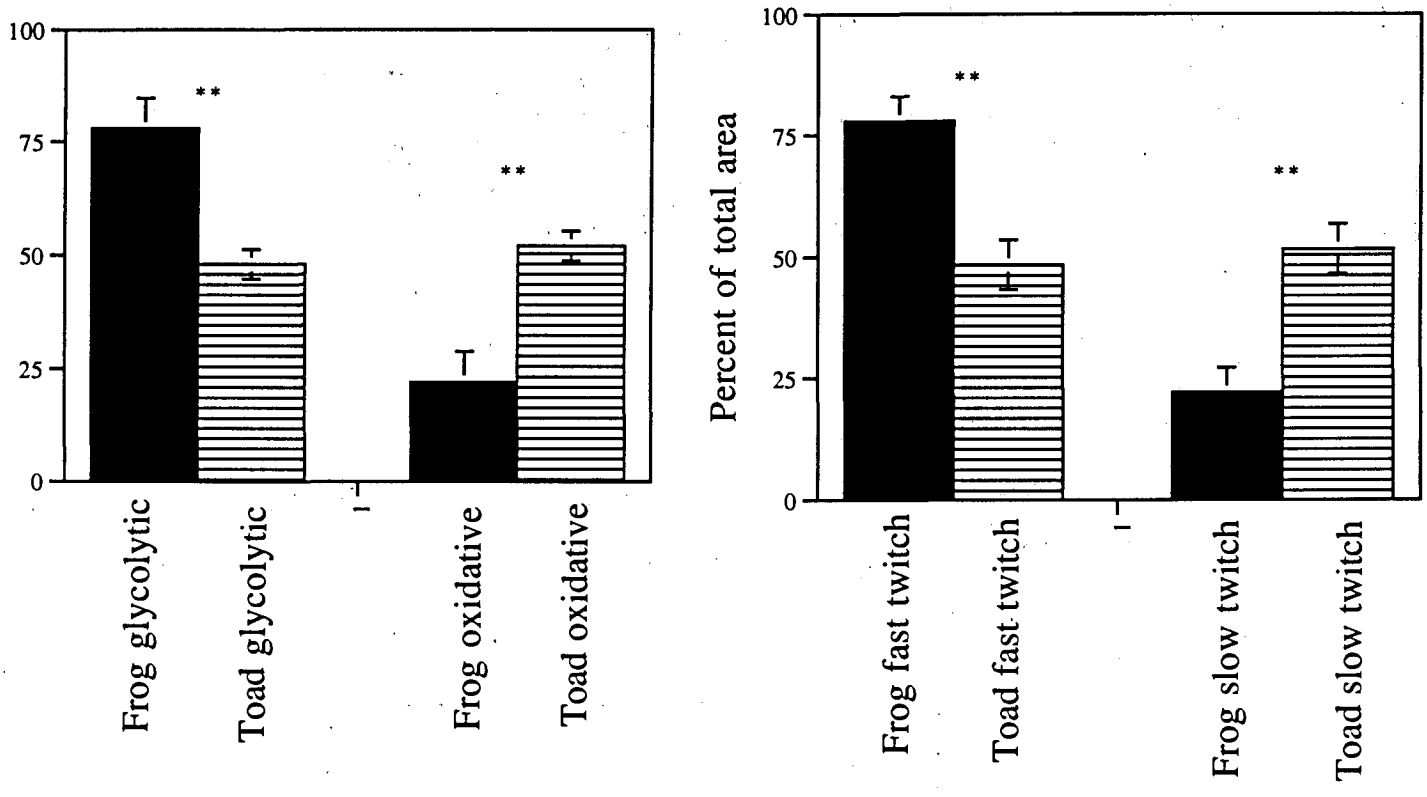

Figure 3: A comparison of glycolytic and oxidativefibers, and fast and slow twitch fibers, as percent of total area, in the frog and toad gastrocnemius (**p<.01) Values represent means ± standard deviation

Sample whole muscle tension traces (Fig. 4) of the frog and toad reveal that the frog gastrocnemius fatigues at a fairly constant rate until this rate increases when approximately 65% of maximum tension is reached (Fig. 5). The toad, however, fatigues fairly rapidly at first, and then maintains low levels of tension with slow fatigue rate (Fig. 5).

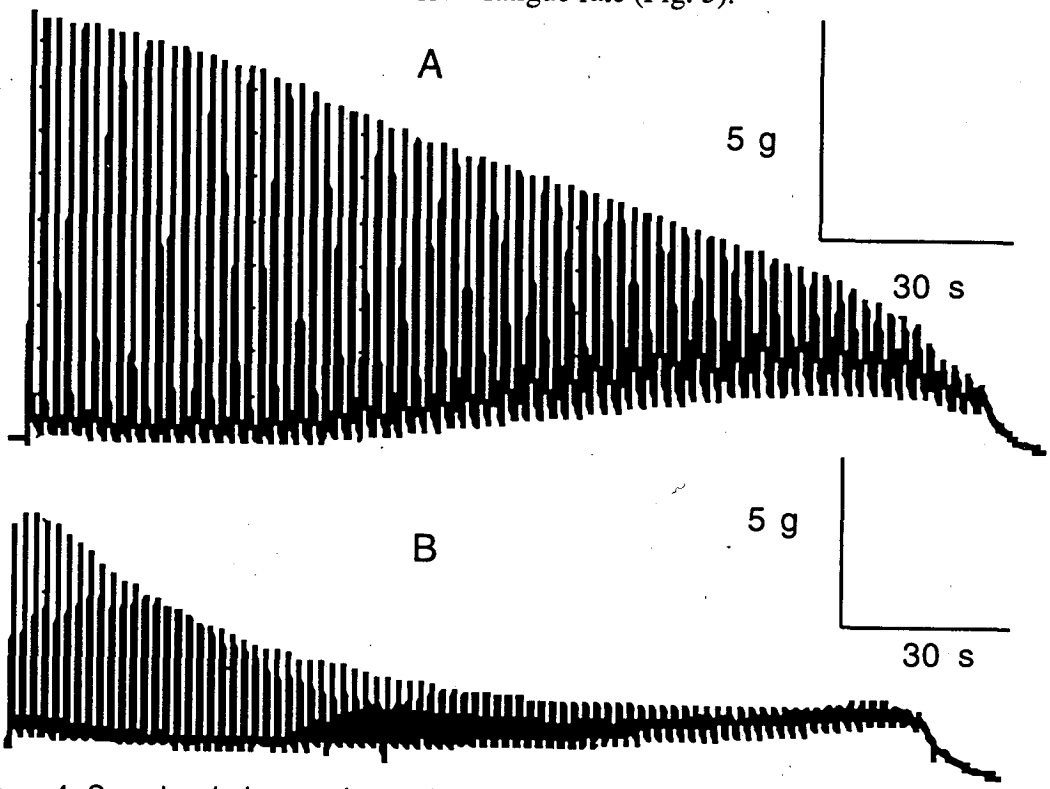

Figure 4: Sample whole muscle tension traces recorded from the isolated gastrocnemius of the frog (A) and toad (B)

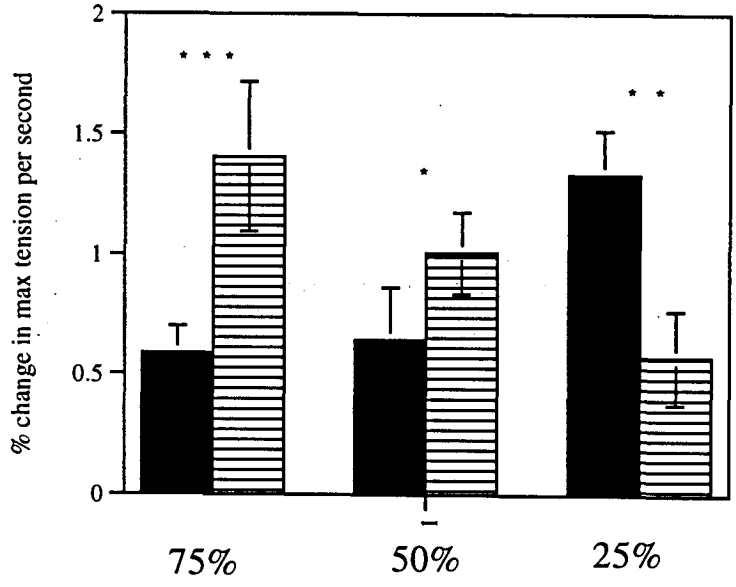

Figure 5: A comparison of fatigue rate (% change in tension per second) at tension values that are 75%, 50%, and 25% of max tension. Solid bars represents the frog gastrocnemius. Striped bars represent the toad gastrocnemius
(* p<.05, ** p<.01, *** p<.001) Values represent means ± standard deviation

DISCUSSION

We have shown that the differences in locomotory behavior that are observed in the frog and toad are clearly reflected in the physiological properties of the gastrocnemius, a principle muscle of movement. The toad gastrocnemius has a significantly larger area composed of slow twitch oxidative fibers, which allows it to maintain lower levels of tension more efficiently than the frog. However, its lack of glycolytic fibers render it incapable of maintaining higher levels of tension for more than a few seconds. The frog gastrocnemius has a significantly larger area composed of fast twitch glycolytic fibers, and can maintain higher tension longer, but fatigues fairly rapidly at lower tension levels.

Fast twitch glycolytic fibers are more capable of quick, powerful contractions than slow oxidative fibers due to their larger area, more developed sarcoplasmic reticulum, and larger amounts of glycolytic enzymes for rapid release of energy through anaerobic glycolysis. The ATPase site of the myosin head utilizes ATP at a faster rate in fast twitch fibers, leading to quicker speed of contraction. Slow twitch oxidative fibers, however, are capable of sustaining low levels of tension for longer periods of time, and typically possess a higher quantity of mitochondria for use in aerobic phosphorylation (Eckert et al. 1988).

Glycolytic fibers are also more prone to fatigue than oxidative fibers due to the production of lactic acid, a byproduct of anaerobic glycolysis. The lactic acid decreases the pH in the muscle cells, and thus alters the effectiveness of enzymes and proteins within the cell leading to reduced storage and release of calcium by the SR, reduced actin/myosin affinity, and reduced troponin/calcium affinity (Eckert et al. 1988). Oxidative fibers, on the other hand, yield considerably more ATP from each nutrient molecule processed, and do not result in lactic acid accumulation. This, coupled with the slow rate at which slow oxidative fibers use ATP and the large number of mitochondria, enable oxidative fibers to maintain tension efficiently for longer periods of time with little fatigue and depletion of energy stores (Eckert et al. 1988).

The histological stains showed that the frog gastrocnemius has significantly more area composed of fast twitch glycolytic fibers than the toad gastrocnemius (Figs. 1 - 3). This pattern is reflected in the results of the physiological experiments, which showed an initial fatigue rate in the frog muscle that was fairly constant, with a more rapid fatigue rate at less $\leq 25\%$ of maximum muscle tension. The toad gastrocnemius, however, fatigued at a more rapid rate early on, but because it was composed of a larger area of oxidative fibers than the frog, it was able to maintain lower levels of tension longer with a significantly slower rate of fatigue (Fig. 5).

Similar differences in histochemical profiles were reported by Sperry (1981) for *Bufo americanus* and *Rana pipiens*. In addition, Mendiola et al. (1991) concluded that the gastrocnemius of the *Rana perezi* contains a significantly larger area of fast glycolytic fibers and a smaller area of slow twitch fibers than the gastrocnemius of *Bufo calamita*. Finally, Bennett (1974) concluded, by measuring activity of glycolytic enzymes, that the toad possesses a maximum lactate production capacity that is 15-25% of the frog, and that differences in the activity patterns of *Rana pipiens* and *Bufo boreas* are not purely behavioral, but are a reflection of the physiological differences within the muscles of these animals. However, Putnam and Bennett (1983) reported no different histochemical profiles in locomotor muscles of *Rana pipiens* and *Bufo boreas* despite these enzymatic activity differences.

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SOCIAL BEHAVIOR IN THE CHILEAN FLAMINGO, *Phoenicopterus chilensis*‡

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INTRODUCTION

Quantitative studies of flamingo behavior have typically attempted to correlate the birds behaviors with biotic or abiotic parameters such as reproduction, flock size, dominance, salinity of habitat, season, or feeding efficiency (Bildstein, et al, 1993; Davies, 1978; Espino-Barros and Baldassarre, 1989a, 1989b; Schmitz and Baldassarre, 1992a, 1992b; Stevens, 1991; Studer-Thiersch, 1974). Little has been done to correlate specific flamingo social displays with other social displays in individuals, except for the work by Stevens (1991) and Studer-Thiersch (1974), who were mainly interested in mating behavior. In addition, these studies of beavhior have focused largely on the Common flamingo, *Phoenicopterus ruber*. Species such as the Chilean flamingo, *P. chilensis,* have been studied much less frequently (Davies, 1978; Bildstein, et al., 1991; Studer-Thiersch, 1974). Also lacking in the study of flamingo behavior are quantitative analyses of the birds' calls. A figure containing sonograms of a "two syllable" call (Studer-Thiersch, 1974) is the only quantification we found of calls by either Common or Chilean flamingos.

The goals of our study were to describe and quantify the occurrence of and correlations among specific social displays in the Chilean Flamingo, and to formally describe the calls associated with specific displays.

METHODS

Observations were made of a captive flock of Chilean flamingos held in approximately one half acre at the Memphis Zoo and Aquarium. Most observations were made while the flock was in a 30' x 40' feeding and nesting area during the months of October to November, and February to May, between 10:00-16:00 hrs., 1995-1997. These time periods were used for convenience, but they also occur "before" and "after" peak courtship and breeding behavior, observed from June to August in this group. This was a fairly stable flock of about 28-32 birds, distributed by age from 2 to at least 33 years old. Birds are marked with visibly numbered leg bands. The sex and minimum age of most birds were known and juveniles could be identified by coloration. Both males and females were observed to give all of the social displays discussed below.

Initially we used the display definitions of Kear and Duplaix-Hall (1975). Then we refined their descriptions to form our own definitions of these stereotyped behaviors. Two methods of observation were used. First, focus individual observations, were done when a known bird was observed for one hour and all defined behaviors were recorded in sequence. Nine focus individuals were recorded. Most social behaviors were relatively short in duration, from 2 to 30 seconds and therefore occurrence rather than duration was measured.

Second, focus behavior observations were made by survey of the entire flock to observe

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any occurrence of a specific behavior. The individuals giving that behavior were then observed in order to record subsequent behaviors. Eight one-hour periods were sampled in this way. Sound recordings were also made by this method. Focus behavior observations were often made by one of us at a time and since some infrequent social displays were given by several individuals close in time, individuals performing specific behaviors were not always identified. This was especially true with some calls given synchronously. In these cases we were often unable to identify the number of birds giving the calls.

Sound recordings were made with a Sony (TCM 5000DEV) cassette recorder at a speed of 0.75 cm/sec. using a short Sennheiser shotgun microphone (ME80). Recordings were digitally analyzed using GW Instrument's Soundscope hardware and software on a Macintosh computer.

Five calls from each call type were analyzed in most cases (one with only 4). In calls with multiple notes, 5 notes (as available) were analyzed and data averaged for the call. Calls had from one to eight notes. Temporal measurements were made with analyses using a wide filter bandwidth (25 msec, 150 Hz), frequency measurements were made with analyses using a narrow filter bandwidth (10 msec, 59 Hz).

RESULTS AND DISCUSSION

- Occurrence of behaviors

Behaviors recorded include the following: Standing Rest, Standing Sleep, Looking, Searching, Preening, Feeding, Walking, Scratching, Sitting Rest, Sitting Sleep, False Feeding, Leg Kick, Wing Salute (WS), Neck Sway (NS), Feather Display (FD), Inverted Wing Salute (IWS) and Beak Fight (BF). Behaviors were split into maintenance and social displays, with social displays having initial codes in parentheses above. The two categories are not always mutually exclusive. Behaviors such as Feather Display and some Preening no doubt can serve both maintenance and social display functions. Since our analysis is not based on duration of behavior, it underestimates long lasting behaviors such as sleeping and feeding. However, since all birds frequently switched behaviors during our observations, and the behaviors which were the longest in duration were also initiated most often, the relative frequency of occurrence should be similar to the relative frequency of duration.

We found that an average of 12% of the behaviors initiated were social displays. Although the definitions of different behaviors and which behaviors are recorded vary among studies, this relatively small percentage of social displays is similar to data from Espino-Barros and Baldassarre (1989) and Schmitz and Baldassarre (1992) who report that 2-10% of the behaviors seen in wild Common Flamingos are social displays. Our 12% is much less than the 25% aggressive behavior seen while feeding in wild Common Flamingos by Bildstein et al. (1991). Of the social displays recorded during our focus studies, we recorded WS, FD, NS, BF and IWS. The most common social display was Neck Sway which represented 44% of the recorded social displays. The next most frequent social display was Feather Display at 41%, followed by Inverted Wing Salute at 10.8%, and Wing Salute at 4.2%. Finally, no Beak Fights were recorded during our focus individual observations. Our 4.2% occurrence of Wing Salute is comparable with the approximately 6.7% of samples with WS found in the Common Flamingo by Stevens (1991). However, in our focus observations Inverted Wing Salute was more common than Wing Salute (10.8% vs. 4.2%, respectively), while Stevens found fewer IWS than WS. It is our observation from non-focus individual observations that IWS is rarer the WS in the Chilean Flamingo.

- "Wing Salute"

We found two types of wing salutes. Perhaps these would be better considered as separate displays. Both are illustrated in Kear and Duplaix-Hall (1975). One position can be described as having neck and head erect with the wings open fully to sides. The second position can be described as having neck and head erect and the wings folded with the wrist forward and hand

pointing down. The lower arm (elbow to wrist) is held parallel to the body but angled a bit up to the front.

Wing salutes by individuals were rare during our study months, occurring in only 30% of the individuals in our focus observations. In flock survey (focus behavior observations), we saw approximately 4/hour in the flock, with a range of 1-8/hr. Wing salutes were given by adults only. Wing Salutes were usually given with a specific call. We did not find Wing Salutes to be correlated with other social displays.

- Agonistic sequence,

In looking for correlations among social displays we were able to quantify an agonistic sequence which includes Feather Display (FD), Neck Sway (NS) and Beak Fght (BF)

Feather Display

Feather Display occurs with the scapular and back feathers erect. Although not described as a separate display by Kear and Duplaix-Hall (1975), they did include this behavior as a part of Hooking, a display we did not observe. We found Feather Display to be a very common behavior and no doubt it is used in preening and feather position maintenance as well as for social display. As a social display it is common in the flock as birds move past other birds and as part of the agonistic escalation we are describing here. Feather Display can occur with the bird's body in various positions. It sometimes occurs with a specific call. Feather Displays were observed in adults and juveniles.

Neck Sway

This display occurs when the neck is held horizontally with a curve or extended straight. The neck and head are swayed back and forth horizontally in wide slow arcs while oriented toward another bird. This display is often given with two or more birds participating and has associated specific call characteristics. Neck Sways were observed in adults and juveniles

Beak Fight

We define this display as a continuation of some neck sways where, bills of two or more birds are in physical contact or where open bills are interdigitated in space. This display also has associated specific call characteristics. Beak Fights were observed in adults and juveniles.

If we look at just these three displays (FD, NS, BF) and their occurrence, we find that they are part of an escalating sequence of agonistic behavior. If we look at all FD and the following social displays, we find that 52% of the FD occur alone. Thirty percent of the FD are followed by NS only and 17% are followed by NS+BF. Schmitz and Baldassarre (1992a) also discuss NS+BF as being more aggressive than NS alone. NS can occur without following FD, (53% of all Neck Sways) but in all of those cases NS were never followed by BF.

- Head Flag/Alert

We observed one other social display which had an associated call, the Head Flag/Alert. Kear and Duplaix-Hall (1975) describe Head Flagging as a group display with erect necks and heads, and the heads turning in horizontal arcs. We did not observe this display in our flock. They describe Alert Posture as similar to Head Flag, but with the head turning to look from side to side. We defined Head Flag/Alert in our study as a stationary posture with the neck extended straight up, independent of head motion. This behavior is sometimes associated with a specific call. Stevens (1991) similarly recorded Head Flags with and without calls.

- Social Display Specific Calls

Analysis of calls associated with each display will allow us to describe differences among calls. The calls associated with WS, FD, and HF/Alert are fairly stereotyped. The calls associated with NS and BF are more variable and can be longer in duration. In addition to these identified social displays with associated calls, short single note calls were given occasionally by birds at different times during other activities. We have not recorded or otherwise studied these latter calls.

CONCLUSIONS

Zoos may be the best place for behavioral studies of animals such as flamingos where small flock size, close proximity, and marked individuals allow for more careful observations and sound recordings. Flamingos show complex social behaviors throughout the year and have specific calls associated with different visual displays. We hope our study will encourage more quantified studies of social behavior, including calls in Chilean and other flamingo species.

Acknowledgments

We are very grateful to Herb Roberts (Curator of Birds), Carol Hesch (Assistant Curator of Birds), and the Memphis Zoo Administration for their help and for allowing us to study social behavior in the Chilean Flamingo. Roger Cicala, Jeremy Jacobs, Carolyn Jaslow, Gary Lindquester, Tom Logue, and Bill Short helped in various technical matters. Thanks to past classes of Bio-105, "Animal Communication" at Rhodes, who investigated various aspects of Chilean flamingo behavior at the Memphis Zoo. NSF grant USE 8952421 to APJ supplied sound recording and analysis equipment. We are grateful to the Biology Department and Academic Dean's Office at Rhodes for supporting undergraduate research.

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IMMUNOFLOURESCENCE LOCALIZATION OF A 25 kDa ENDO-(1,4)-ß-GLUCANASE AND MICROTUBULES IN GERMLINGS OF THE OOMYCETE FUNGUS *Achlya ambisexualis*‡

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ABSTRACT: The purpose of this investigation was to determine the distribution of an endoglucanase enzyme within cells of the fungus *Achlya ambisexualis*. This was accomplished by producing an immune serum in mice for use in immunoflourescence microscopy. Endoglucanases are thought to soften cell walls of fungal hyphae at points of branch initiation and possibly growing apices.

In this investigation, the endoglucanase was strongly located in cell walls of germinating spore cysts, and it was concluded that the enzyme most likely functions to facilitate swelling of those cells. The absence of a significant association with points of apical growth or branch initiation is discussed in light of competing hypotheses explaining the mechanisms of hyphal growth, as well as the limitations of this experimental methodology.

SIGNIFICANCE

Fungi play an essential role in the environment as decomposers that breakdown wastes and environment detritus as they grow and develop. Fungi are also useful to humans as producers of antimicrobial agents and other economically valuable products. At the same time, other fungi can be detrimental, in the form of crop destroying plant pathogens or as destroyers of grain stores (Webster 1993).

Not only are fungi important in their own right, but studying some of the basic molecular events involved with spore encystment, germination, and cell growth can be important in improving current understanding of the biochemical controls associated with cell growth and differentiation in the cells of other organisms (MacLeod and Horgen 1979). In the studies of fungi, *Achlya* is often used as a model system for the investigation of many important developmental questions because of its highly synchronous and short generation life cycle (MacLeod and Horgen 1979).

INTRODUCTION

Achlya ambisexualis is an oomycete, which is a unicellular water mold, considered to be a primitive type of fungus. Its asexual life cycle begins with an encysted spore, which germinates by means of a germ tube, an initial finger like projection of the spore cell wall called a hypha (Thomas, 1989). The extension of the hyphae during growth occurs only through apical growth, whether it be a germ tube, a hypha, or a hyphal branch (Thomas and Mullins, 1969; Bartnicki-Garcia, 1973; Hill and Mullins, 1980). Apical growth is the process by which walled cells grow longitudinally by expanding predominantly or exclusively at the

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very tip of the cell. The result of this growth at the apex is a tubular shaped hyphal cell (Heath 1990). *Achlya* possesses the potential for unlimited growth as long as necessary nutrients and environmental conditions are present. If those conditions or nutrients become scarce, *Achlya* will use stored energy to complete its life cycle by forming motile biflagellate zoospores (Thomas, 1989), which can reinitiate hyphal growth upon encountering a nutrient substrate (Heath and Harold, 1992).

If *Achlya* is forced to endure nutritional starvation for only a short period of time followed by the introduction of a nutrient rich growth medium, it will respond by forming numerous hyphal branches (Mullins 1973). Mullins noted, in his observations, a marked increase in the presence of β-1,4-D-glucanase (also known as cellulase) enzymes with this increase in branching (Mullins 1973; Mullins 1979). He hypothesized that the glucanase must weaken the glucan bonds in the cell wall, allowing the internal turgor pressure of the fungal cell to push out and form a new hyphal branch.

My research project aimed to explore further the hypothesis of Mullins, by developing an antibody against an endo-(1,4)-β-glucanase from *Achlya ambisexualis*. An antibody is a Y-shaped protein that functions in the vertebrate immune system where it binds to foreign proteins. Antibodies are extremely specific as to which foreign protein they will bind, namely the antigen that caused their initial formation within the organism's body. They can therefore be used for myriad purposes (Hoson and Nevins 1989), such as the localization of a speific protein within the cell of a fungus through immunofluorescence microscopy (Kritzman *et al.* 1978).

Work by Hill (1995) has shown that *Achlya ambisexualis* produces at least five distinct polypeptides that exhibit glucanase activity with molecular weights ranging from 25 kDa to 97 kDa. Dr. Terry Hill and Dr. Darlene Loprete, of the Rhodes College Biology and Chemistry departments, respectively, have begun the process of isolating the individual

endo-(1,4)-β-glucanases. They were able to provide a relatively pure form of the 25 kDa glucanase (unpublished data) for the production of an antibody. It was the original intent of this project to try to develop a particular kind of antibody, called a monoclonal antibody (mAb), which exhibits antigen binding of the highest possible specificity. Although this particular goal was not realized, I was able to produce an immune serum containing polyclonal antibodies (pAb's). While the binding specificity of pAb's is in principle not as great as that of a mAb, nevertheless their specificity is high enough to permit their use in immunolocalizations of the type performed in this research (Hoffert *et al.* 1995; Hoson and Nevins 1989).

The immune serum raised to the 25 kDa glucanase enzyme was used to investigate several questions. What is the location of the 25 kDa endo-(1,4)-β-glucanase within encysted zoospores, germ tubes, hyphae, and within branching hyphae? Can a conclusion, about the function of the 25 kDa glucanase be drawn based on its subcellular distribution? Is the subcellular distribution of glucanase consistent with a role in the branching and extending of hyphae as proposed by Mullins (1979)?

In addition to the questions about glucanase, this paper also investigated some other areas of related interest. In the process of utilizing an untried Ab it is necessary to include a positive control to ensure that the procedure is working and to ensure that the Ab has full access to the interior of the hyphal cell. To this end, a commercially developed monoclonal Ab against α-tubulin, a component of microtubules, was utilized. Microtubules (MT's) are fine tubelike structures within cells approximately 25 nm in diameter with walls approximately 5 nm thick and they are thought to play a role organelle movement and in general cell polarity (Wolfe 1993). Though structures of that size are below the limits of resolution of the light microscope, aggregates of MT's can be well visualized. Therefore, in

addition to fulfilling a role as a positive control, the microtubule mAb allowed me to investigate the structure of the microtubule cytoskeleton within *Achlya ambisexualis* cells. To my knowledge, this has not yet been investigated.

Materials and Methods

Production of Antibodies

Antibody production procedures were modifications of those described by Cryer and Liddell (1991).

A sample of concentrated and purified 25 kDa glucanase was provided by Dr. Loprete and Dr. Hill, who purified the enzyme to electrophoretic homogeneity by ion exchange chromatography so that the protein concentration was 0.72µg per µl. The production of all Ab's involved the immunization of BALB/c male mice 8 weeks of age. In the primary injection, given interperitoneally, the mouse received 25 µg of purified glucanases in phosphate buffered saline (PBS) with Freund's Complete adjuvant, in a total volume of 200 µl. Booster shots were given to the mouse at four weeks and then again at seven weeks after the primary injection. In the booster shots the mouse received 13 µg of the purified glucanases in PBS with Freund's Incomplete Adjuvant, in a total volume of 200 µl.

Two weeks after the first booster shot, the mouse was tail bled, which involves the subsequent separation of the immune serum from the clotting agents and cellular components of the blood through a clotting and centrifugation process. Sera were stored at -20°C in small aliquoted volumes. Each serum was tested by the dot-blot technique (see description below), to ensure that it had an immune response to the antigen, and the test

yielded positive results, confirming immunity to the antigen. This reflects the presence of several antobodies against the antigen, hence the term "polyclonal" serum.

At this point, an attempt was made to obtain a monoclonal Ab. In this procedure the spleen of the mouse was removed and its cells were separated and then caused to fuse with a stable line of myeloma cells through brief exposure to polyethylene-glycol (Kohler and Milstein 1975). The mixture of hybrid cells that resulted (hybridomas) was shown by dot-blots to have some cells among them that were producing mAb, and attempts were made to clone the antibody-producers by limiting dilution to produce a stable isolated lineage. Despite four such attempts, however, no stable clones were obtained. Therefore I decided to perform all further investigations with the immune serum that contained the pAb with the most positive dot-blot test result.

Immuno-Dot-Blot

The dot-blot technique, which was used to semi-qualitatively detect the presence of antibodies, was carried out in a Bio-Rad dot-blot Apparatus following the manufacturer's recommended procedures. Each well received 1µg/5 µl of the purified glucanase enzyme. Blocking was done for one hour with a 4% Carnation milk powder (MP) in PBS solution. The wells were rinsed in PBS plus Tween-20 (PBST), and the Ab-containing serum (buffered in a 0.5% MP in PBS) was added for 1 hour. The wells were rinsed, as already described, and a secondary Ab was added. The secondary Ab consisted of a 1:1000 dilution of Goat Anti-mouse Peroxidase-conjugated IgG antibody, buffered in a 0.5% MP in PBS. The secondary Ab binds to any mouse antibody that is bound to the nitrocellulose membrane. Unbound secondary Ab was rinsed away with PBS, and the antigen-antibody complex was visualized using a freshly prepared solution consisting of 50 ml PBS, 30 µl of 30% H_2O_2, and 30 mg of 4-chloro-1-naphthol dissolved in 10 ml of methanol. Any

antibody present on the nitrocellulose membrane turned purple. All steps were carried out at room temperature.

Western Blot Procedure

The Western Blot procedure is a technique in which proteins of an antigen mixture are first separated on the basis of molecular weight by electrophoresis. Those proteins are then transferred to a nitrocellulose membrane, which is then carried through an immunoblotting procedure like that described for immuno-dot-blot in order to determine which of the separated proteins is recognized by the antibody.

Electrophoresis/Transfer : The procedure employed is a modification of the procedure of Laemmli (1970). Samples of the purified glucanases were diluted to the desired concentrations in 4X sample buffer. These were electrophoresed on a 10% polyacrylamide gel for 45-60 minutes at 150 volts. Proteins were then electrophoretically transferred from the acrylamide gel to an nitrocellulose membrane at 200 mA for 5-6 hours. The nitrocellulose membrane was immersed in 0.5% Ponceau dye to stain protein bands and then cut into strips corresponding to the protein lanes.

Probing: Strips were immersed in an agitated suspension of 4% MP in PBS at 37°C for 1 hour as a blocking step to reduce any unspecific binding. Next, strips were rinsed PBST and exposed to the primary antibody, buffered in a 0.5% MP in PBS with agitation at room temperature for one hour. The rinse was conducted as in the previous step. In the secondary Ab step, the strips were placed in a 1:1000 dilution of Goat Anti-mouse Peroxidase-conjugated IgG antibody, buffered in a 0.5% MP in PBS, with agitation for 1 hour at room temperature. Next the strips were rinsed in PBS. Tween-20 was not used because it interferes with the enzymatic visualization step. Finally the Ab's were visualized using a freshly prepared solution of 50 ml PBS, 30 µl of 30% H_2O_2, and 30 mg of 4-

chloro-1-naphthol dissolved in 10ml of methanol. Any secondary Ab bound to primary Ab present on the nitrocellulose membrane turned purple. This procedure is adapted from the Western Blot procedure as described by Cryer and Liddell (1991).

Immunolocalization

Organism/Culture/Sporulation

The organism used in these experiments was *Achlya ambisexualis* Raper strain E87-male (ATCC 11399). Petri plate cultures were grown on peptone-yeast extract-glucose agar (PYG agar; Cantino and Lovett 1960). Liquid cultures were grown in a defined liquid medium, referred to as JTM. The JTM was modified from the original recipe of Mullins and Barksdale (1965) in that the glucose and monosodium L-glutamate concentrations were made to be 0.2% and 0.08% weight by volume, respectively.

Sporulation was achieved by cutting three 48 hour old colonies from the PYG medium and aseptically placing them in 100 ml of JTM in a 250ml Erlenmeyer flask, which was then placed on a rotary shaker at room temperature for 2 hours as a rinsing step. The JTM medium was then aseptically replaced with 100 ml of sterile calcium gluconate solution, (100 mg per L), and incubated for 12-14 hours at room temperature on a rotary shaker. Swimming zoospores were then harvested for use in subsequent observations.

Procedure and time course of germination

Glass coverslips, 18 by 18 mm, were cleaned and sterilized by immersion in acetone, followed by flaming. The coverslips were then placed in humidity chambers made with small Petri plates, a Whatman #1 filter to hold the sterile type 1 water, and a bent glass rod sealed at both ends which served as a support. Once the coverslips were in place, they received 1 drop of the spore suspension from the sporulation step described above,

followed by 2 drops of the desired growth medium (JTM or PYG). Drops were yielded from a standard long stem Pasteur pipette. The spores adhered to the coverslip as they encysted, prior to any evidence of germination, which is commonly seen in both oomycetes and true fungi (Durso *et al.* 1993; Hardham and Gubler, 1990).

Zoospores were allowed to incubate at room temperature for up to 7 hours. The coverslips were then drained of the growth medium, and the adhering germinated spore cysts and spores were fixed in 6% formaldehyde solution (made from paraformaldehyde in 60 mM PIPES buffer pH 7.0) at room temperature by complete immersion of the coverslips. Coverslips were removed and fixed after various times of incubation, usually 3 hours, for use in the subsequent immunofluoresence procedure described below.

For observations of spore swelling, diameters were measured with an ocular micrometer using a 100x oil objective lens on a phase contrast microscope. Initial measurements were taken after 5 minutes of incubation, when spores were first beginning to adhere to the coverslips, and after 45 minutes of incubation, when most spores had adhered.

Procedure for initiation of lateral branching

Spores were incubated on coverslips in JTM growth medium, as described above, for either 3 or 7 hours. Then the JTM was drained off and the coverslips rinsed with JTM balanced salt solution (contained only the inorganic constituents from the JTM medium). Then four drops of JTM balance salt solution, enough to cover the coverslip, were placed on each coverslip in order to provide a 3 hour period of nutritional starvation. The coverslips were then drained, and four drops of a 0.2% (w/v) peptone in JTM balanced salt solution were added to the coverslip to encourage branching. One hour after the introduction of the peptone solution the coverslips were drained, fixed as described above, and examined by immunofluorescence microscopy.

Immunofluorescence Microscopy

This procedure is modified from those outlined in Durso *et al.* (1993), Hardham *et al.* (1986), and Kaminskyj and Heath (1994).

Coverslip cultures were incubated and fixed as already described. The formaldehyde was then drained off, and 20 µl of Driselase solution (10 µg/ml of driselase in 100 mM MES buffer pH 5.8 containing 10 mg/ml Bovine Serum Albumin and 50 µg/ml leupeptin) was added directly to each coverslip for 5 minutes. This step was performed in order to partially degrade the cell wall enough for the antibodies to have access to the interior of the cell. The Driselase was then drained off, and the coverslips were rinsed by dunking in 100 mM MES buffer at pH 5.8. The coverslips were then immersed in 1% Triton X-100 detergent, in 60mM PIPES buffer pH 7.0, in order to permeablize the plasma membrane to allow antibodies further access to the interior of the cell. The coverslips were drained and rinsed by dunking in PBST, and nonspecific binding sites were blocked by incubation for 30 minutes at 37°C in a solution of 5% ovalbumin and 2.5% MP in PBS. The coverslips were rinsed in PBST followed by incubation with a 1:60 dilution of primary Ab in a solution of 1% ovalbumin and 0.5% MP in PBS for 1 hour at 37°C. After rinsing, the coverslips were incubated in a 1:40 dilution of secondary Ab, a Goat Anti-Mouse FITC conjugate, for 30 minutes at 37°C. The coverslips were given a final rinse in PBST and then mounted on slides with n-propyl gallate, which was used to retard photobleaching in the visualization step (Giloh and Sedat, 1982).

The microscope used for the immunofluorescence observations was an Olympus immunofluorescence microscope model BH-2-RFCA. The mercury lamp burner was an Olympus 100W high pressure mercury burner model BH2-RFL-T3. Photographs were

taken with a type 12 Olympus SC 35mm camera, using Kodak T-max 3200 black and white film.

Purchased Antibodies

The following commercial antibodies were used in this research. The Sigma chemical company (St. Louis, Mo.) T-9026 mouse anti-α-tubulin IgG antibody developed from mouse ascites fluid was used as the primary Ab in immunofluorescence microscopy to localize the microtubule cytoskeleton within germ tubes and hypha

The Sigma F-1010 Goat-anti-mouse polyvalent (IgG, IgM, IgA) immunoglobulins-FITC conjugate was used as the secondary antibody in the immunofluorescence procedure described above.

The Sigma A-0412 Goat-anti-mouse polyvalent (IgG, IgM, IgA) immunoglobulins peroxidase conjugate was used as the secondary antibody in both the Western Blot and in the dot-bot procedures described above.

Results

Investigations of the 25 kDa polyclonal antibody

Four different mice provided four different polyclonal sera exhibiting an immune reaction to the 25 kDa glucanase, as confirmed by dot-blot analysis (Fig.1). These were designated respectively, mouse A, mouse B, mouse RE, and mouse LE. The pattern of cell labeling for each serum was evaluated by immuno-fluorescence. The sera from mouse RE and mouse LE were weak. The sera from mouse A and mouse B were comparatively brighter, with mouse B serum being the brighter of the two. The sera from mouse A and from mouse B yielded the same staining pattern, namely staining concentrated in the

cell wall of the spore cyst with little or none in the hypha.A Western Blot was conducted to determine which of the *Achlya* secretory proteins the polyclonal antibodies in therespective sera bound (Fig. 2). The serum from mouse A was very specific for the 25 kDa glucanase protein, with a possible light band in the 64 kDa range. Mouse B had a very strong band to the 25 kDa glucanase protein with a weaker band to the 64 kDa protein and possibly a very faint band to a heavier protein. Based on the fact that both sera yielded the same banding pattern and the fact that the mouse B sera yielded a brighter stain, the mouse B serum was used as the polyclonal antibody probe for all further investigations into the localization of the 25 kDa glucanase.

The staining pattern yielded by immunofluorescence microscopy, utilizing the mouse B serum, localized the 25 kDa glucanase in the cell wall of the encysted zoospore. This staining pattern was very distinct and easily differentiated from the more general background glow and

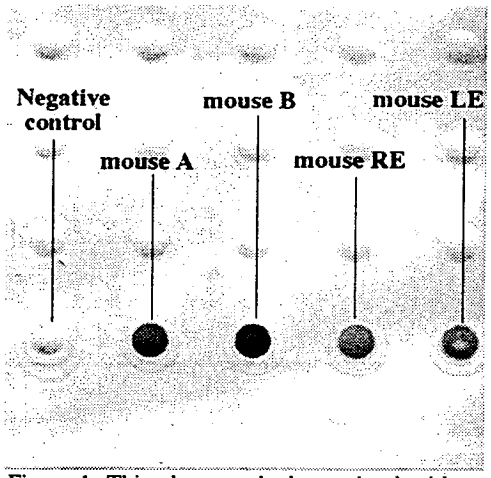


Figure 1: This photograph shows the dot blot results for the tail bleed sera of the four mice. From right to left 1) negative control, 2) mouse A, 3) mouse B, 4) mouse RE, and 5) mouse LE. Mouse B had the most positive result.

Figure 2: Photograph of the Western Blot results of mouse A and mouse B sera against *Achlya* proteins. Sera in lanes from left to right Lo) low molecular standards, 1) mouse A, 2) mouse B, 3) non-immune mouse sera, 4) proteins stain with ponceau dye, and 5) mouse B. Mouse B had a very strong band at the 25 kDa molecular weight.

occasional artifactual bright spots. The earliest observations were of spores which had been incubated in growth medium for 5 minutes (Fig. 5). The high cyst wall concentration pattern remained consistent throughout the germination of spores and the growth of the resultant hyphae (Fig. 5-8). There was no significant staining of the hyphal cell wall (Fig. 8). The pattern of cyst cell wall staining was seen in both JTM and PYG incubated spores, but it was more apparent in the JTM incubated spores as opposed to those spores incubated in PYG (Fig.9 and Fig.10). Perhaps more important, there was also no significant staining in the hyphal tip (Fig. 9), nor was there significant staining in the branching hyphae, in the tips of new branches,or in the points of early branch initiation (Fig. 11-14).

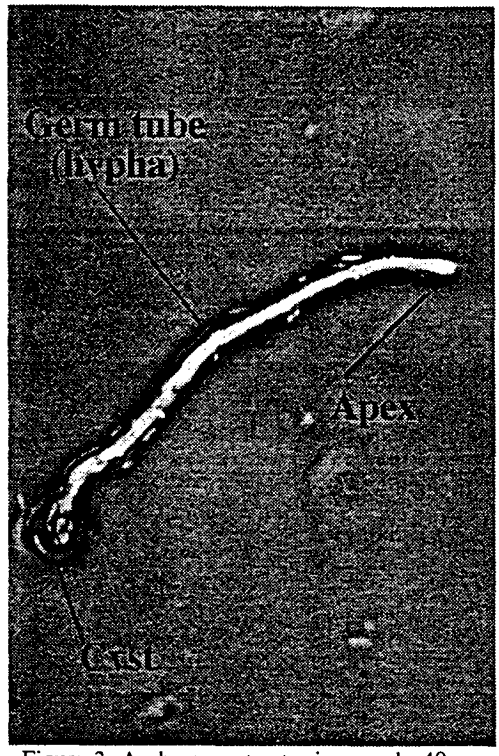


Figure 3: A phase contrast micrograph, 40x oil, of a spore germinated 180 min in JTM.

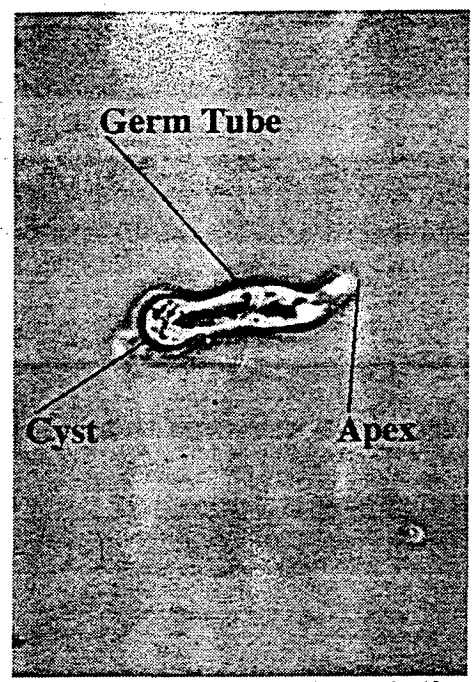


Figure 4: A phase contrast micrograph, 40x oil, of a spore germinated 180 min. in PYG growth medium.

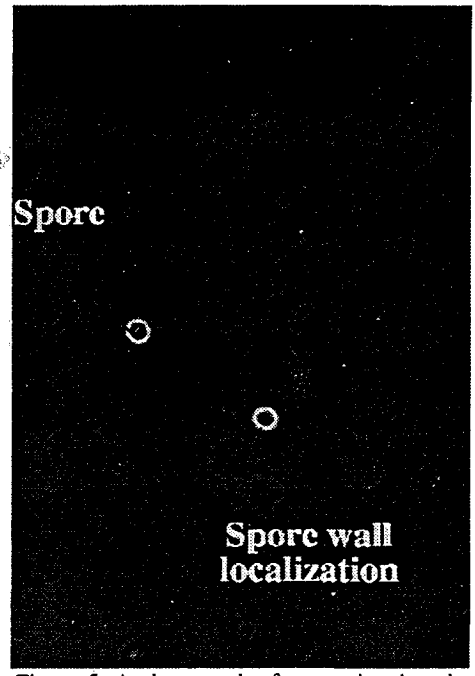


Figure 5: A photograph of spores incubated 5 min. In JTM prior to fixation. Note the localization of the 25 kDa glucanase in the encysted spore wall.

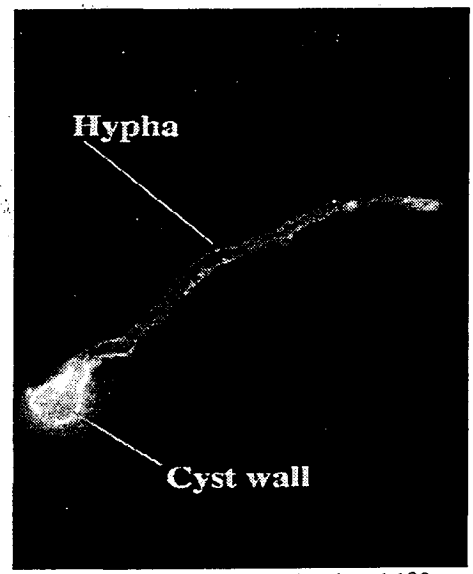


Figure 6: Picture of a spore incubated 180 min. In JTM and stained utilizing mouse B polyclonal serum. Note the concentration of the 25 kDa glucanase in the cyst wall and no significant localization in the hypha

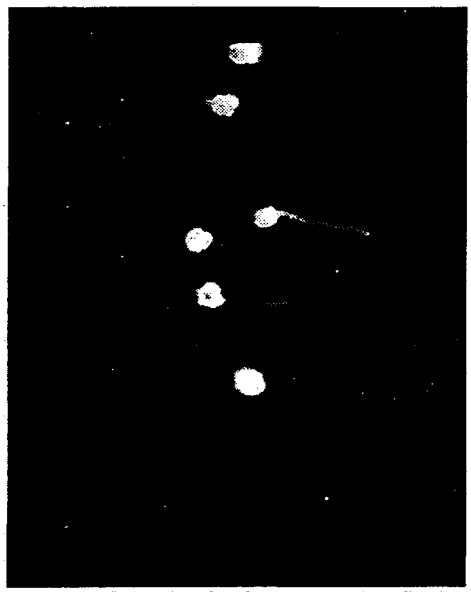


Figure 7: Photograph of a low oil 40x field of spores incubated 90 min in JTM. Note the continued localization oc the glucanases in the cyst wall.

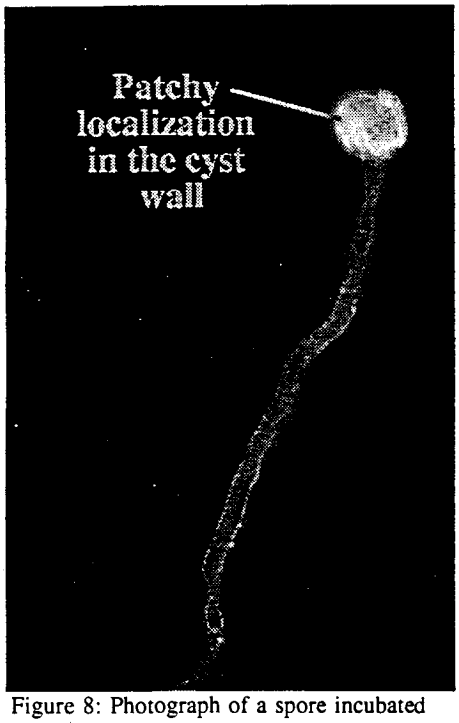


Figure 8: Photograph of a spore incubated 180 min. In JTM. Note that the glucanase is still localized in the spore cell wall and also not the patchy appearance of the stain.

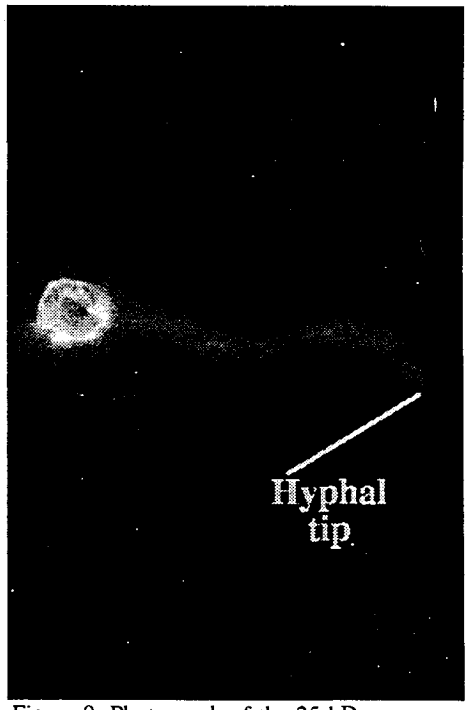


Figure 9: Photograph of the 25 kDa glucanase localization in a germinated *Achlya* spore. Note no significant staining in the hyphal tip.

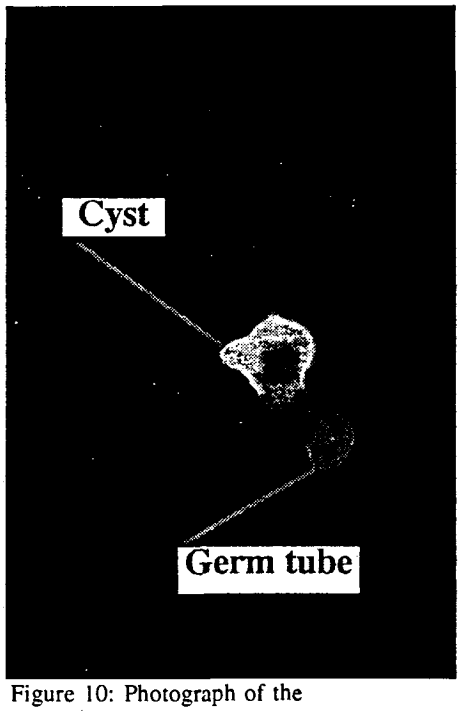


Figure 10: Photograph of the immunofluorescence localization showing the 25 kDa glucanase localized in the cyst cell wall of a spore germinated in PYG for

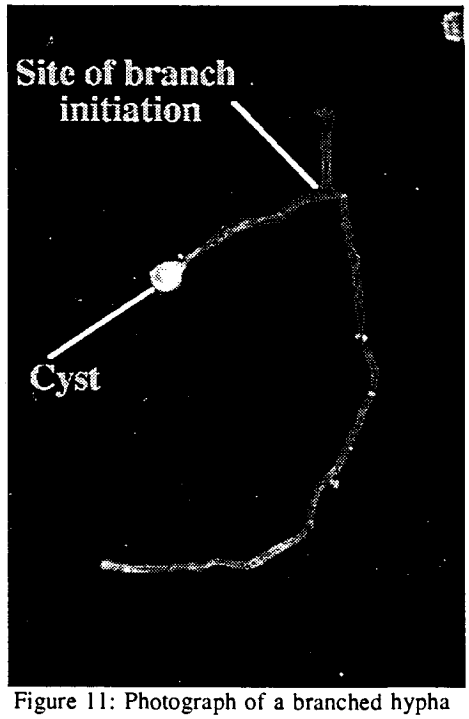


Figure 11: Photograph of a branched hypha of *Achlya* incubated 7 hrs. Note the glucanase located in the spore cell wall, not the hypha or site of branch initiation.

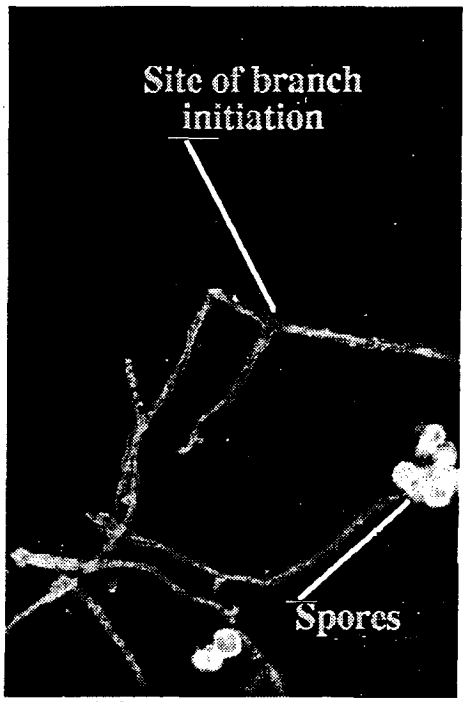


Figure 12: Low oil 40x field photograph of several branched hyphae. Note the lack of glucanase localization at all sites of branch initiation.

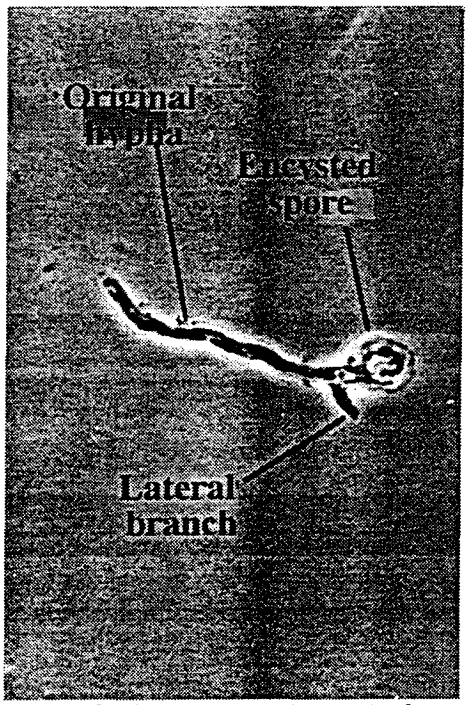


Figure 13: Phase contrast micrograph of a branched hypha from a spore incubated 3hr in JTM prior to branch induction.

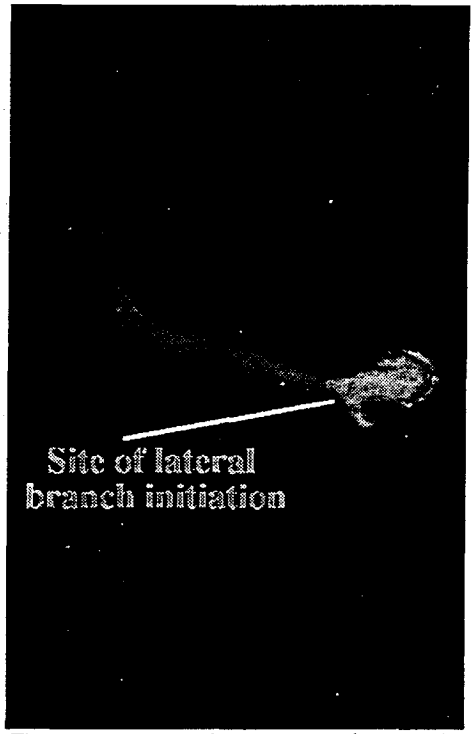
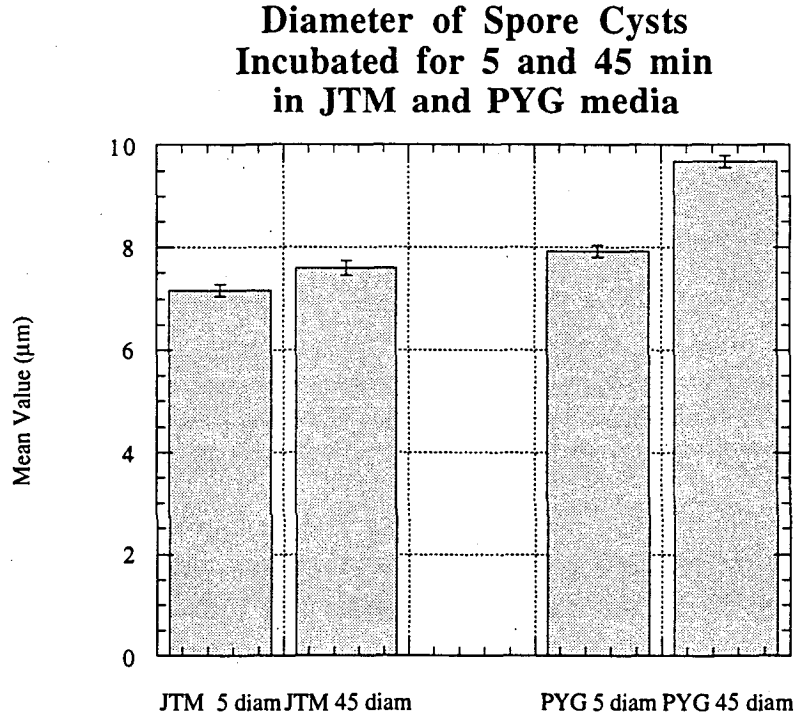


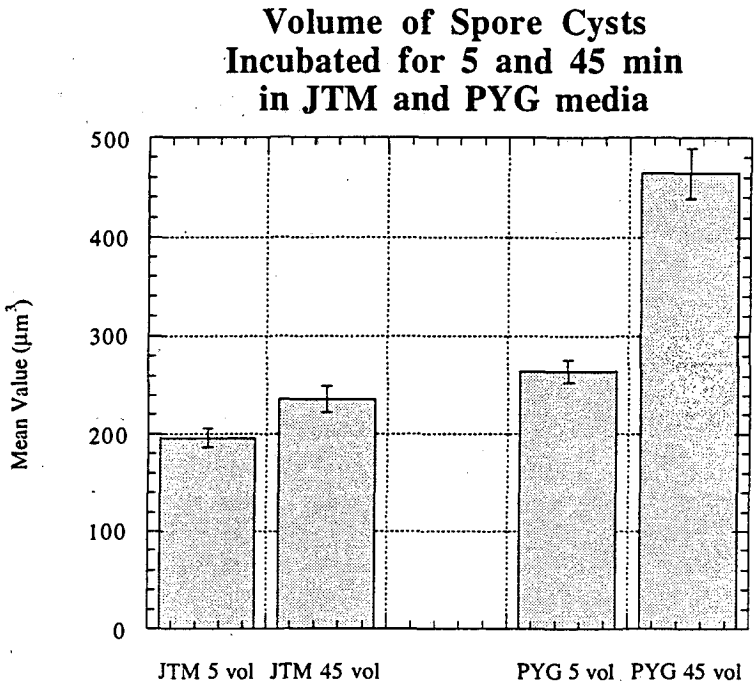
Figure 14: Immunofluorescence photograph of figure 13. Note the glucanase localization in the spore walls not at the hyphal tip or at the site of branching.

Spore Swelling

There was a 15 minute time period during which spore adherence to the coverslips occurred, extending roughly from the 5 minute mark in incubation until the 20 minute mark (data not shown). During this period there was about a 6% increase in spore diameter, for spores incubated in JTM. The corresponding increase in spore volume was about 42%. In the spores incubated in PYG growth medium, there was approximately a 22% increase in spore diameter, which corresponded to about a 75% increase in spore volume. See graph 1 and graph 2 below for a graphical representation of the statistical data.



Graph 1: This Graph shows the man values of the diameters of the spores (in µm) incubated at 5 min vs. 45 min for both JTM and PYG growth media. Each mean value is accompanied by a bar showing one standard error above and below the mean. The JTM 45 is statistically significantly different in diameter from JTM 5 min by approximately 6%. The PYG 45 is statistically significantly different in diameter from the PYG 5 by approximately 22%.



Graph 2: This graph shows the mean values of the volumes of the spores (in μm^3) incubated at 5 min vs. 45 min for both JTM and PYG growth media. Each mean value is accompanied by a error bar showing one standard error above and below the mean. The JTM 45 is statistically significantly different in volume from JTM 5 min by approximately 42%. The PYG 45 is statistically significantly different in volume from the PYG 5 by approximately 75%.

Microtubules

Microtubules in germ tubes of *Achlya ambisexualis* formed extensive arrays running parallel to the longitudinal axis of the germ tube hypha, but were not confined to a single focal plane within the cell (Fig. 15). The bundles of microtubules appeared to have foci from which clusters of microtubules radiate. These foci appear to always be located cortically within both the cyst body and the hyphal extension (Fig 16). Microtubule fibers and the location of the foci along the cell wall showed no obvious pattern. There were, however, more foci located in the same horizontal plane as that of the germ tube, protruding from the spore, than elsewhere in the spore (Fig. 17). In the limited 1, 2, and 3 hour time course incubation experiment conducted for immunofluorescence, microtubules were only visualized in the cells that had been incubated for three hours (Fig. 18).

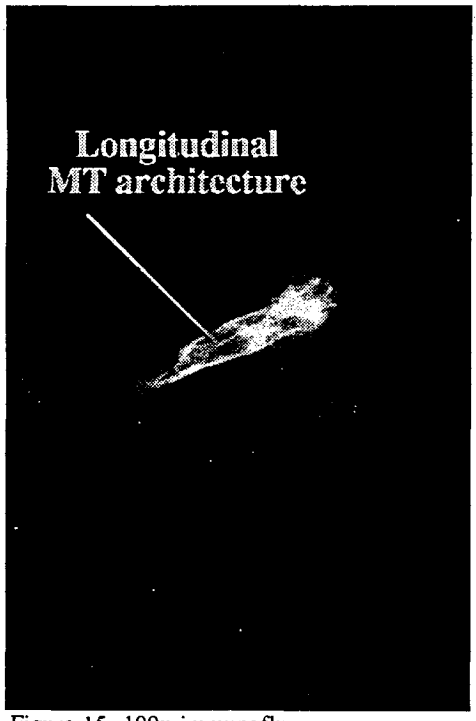


Figure 15: 100x immunofluorescence photograph of MT's localized in a germling incubated in PYG. Note that MT's run longitudinally within the hypha.

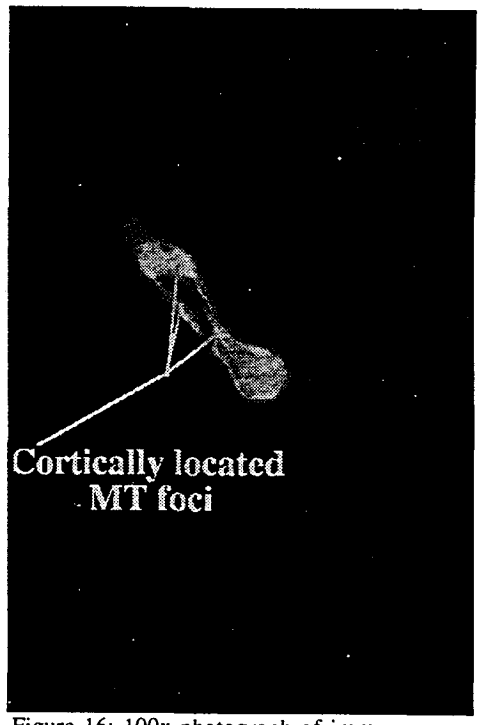


Figure 16: 100x photograph of immuno-localization of Mt's within a germinated spore. Note the cortically located foci.

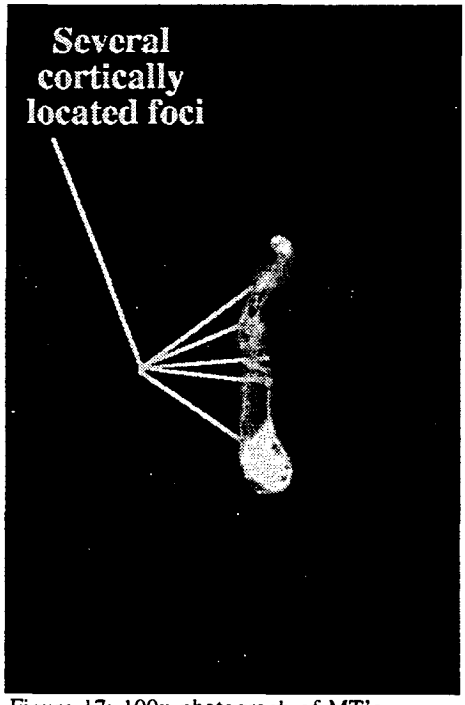


Figure 17: 100x photograph of MT's localized in a PYG germling. Note that there are five foci in the focal plane of the hypha, which is the focal plane with the most foci.

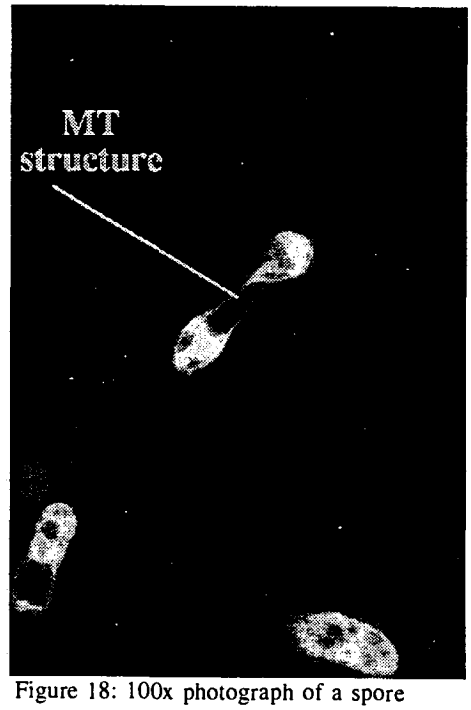


Figure 18: 100x photograph of a spore germinated in PYG. Note the MT architecture seen after 3 hours incubation.

Discussion

Glucanase

Before this paper addresses the significance of the various results that the investigations with the mouse B serum yielded, it is important to explain the current state of understanding and debate about the mechanism of hyphal apical growth and branching, as well as the currently accepted role(s) of glucanase in those processes. Hyphal walls of *Achlya ambisexualis* are composed of 15% microfibrillar cellulose, 84% glucans, and 1% proteins and lipids (Mullins 1973). By their nature, cell walls are rigid and serve to confer shape and protection against osmotic bursting. An early model to explain how a hypha can grow and at the same time continue to synthesize new cell wall at the point of extension was given by Bartnicki-Garcia (1973), who proposed an integrative model for the mechanism of apical growth in which growth is viewed to result from a harmonious balance of wall synthesis and degradation in the hyphal apex. He was able to show by autoradiographic studies that new wall materials are carried in vesicles and deposited at the tips of hyphae. He reasoned that the cell wall at tips of hyphae would have to undergo some lytic digestion by enzymes like glucanase in order to be plastic enough for the wall to be "pushed" forward by internal turgor pressure during growth.

Recently, the Bartnicki-Garcia model for growth has been joined by a competing and more widely accepted "steady state" model proposed by Wessels (1988). In Wessels' model, there is no provision for hydrolytic enzymes in maintaining plasticity of cell walls in growing hyphal apices. He proposed that the cell wall material in cytoplasmic vesicles is delivered to growing apices in a highly "visco-elastic" state. The "visco-elastic" material is only slightly polymerized at the plasma membrane as it is incorporated into the wall, and thus the nascent wall is intrinsically capable of stretching (essential to growth) before further cross linking and cell wall rigidification occurs.

While these two competing models differ in their conception of the role (or non-role) of hydrolytic enzymes in growth of existing apices, it is accepted by both sides of the debate that wall degradative enzymes like glucanases must be involved in the initiation of new apices in the process of hyphal branching. This is based on several different points of experimental data. The first observation is that there are increased levels of secreted glucanases correlated to an increased rate of branching, which suggests that glucanases play a role in that process (Thomas and Mullins 1969). Aggregations of vesicles, presumably containing glucanases, have been correlated to areas of cell wall thinning in *Achlya* (Mullins and Ellis 1974) where lateral branching from the hypha is observed to occur.

The 25 kDa glucanase was, clearly, localized in the cell wall of the zoospore cyst and there was no evidence of significant amounts of it in the cytoplasm (Fig.6 & 9). In fact, when one focused up and down vertically through the focal planes of the spores, it was possible to observe wrinkling or dimpling in the cell surface, which is consistent with the amorphous nature of the layered cyst wall structure as recorded by Burr and Beakes (1994). The 25 kDa glucanase was not localized in the hyphal tips or at the sites of lateral branch initiation. This result fails to support the model of Barnicki-Garcia, which assumes the involvement of glucanases in the weakening of the cell wall at the hyphal apex. In addition, no labelling was seen in hyphae in which branch initiation had been induced, and this result fails to support the hypothesis of Mullins (1973). However, it is still possible to consider a number of complicating factors which argue against too hasty a conclusion that the Bartnicki-Garcia and Mullins hypotheses have necessarily been invalidated.

Money and Hill have evidence (unpublished data) that glucanase is secreted in easily measurable amounts when hyphae are grown under conditions of reduced turgor, but the levels they measured became much smaller in hyphae growing under the "normal" (full turgor) conditions employed in this study. It has also been observed by Polacheck and

Rosenberger (1978) that newly formed cell walls have a very high susceptibility to degradation by glucanases. It is therefore reasonable to suggest that even if glucanases do play a role in rendering hyphal apices and branch points plastic enough for growth, they may be needed in only very small, almost insignificant amounts in conditions of normal turgor. Glucanase in tips and branch points may, therefore, be present in amounts simply too small to be detectable by immunofluorescence with the mouse B serum used in this study.

On the other hand, the patterns of immunofluorescence seen in this study must be considered at face value; the 25 kDa glucanase was seen in the spore cell wall. It was not localized in the tips of growing hyphae or in the points of branch initiation and the simplest explanation for this observation is that the 25 kDa glucanase is simply not involved in either of those processes. If apical growth occurs through the Wessels' steady state model, discussed earlier, then it is reasonable that glucanase would be absent in the tips. Although this argument provides a possible explanation as to why there is a lack of localization of the 25 kDa glucanase in the hyphal tip, it does not address this lack of glucanase at points of lateral branch initiation in the hyphae.

One reason for this lack of glucanase localization at the sites of branch initiation, is that this research investigated only one of the five glucanases reported by Hill (1995), and it is therefore entirely possible that one or more of the glucanases not studied, may be the one(s) involved in plasticizing the cell wall and the hyphal tips.

Further investigations should continue with the other glucanases and the immunofluorescence procedure, used in this study, to determine their location in the germinating spore and in growing hyphae. It might also be beneficial to employ electron microscopical procedures in order to attempt to detect minute amounts of glucanases below the limits of the immunofluorescence microscopy procedure.

As mentioned in the results, there was marked swelling in spores germinated in

both the JTM and in the PYG Medium. A statistical t-test shows that the increase in the diameter and the volume (6% and 20% respectively) of the spores germinating in JTM medium is significant to the 0.03 level of probability. The same test for spores germinating in PYG medium shows statistical significance at the 0.01 level of probability that there were increases of 22% and 75% in spore diameter and volume, respectively.

Cyst swelling is not an unusual phenomenon in the germination of fungal spores (Durso *et al.* 1993) although it was a mild surprise in the case of *Achlya ambisexualis* because MacLeod and Horgen (1979) reported no significant swelling in the germination of spores from *Achlya bisexualis*, a very close cousin to the species with which this study worked. Spore swelling is commonly interpreted as resulting from the osmotic uptake of water during germination (Heath 1990). What is significant in this study is the fact that swelling implies a reduction in the capacity of the cell wall to contain the outward expansive force of turgor within the cell. Since glucanases are thought to be capable of weakening cell wall linkages (Mullins 1979), it is natural to propose that this is the explanation behind the localization of glucanases in the cyst cell wall. The localization of the glucanase in the spore cell walls, as observed in this study, suggests that it is involved in the breaking of the glucan cross linkages of the encysted zoospore cell wall so that the swelling, noted in the results and discussed above, is able to occur.

The younger spores, those incubated between 5 and 20 minutes, appeared much more uniform in staining than did the cyst walls in the spores that had been incubated longer (i.e. 3 hours) and had undergone germination. This could be due to the fact, that as the spores swell they must incorporate new wall matrix and synthesize new cell wall to contain their new volume of cytoplasm. This newly synthesized wall would not be expected to have appreciable levels of glucanase in it, as this would be counter productive to wall rigidification. Thus, the more patchy staining of the older spore walls is reasonable.

The fact that the glucanase was present in the cyst walls from the earliest possible staining of the spore, after a 5 minute incubation, calls into question whether or not the glucanase is synthesized de novo during swelling or if it is a constitutive product of the cell wall in *Achlya,* as are similar enzymes in the yeast *Saccharomyces cerevisiae* (Larriba, *et al.* 1984). However, all of the spores examined in this investigation were incubated in growth media even during their attachment to the coverslips. As a result, germination specific events had probably already begun in even the youngest spores observed. Given the time constraints on this research, further investigation into this question was not possible. Further research should, however, investigate this point by harvesting zoospores from the sporulation medium and incubating them in a balanced salt solution, devoid of nutrients, until such time has they have encysted and adhered to the slide coverslips. Since the spores would be incubated in a non-growth medium, it is assumed they would be able to attach without also undergoing swelling or other germination related processes. Localization of glucanase at this point, depending on whether or not it is present in the cell wall, would answer the question of whether glucanase production in the cell wall is a swelling and germination related event or if it takes place during encystment as well.

Microtubules

An axial architecture of microtubules (MT), as observed in this study, is not unusual in long apically growing cells. Similar MT architecture is seen in yeasts, (McLaughlin *et al.* 1996; Gabriel and Kopecka 1995) pollen tubes (Tiezzi 1991), true fungi (Roberson *et al.* 1989; Jochova *et al.* 1993), and oomycetes (Temperli *et al.* 1990) so it was not unexpected that the MT architecture of *Achlya* would also be along the axis of the hyphae. Nevertheless, to this author's knowledge, this is the first time that this basic architecture has been confirmed in the hyphae of *Achlya ambisexualis*.

The MT foci in both the hyphae and the cysts of *Achlya* were always observed to be located cortically. Other works with fungi have noted foci for microtubules which can

be, but are not necessarily, cortically located (Temperli *et al.* 1990; Roberson *et al.*1989; Kaminskyj and Heath 1994). These other MT foci, as observed for instance by Temperli *et al.* (1990) working with the oomycete *Phytophthora infestans*, appear to be located only in relation to the cell nuclei, which have no particularly consistent placement within the cells of that fungus. MT foci are also seen in yeasts, but only when nuclei are undergoing division or migration (McLaughlin *et al.* 1996).

A possible explanation of the cortical distribution of MT foci observed in this study comes from electron microscopical work done by Heath and Kaminskyj (1989), who noted that the nuclei of the oomycete *Saprolegnia ferax* are almost exclusively peripherally located, and furthermore that the centrioles of the nuclei are located on the side of the nucleus closest to the plasma membrane. Centrioles are known to act as organizing centers from which microtubules extend in many types of higher cells (Wolfe 1993) and this has been confirmed for fungi as well (Kaminskyj *et al.* 1989). It is likely then, that the MT foci observed in this paper are in fact organized by centrioles associated with nuclei. Future experiments should employ both a nuclear immunofluorescence stain and the anti-MT Ab to compare the location of the nuclei and the MT foci. If the foci prove to be in association with the nuclei, then it would further the hypotheses, put forward by others (Hyde and Hardham 1993; Steinburg and Schliwa 1993; Raudaskoski *et al.* 1987), that MT's are involved in regulating organelle migration, especially nuclei, in apically growing cells .

Regardless of whether associated with nuclei or not, these observations have a possible bearing on questions of organelle migration in fungal hyphae. This is of some importance to an understanding of the role of glucanase in *Achlya* hyphae, because there is evidence that at least some of the wall vesicles aggregated in the hyphal apex contain endo-(1,4)-β-glucanases (Hill and Mullins 1980), and these vesicles may be transported by MT's (Bartnicki-Garcia 1990).

There are essentially two different schools of thought on the question of vesicle movement: those who propose that microtubules are involved in vesicle transport and secretion and those who propose that they are not. Jochova *et al.*(1993) have shown that when the fungus *Aspergillus nidulans* is exposed to anti-microtubule agents there is a significant reduction in enzyme secretions. This observation, along with the ultrastructural investigations that show a close association of vesicles with microtubules, has lead them to conclude that there is compelling circumstantial evidence for MT involvement in the long-distance transport of secretory vesicles. In similar work, it was shown that MT's are involved in the cellular spacing and positioning of three different types of vesicles (Hyde and Hardam 1993). Although Jochova *et al.*, (1993) could not state conclusively that MT's were involved in the secretory pathway, they felt it likely.

At the other end of the debate, Heath and Kaminskyj (1989) showed that the Golgi apparatii in the oomycete *Saprolegnia ferax* are located axially within the hyphae and they propose that vesicles move radially from the Golgi out to the periphery of the cell, where they then proceed to move longitudinally towards the hyphal tip. They suggest moreover, that it is the actin cytoskeleton that provides the radial movement, not microtubules. This conclusion is based mainly on electron microscopical evidence that implies that the MT structure is too short to provide a sufficient "track" to support the longitudinal movement. That conclusion is at odds with the observations of this study, however, which noted long microtubule fibers, some reaching all the way from a focus in the cyst to a focus near the hyphal tip. Furthermore, it is likely that the immunofluorescence used in this study gives a truer indication of the architecture of the oomycete MT cytoskeleton than does electron microscopy, because this study employs whole cells while electron microscopy employs only cell sections.

Summary

Although this study was unable to realize the goal of producing a monoclonal antibody, it was able to use a polyclonal serum, with a demonstrated high degree of specificity, to visualize the location of the 25kDa glucanase in the encysted zoospores and germlings of *Achlya ambisexualis*. The 25 kDa glucanase was localized in the cyst wall of germinating spores through immunofluorescence. There was no significant localization in the tips of hyphae or at the sites of lateral branch initiation. This could be due to the fact that only one of several glucanases was investigated in this study or to the fact that the glucanase was simply not present, or present in such small amounts that it was not detectable by the polyclonal Ab used in the immunofluorescence microscopy procedure. It was not possible to rule out all glucanase involvement in hyphal extension or in lateral branch initiation in *Achlya*, but it is reasonable to propose that the 25 kDa glucanase is not involved in these processes, based on its localization in the cell.

This study noted that there was a statistically significant amount of swelling in spores germinated in both JTM and PYG growth media. For this swelling to occur, a change in the cell wall must take place and, since the 25 kDa glucanase was localized in the spore cell wall, this author feels it reasonable to propose that a role of this enzyme is to mediate spore swelling. It is this author's proposal that the glucanase acts to degrade the cross linkages between the glucans in the cyst cell wall, and it is this degradation that allows the encysted spores to swell during the germination process.

Finally, the observations made in this paper form a good basis for the start of a complete characterization of the microtubule architecture in *Achlya ambisexualis,* which may ultimately yield important information on their role in cellular polarity and the migration of organelles. Though the debate over the role of microtubules in vesicle transport is far from a resolution, this research has at least been able to show that

microtubules are longitudinally arranged in the periphery of the *Achlya ambisexualis* hyphae and that they are longer than Heath and Kaminskyj (1989) thought them to be. Microtubules in *Achlya*, as characterized by this study, are in a structural position to provide vesicles, essential to hyphal extension, with a pathway to the apex.

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EFFECT OF WATER TEMPERATURE ON DIVING REFLEX-INDUCED BRADYCARDIA IN HUMANS

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ABSTRACT: Cold water facial irrigation elicits bradycardia induced by the diving reflex. This study explores the relationship between water temperature and the level of diving reflex-induced bradycardia in humans. The diving reflex has gained clinical significance through rapid reductions in tachycardia in emergency room medicine. To investigate the temperature dependence of bradycardia, heart rate changes in ten healthy subjects were monitored during thirty second, breath-hold facial immersions in 37.0°C, 25.6°C, 14.3°C, and 3.0°C water baths. The results show a significant relationship between water temperature and the degree of heart rate decline ($p<0.0001$). Mean heart rate depressions for the 37.0°C, 25.6°C, 14.3°C, and 3.0°C water baths were 9.51%, 15.44%, 20.31%, and 29.39%, respectively. These results demonstrate the highly temperature dependent nature of diving reflex-induced bradycardia.

INTRODUCTION

The diving reflex results in a decrease in heart rate (bradycardia), and peripheral vasoconstriction. The reflex is initiated by the application of cold water to the face during breath hold (apnea). The diving reflex is frequently employed by a variety of diving mammals to extend bottom time. In a way, it makes the animal much smaller- the oxygen is reserved for a small part of the body- the organs for which oxygen is most essential (Schmidt-Nielsen, 1994). The diving reflex also has clinical significance. It has been implicated recently in such phenomena as Sudden Infant Death Syndrome (SIDS) where water inadvertently applied to the face of a sleeping infant could be the cause of life-threatening apnea (Lobban, 1991). Diving reflex-induced bradycardia is used to treat paroxysmal supraventricular tachycardia in emergency medicine (Marsh *et al.*, 1995). A more accurate understanding of the relationship between water temperature and the degree of bradycardia could yield improvements in emergency medicine care.

To investigate the temperature dependent nature of bradycardia, heart rate changes in ten healthy subjects were monitored during voluntary breath-hold facial immersions in water baths ranging from 37°C (thermoneutral water) to 3°C (ice water). The most frequently studies temperature range is 15-20°C (Arndt and Stock, 1993). The scope of this study goes beyond this range to see if colder water results in a more severe bradycardia.

METHODS

Ten healthy subjects were used in this study. Volunteers were screened for smoking and drugs which alter cardiac activity. Caffeine intake was not allowed within six hours of the experiment. Subjects were placed prone on a pad until their ECG monitored heart rate stabilized. The prone position has been acknowledged as the best subject orientation, because subjects can immerse their faces with the least movement (Marsh *et al.*, 1995).

Water baths of 37.0°C, 25.6°C, 14.3°C, and 3.0°C were prepared and monitored by digital thermometer. Immersions occurred in a random order and the subjects had no knowledge of the temperature of the water bath before immersion. Volunteers were asked to take a breath and exhale until an intermediate lung volume was obtained prior to immersion. Lung volume was monitored

via pneumotrace. It has been shown that different breath hold lung volumes result in different levels of bradycardia (Hong *et al.*, 1970). Subjects then submerged their faces in the water bath for a 30 second interval. There was an equilibrium period of no less than 3 minutes between immersions.

Changes in heart rate were averaged over two trials. The base line heart rate was defined as the mean heart rate over a 10 second interval, 5 seconds before the subject was asked to being the immersion procedure. The final heart rate was an average of the last 5 seconds of immersion.

RESULTS

In exceedingly colder water bath, a greater percent decline in heart rate was seen (Table 1, Figure 1).

Water Bath Temp	Mean % change in HR	Standard Deviation
37.0 °C	- 9.51	6.65
25.6° C	-15.44	7.48
14.3° C	-20.31	10.42
3.0° C	-29.39	8.03

Table 1. Mean percent changes in heart rate and standard deviations for each water bath temperature treatment.

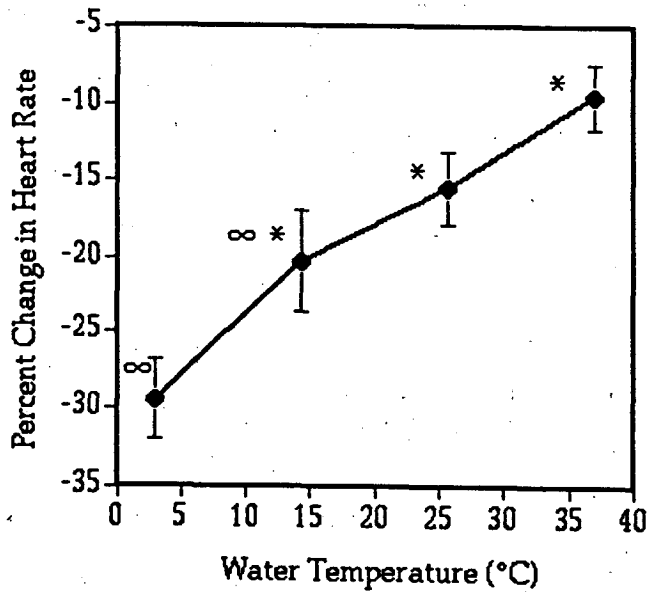

Figure 1. Percent changes in heart rate as a function of water bath temperature. Data points shown represent means of ten subjects (±1 SE). Means with a common superscript are not significantly different from each other (Scheffe ' test with 0.05 level of significance).

Analysis of variance test reveals that temperature combined with voluntary apnea significantly decreases heart rate ($p<0.0001$).

Mean percent heart rate change for immersion in the 3.0°C bath (-29.39) was significantly different from immersions in 37.0°C (-9.51) and 25.6°C (-15.44) water baths, while not being significantly different from the mean value of the 14.3°C was not significantly different from the values obtained from the three other treatments.

A sample ECG trace illustrates the decline in heart rate after immersion (Figure 2).

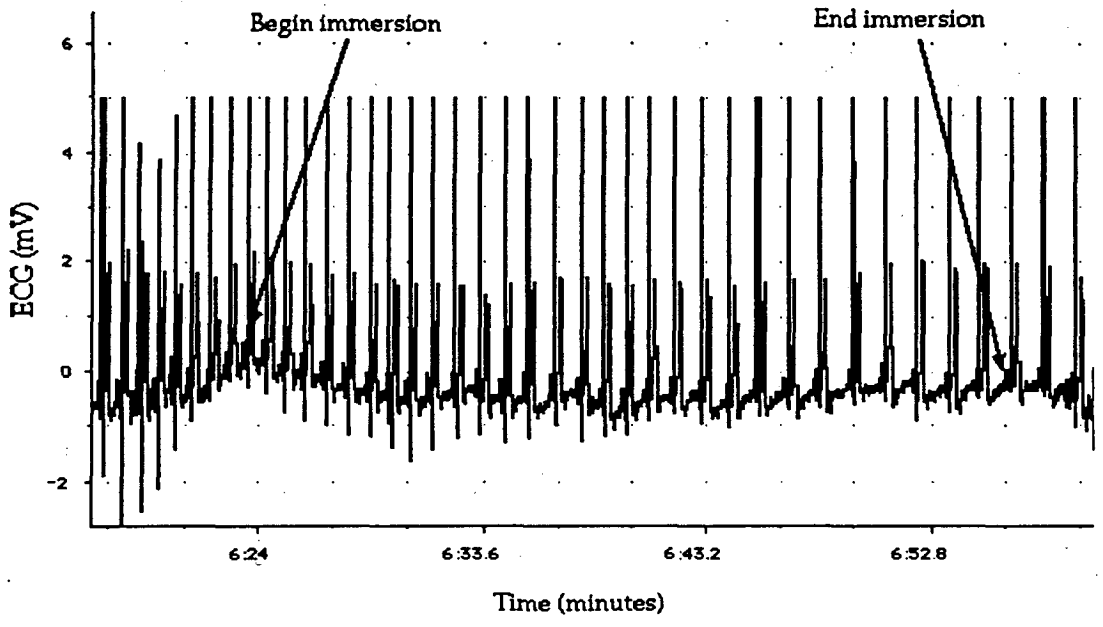

Figure 2. Typical ECG trace during voluntary apnea and facial immersion in a 3.0°C water bath. In this example, heart rate declined 30.4%, from 63.6 to 44.2 beats per minute (BPM).

DISCUSSION

The temperature dependent nature of the bradycardia response to face immersion and voluntary apnea was well illustrated by the data in this study ($p<0.001$). As water bath temperature was decreased in the experiment, a greater decline in heart rate was seen.

The diving reflex-induced bradycardia is initiated by the trigeminal nerve receptors around the nose (McCulloch *et al.*, 1995). As water temperature decreases, stimulation of the receptors is enhanced and the severity of the bradycardia increases (Arndt and Stock, 1993). The enhanced stimulation of these receptors with cold water results in an inhibition of the cardiovascular center and a subsequent reduction in heart rate through both enhanced parasympathetic output and reduced sympathetic output to the heart.

The diving reflex-induced bradycardia is also integrated with the three types of temperature receptors described by Guyton (1991). The effect of these receptors is differentially linked to bradycardia throughout the temperature range. According to Guyton (1991), at 37.0°C a combination of both warm and cold receptors are being stimulated. As the temperature drops to 25.6°C, cold receptors are predominantly stimulated. At 3.0°C, cold-pain receptors are principally stimulated. The enhanced activity of the cold-pain receptors at 3.0°C may be responsible for the more pronounced bradycardia compared to the levels recorded at 37.0°C and 25.6°C.

This study demonstrates the significant effect water temperature and voluntary apnea have on heart rate: heart rate declines as the temperature of the water bath is decreased. May questions still remain, however. One area which has not yet been investigated is the rate at which heart rate declines when the individual is exposed to water baths of different temperatures. This experiment could be improved by providing a higher capacity water bath which circulates the water. This would reduce the effect of water, warmed by the face, from rising and forming a later along the skin during the immersion.

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GEL EXCLUSION CHROMATOGRAPHY OF CELLULASES SECRETED BY *Achlya ambisexualis* GROWING UNDER OSMOTIC STRESS IN A DEFINED LIQUID MEDIUM‡

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INTRODUCTION

The importance of fungi ranges from their essential role in decomposing organic material in the environment to producing valuable medicinal products and antimicrobial agents. In addition, the potential value of fungi extends to studies involved with fungal growth, which may further our current understanding of enzymatic activity associated with cell growth in higher eukaryotes. To this extent, the fungus *Achlya ambisexualis* has been used as a model system for research investigating various aspects of cell growth.

Achlya ambisexualis is a filamentous, aquatic Oomycete of the family Saprolegniaceae. *Achlya* growth occurs by means of outward extension and branching of hyphal cell walls (Gow and Gadd, 1995). Proper hyphal growth and branching can be attributed to the secretion of cellulase enzymes, which function to disrupt the lateral wall bonds of the hyphae (Thomas and Mullins, 1967). It has been found that three distinct peaks of cellulase activity are present during *Achlya* growth without osmotic stress (Hill, 1996). Designated E-I, E-II, and E-III, respectively, the three peaks correspond to molecular masses calculated to be about 245, 92, and 30 kDa. Furthermore, it was concluded that the E-III form of the cellulases consists solely of subunits of the E-I and E-II multimers, which have dissociated as a result of age or changing culture conditions.

It has recently been found that *Achlya* cultures exposed to osmotic stress exhibit a maximum of 70-fold increase in cellulase activity over levels present in unsupplemented media (Money and Hill, 1997). In light of these findings, this experiment was conducted to investigate cellulase activity and protein content in media under conditions of osmotic stress. The findings of this experiment could prove to be instrumental to the success of ongoing efforts to isolate the various forms of cellulases in *Achlya ambisexualis*.

METHODS and RESULTS

Cultures of *Achlya ambisexualis* were grown in a defined liquid medium supplemented with 0.4 M sorbitol to induce osmotic stress and incubated on a reciprocal shaker at room temperature for periods of 3, 4, 5, 6, 7, and 8 days. The cultures were harvested by means of vacuum filtration through Whatman filter paper (GF/C, 9.0 cm, qualitative), and the collected growth media filtrates were stored at -80°C.

Protein concentrations of the growth media filtrates were determined according to the method of Bradford (1976). Cellulase activity in the media filtrates was assayed viscometrically, using carboxymethylcellulose as a substrate, at pH 6.6 and 30°C (Hill, 1996).

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For separation of cellulases by gel exclusion chromatography the media filtrates were concentrated in a high pressure filtration cell under approximately 0.4 MPa of nitrogen gas pressure. Proteins in the concentrated media were precipitated with ammonium sulfate and resuspended in 300µl of buffer. These samples were chromatographed on a column of Sephadex G-100, and 1.5 ml fractions were collected and viscometrically assayed for cellulase activity. The molecular mass of each resulting chromatographic peak was qualitatively estimated by comparison to calibration standards of alcohol dehydrogenase (150kDa), carbonic anhydrase (29kDa), and cytochrome C (12.4 kDa), all of which were run on a different but nearly identical column, because the original column had been damaged after chromatographic separation of the cellulases.

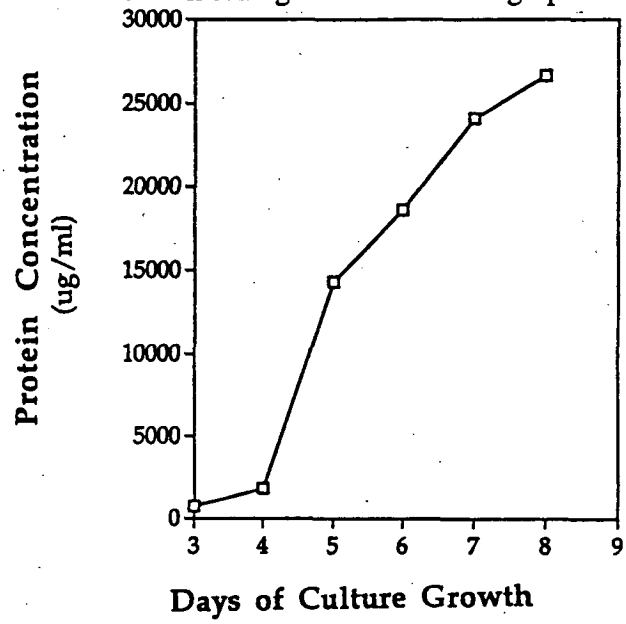

Figure 1: Protein concentration in media was determined after 3, 4, 5, 6, 7, and 8 days of *Achlya* growth under osmotic stress. Relatively low levels of protein concentration were detected at 3 days of culture growth. Increasing levels of protein concentration were observed with each successive day of growth, with the greatest rate of increase occurring between 4 and 7 days.

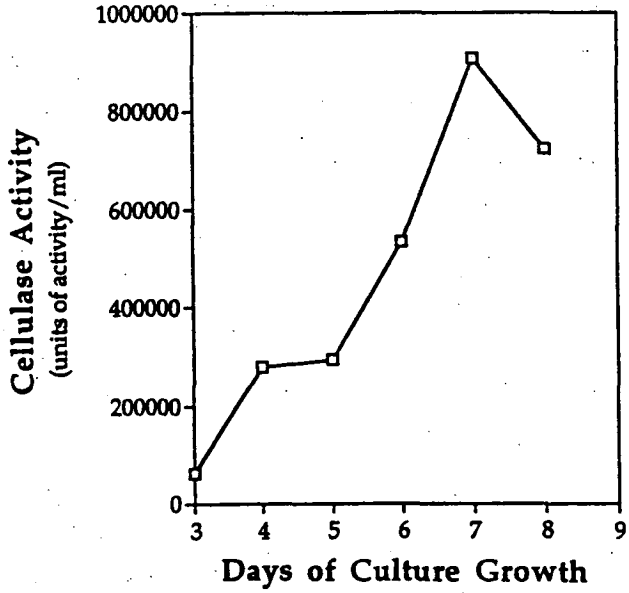

Figure 2. Cellulase activity in media was determined after 3, 4, 5, 6, 7, and 8 days of *Achlya* growth under osmotic stress. The greatest increase in cellulase activity was observed between 5 and 7 days. Activity declined after 7 days of culture growth.

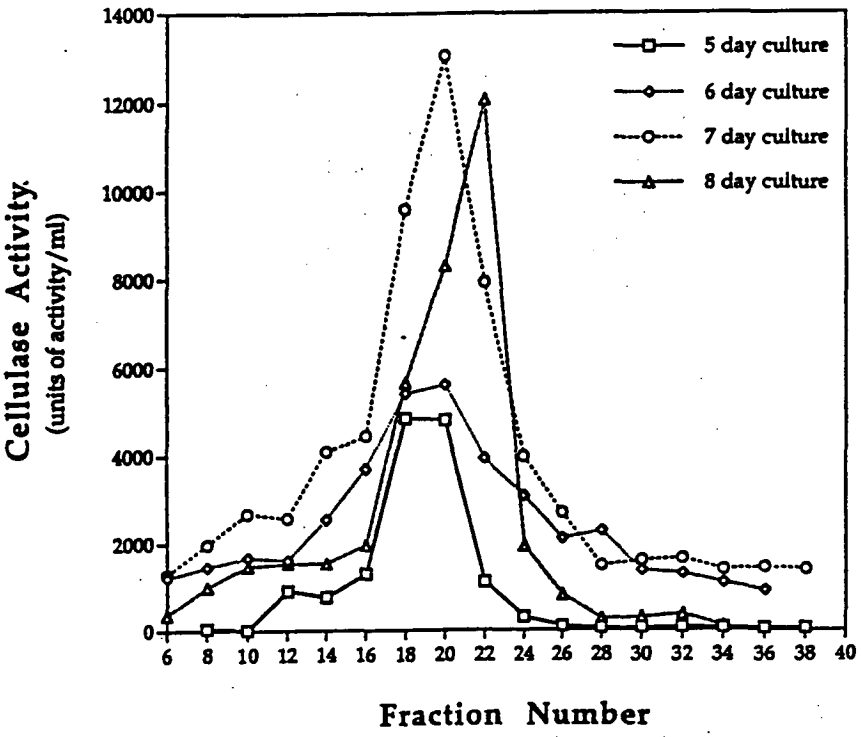

Figure 3. Gel exclusion chromatographic separation of proteins exhibiting cellulase activity was carried out with media after 5, 6, 7, and 8 days of culture growth under osmotic stress. Each culture was found to contain a single activity peak.

DISCUSSION

Under the conditions of this experiment, it was revealed that the concentration of protein in the media increased with each successive day of growth, as shown in Figure 1. The greatest levels of cellulase activity were detected at 7 days of growth, as illustrated in Figure 2. Thus, it was determined that a growth period of approximately 7 days would be optimal in producing the greatest levels of cellulase activity for future experimentation with *Achlya* under conditions of osmotic stress.

This experiment marked initial efforts to separate *Achlya* cellulases secreted under conditions of osmotic stress. Prior to this experiment, it was unknown what cellulase forms were present in culture media with osmotic stress. Figure 3 reveals that a single activity peak was detected for each culture in which cellulases were separated by gel exclusion chromatography. Furthermore, all of the activity peaks were identified within fraction number 20, except for the activity peak of the 8 day culture which was observed shortly thereafter. This slight deviation might have been due to minor variances involved with each trial. Thus, the uniformity of the observed activity peaks found in the cultures suggests that single cellulase form was present under conditions of osmotic stress.

Hill (1996) demonstrated that *Achlya* cultures grown in the absence of osmotic stress contained three distinct peaks of activity with molecular masses calculated to be about 245 kDa, 92 kDa, and 30 kDa. These three peaks were designated E-I, E-II, and E-III, respectively, as illustrated in Figure 4.

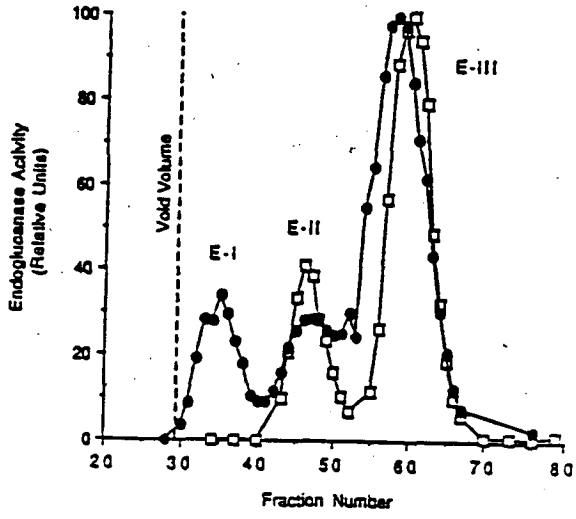

Figure 4: Gel exclusion chromatographic separation of *Achlya* cellulases secreted during growth in the absence of osmotic stress. From Hill (1996)

In order to establish which of the three activity peaks above corresponded to the identity of the single peak found under conditions of osmotic stress, the molecular mass of the single activity peak was qualitatively estimated. By comparison to calibration standards of alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa), the single activity peak found under osmotic stress was determined to behave as low molecular proteins much more consistent with those expected of the E-III monomers, rather than the aggregate, high molecular weight E-I and E-II forms. Thus, it was concluded that the single activity peak present in this experiment corresponded to the E-III peak observed by Hill (1996).

Whereas the various E-I, E-II, and E-III forms are normally present during growth without osmotic stress, this study revealed a uniformity of cellulases corresponding to the approximately 30 kDa E-III form. The absence of the aggregate E-I and E-II forms in this study leads to the proposal that *Achlya* cellulases secreted under conditions of osmotic stress are produced entirely in their monomeric forms.

It will be important to work aimed at characterizing the roles of cellulases in fungal growth that all the known types be isolated and characterized for identification of substrates and location in the cell. The results of this experiment suggest that, although *Achlya* growth under osmotic stress may produce heightened levels of cellulase activity, it may not be the most effective means of isolating all the different cellulase enzymes, because the E-I and E-II forms are not present under these conditions.

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MECHANISMS OF SYNAPTIC INHIBITION AT A CRAYFISH NEUROMUSCULAR JUNCTION‡

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INTRODUCTION

Functionally, the nervous system receives, integrates, and responds to stimuli of the environment, Thus, it specializes in receiving inputs, interpreting these inputs, and then creating the according outputs (Starr and Taggart, 1992). This experiment aims to examine this complexity with the investigation of synaptic communication in a crayfish. In particular, the goal of this experiment is to investigate the specific receptor mechanisms of synaptic inhibition at a crayfish neuromuscular junction (NMJ). The crayfish has been chosen because the crayfish NMJ shows striking similarities to the vertebrate central neuron (Katz, 1966).

The NMJ of the opener muscle in the first walking leg of the crayfish consists of two presynaptic components, the opener excitor (OE) axon and the opener inhibitor (OI) axon, and a single postsynaptic component (the opener muscle fiber). When stimulated above threshold, the OE axon releases the neurotransmitter glutamate which causes excitatory postsynaptic potentials (EPSPs) in the muscle fiber (Katz, 1966). This occurs as postsynaptic glutamate receptors open to allow a sodium ion influx to depolarize the muscle fiber (Atwood, 1976). In the absence of stimulation, transmitter release still occurs to produce minor depolarizations of the muscle fiber known as miniature end plate potentials (MEPPs). The OI axon provides postsynaptic inhibition at this NMJ by releasing the neurotransmitter γ-aminobutyric acid (GABA) onto the opener muscle fiber to produce inhibitory postsynaptic potentials (IPSPs). This inhibition occurs as postsynaptic $GABA_A$ channels increase chloride conductance to maintain the muscle fiber in a negative hyperpolarized state and thereby decrease muscle contraction (Atwood, 1976). However, the OI axon also provides presynaptic inhibition. This inhibition occurs as a presynaptic inhibitory neuron depresses the transmitter release from a second neuron (Kandel, 1991). In this case, the OI axon synapses directly onto the axon terminal of the OE axon. Through this axoaxonal synapse, the OI decreases the amount of presynaptic depolarization and thus decreases the amount of neurotransmitter release (Katz, 1966).

The exact mechanisms of this presynaptic inhibition are still questioned. While GABA is involved in the inhibition, the specific GABA receptor to which this neurotransmitter binds on the presynaptic axon is not known. There are two GABA receptor subtypes. Specifically, there is a $GABA_A$ subtype receptor and a $GABA_B$ subtype receptor. In the past, presynaptic inhibition has been attributed to a GABA mediated chloride conductance via a $GABA_A$ receptor (Atwood, 1976). However, other evidence seems to indicate that presynaptic inhibition is not entirely due to $GABA_A$ mediation of chloride channels. Namely, some have found that the $GABA_B$ agonist baclofen mimics the effects of presynaptic inhibition (Barry, 1984). Accordingly, presynaptic inhibition could result from a combination or both $GABA_A$ and $GABA_B$ receptors, or even a third GABA receptor subtype (Blundon, 1992).

This study examined the specific receptor mechanisms of both pre- and postsynaptic

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inhibition in the crayfish NMJ. In particular, the effects of various agonist and antagonist drugs on pre- and postsynaptic inhibition were examined.

METHODS and RESULTS

Young crayfish (*Procambarus clarkii*) were selected to expose the neuromuscular junction of the crayfish first walking leg for electrophysiological study. To begin, the first walking leg of the crayfish was removed and placed dorsal side down in a microdissection dish filled with Van Harreveld's saline. With the leg pinned out, the ventral half of the propodite, the closer muscle, connective tissue, blood vessels, and the sensory nerve bundle were all removed to expose the opener muscle fiber. The next step was to remove the ventral portion of the meropodite and the flexor muscle. The nerve bundle containing the sensory bundle, the opener excitor motor neuron, and the opener inhibitor motor neuron could be seen. The sensory bundle was removed and the OE and OI nerves were separated and put on separate platinum wire hooks each with their own stimulator. Individual muscle fibers of the opener muscle were then penetrated with glass microelectrodes (5-10 Ω) filled with 4M potassium acetate. The resting potential of the muscle fiber, MEPPs, EPSPs, and IPSPs were all found using an oscilloscope and conventional amplification. Stimulation of the opener excitor alone beyond threshold caused the formation of EPSPs in the muscle fiber. On the other hand, stimulation of the opener inhibitor alone beyond threshold evoked IPSPs in the postsynaptic muscle fibers. All traces were collected at a stimulus frequency of 20 Hz and the signal averaged.

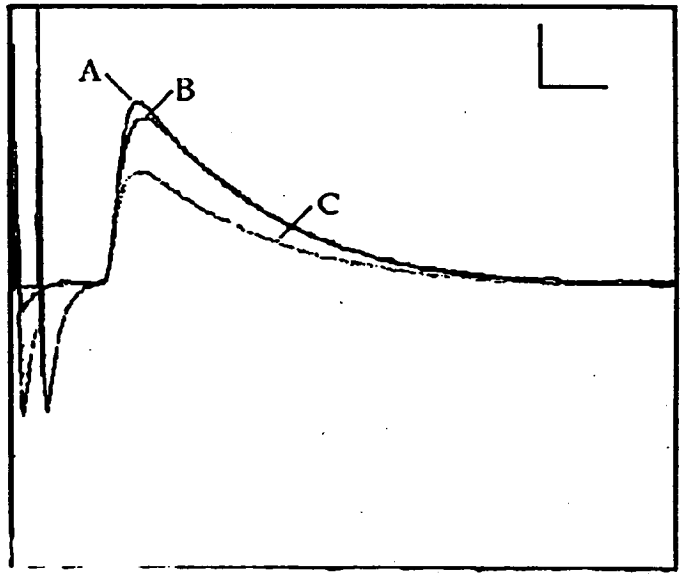

Figure 1. Excitation of the OE nerve alone (A) gave a greater depolarization than the simultaneous excitation of the OE and OI nerves (postsynaptic inhibition) (B). Postsynaptic inhibition appeared as a reduction in EPSP amplitude by less than 10%. However, excitation of the OI 2.0 ms before excitation of the OE allowed pre- and postsynaptic inhibition to occur. This 2.0 ms delay gave the inhibitory neurotransmitter time to cross the axoaxonal synapse and modulate the opener excitor as it is stimulated (Blundon, 1992). Pre- and postsynaptic inhibition decreased EPSP amplitude by about 35% (C). This reduction in EPSP amplitude was greater than the reduction in EPSP amplitude due to postsynaptic inhibition alone.

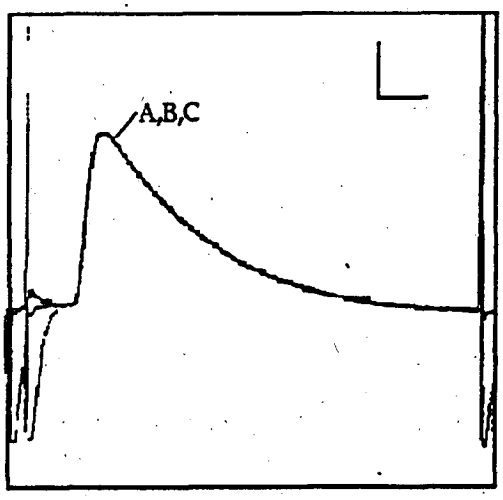

Figure 2. After observing postsynaptic inhibition and pre- and postsynaptic inhibition in the crayfish NMJ, the $GABA_A$ antagonist picrotoxin was added to the crayfish saline. The addition of 100 micromolar picrotoxin appeared to completely eliminate postsynaptic inhibition as well as presynaptic inhibition. This was seen as all three traces had exactly the same level of depolarization (A, B, C). The inhibitory actions seen in Figure 1 could no longer be elicited. Accordingly, picrotoxin also eliminated all IPSPs.

	control mean ± SD	Baclofen mean ± SD	$\mu_1=\mu_2$ Effect of Baclofen
MEPP Amplitude	256.2 μV ± 85.75	202.4 μV ± 46.06	* p= 0.0221
MEPP Frequency	9.3 M/20s ± 6.00	7.7 M/20s ± 5.36	** p= 0.0026
EPSP Amplitude	78.4 μV ± 86.44	81.2 μV ± 71.60	NS

Table 1.There was significant difference in the means of MEPP amplitude and the frequency with the addition of 200 micromolar baclofen, a $GABA_b$ agonist. There was no significant difference in the means of EPSPs with baclofen. (*Note: μ_1 corresponds to trial before the addition of baclofen, while μ_2 corresponds to trial after the addition of baclofen.) Thus, baclofen caused a reduction in MEPP frequency and amplitude, but had no effect on EPSP amplitude. Statistics were found using 13 trials to compare means using a paired T-test. In addition, the addition of 200 micromolar baclofen did not seem to affect pre- or postsynaptic inhibition.

DISCUSSION

The results of this study reaffirm that both pre- and postsynaptic inhibition exist at the crayfish NMJ. These results also indicate that presynaptic inhibition decreases the amplitude of postsynaptic EPSPs more than postsynaptic inhibition. These observations support Blundon and Bittner's findings that presynaptic inhibition can reduce EPSPs by up to 70% while postsynaptic inhibition reduces EPSPs by less than 10% (Blundon and Bittner, 1992).

The next step in the experiment examines the mechanisms of pre- and postsynaptic inhibition at the crayfish NMJ. To begin, the addition of picrotoxin, a $GABA_A$ antagonist, completely eliminates IPSPs and postsynaptic inhibition. This indicates that postsynaptic inhibition is due to the release of the inhibitory neurotransmitter GABA from the OI axon. Subsequently, GABA increases the chloride conductance of the postsynaptic muscle fiber via a $GABA_A$ receptor. These findings parallel past studies that link chloride conductance across the postsynaptic membrane as the most likely mechanism of postsynaptic inhibition (Keynes and Aidley, 1985). In addition, this study has found that picrotoxin completely eliminates presynaptic inhibition. This suggests that presynaptic inhibition is also mediated solely through $GABA_A$ receptor channels. Thus, an increases in chloride conductance would be the mechanism by which the OI depresses transmitter release from the OE. Several other lines of evidence support the role of the neurotransmitter GABA and the $GABA_A$ receptor in presynaptic inhibition. Namely, the $GABA_A$ agonist muscimol has similar effects to presynaptic inhibition, while the $GABA_A$ antagonist bicuculline or chloride free saline decrease presynaptic inhibition (Blundon, 1992).

It is apparent that the $GABA_A$ channel does play a role in presynaptic inhibition at the crayfish NMJ. However, evidence also suggests that presynaptic inhibition may involve a combination of $GABA_A$ and $GABA_B$ receptors. Namely, presynaptic inhibition cannot be completely eliminated with the addition of the $GABA_a$ antagonist bicuculline or with chloride free saline (Blundon, 1992). Our picrotoxin data does not support this hypothesis. In addition, this experiment has shown that the addition of baclofen, a $GABA_B$ agonist, has no effect on EPSP amplitude. If $GABA_B$ channels play a role in presynaptic inhibition, one would expect to see a reduction in EPSP amplitude with the addition of baclofen, but this is not seen. This strengthens the case that presynaptic inhibition is mediated solely through $GABA_A$ receptors. Additionally, baclofen does not seem to have an effect on pre- and postsynaptic inhibition. However, this experiment has also shown an increase in MEPP frequency and amplitude with the addition of baclofen. In contraction to the results above, this suggests that $GABA_B$ receptors may still play some role in pre- and postsynaptic inhibition. Finally, this evidence does not eliminate the notion that a third type of GABA receptor ($GABA_C$) may be sensitive to the drugs studied so far and also play a role in presynaptic inhibition (Blundon, 1992). In conclusion, more research must be done on the actual mechanisms driving presynaptic inhibition before concrete conclusions can be made. Future directions include further work with chloride free saline, bicuculline, and muscimol.

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PROBING THE INTERACTION OF THE ACIDIC TAIL OF HMG-D IN THE HMG-D DNA COMPLEX‡

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ABSTRACT: The *Drosophila melanogaster* high mobility group protein, HMG-D, is a non-histone chromosomal protein that binds DNA and is thought to aid in the formation of higher order nucleosome complexes. HMG-D is a 112 amino acid protein composed of a highly conserved domain, known as the HMG-box, and a basic region followed by C-terminal acidic tail. The goal of this study is to assess the function of the acidic motif in the binding of HMG-D to DNA. Structural studies of HMG-D mutants should indicate if the acidic tail facilitates binding to DNA. The 112c mutant generated for this study is a full length HMG-D with a C-terminal cysteine. Electrophoretic mobility shift assays are performed to quantitatively compare the binding affinity between the mutant and the wild type full length protein. In future studies, the 112c mutant can be utilized to map the position of the acidic tail in DNA HMG-D complexes. Site-specific DNA cleavage will indicate the point on the DNA at which the tail binds. Determination of the importance of the acidic tail will lend greater understanding of how HMG-D binds DNA, and propagate additional ideas about the function of HMG proteins in forming higher order nucleoprotein complexes in chromatin.

Eukaryotic DNA exists primarily in a condensed form due to chromosomal proteins which compact the DNA into higher order nucleoprotein complexes (figure 1A) . These proteins are thought to assist in the preparation of the DNA for replication, recombination, and gene expression (Alberts *et al.*, 1994). The high mobility group (HMG) family is an abundant type of non-histone chromosomal protein that aids in this bending and packaging of DNA. Studies of the HMG family are crucial to the complete definition of chromatin structure (Churchill *et al.*, 1995). The focus of this study is HMG-D, a chromosomal protein of this family found in *Drosophila melanogaster.* HMG-D shows homology with regions of vertebrate HMG proteins, HMG1 and HMG2, and to stretches of Histone H1. Due to its remarkable homology, HMG-D serves as an excellent model for cognate chromosomal proteins (Churchill *et al.*, 1995; Ner *et al.*, 1994; Murphy, 1997).

The 112 amino acid HMG-D consists of 3 alpha helices, a basic region, and an acidic tail (figure 1D). The first 74 N-terminal helices comprise the HMG-box, a highly conserved domain common to all HMG proteins. The ~17 amino acids following the HMG-box constitute the basic region of the protein, which is homologous to Histone H1. The final 12 residues at the C-terminal form an acidic tail homologous to vertebrate HMG proteins. The approximate location on the DNA to which the HMG-box binds has been determined, while the position of the two succeeding regions remains unknown. Of interest in this study is the assessment of the C-terminal acidic residues in the HMG-D-DNA interaction (Churchill *et al.*, 1995; Ner *et al.*, 1994; Murphy, 1997).

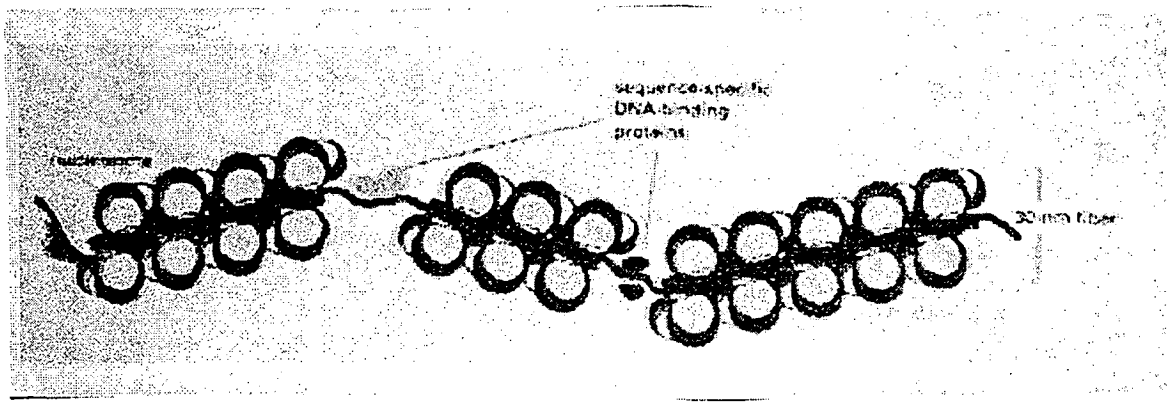
The function and location of the acidic tail in the DNA complex with the wild type protein is unknown. Therefore, a mutant protein containing a sequence alteration in the acidic motif is generated to provide a comparison with the wild type. The mutant utilized in this research is the full length protein with an additional C-terminal cysteine. The cysteine serves as an end marker on the protein, crucial for subsequent mapping experiments (Travers *et al.*, 1994; Churchill *et al.*, 1995). The 112c mutant protein is generated through several steps (figure 2). The mutant sequence is acquired, amplified through PCR, and inserted into a plasmid. The mutant plasmid is

‡Poster presentation

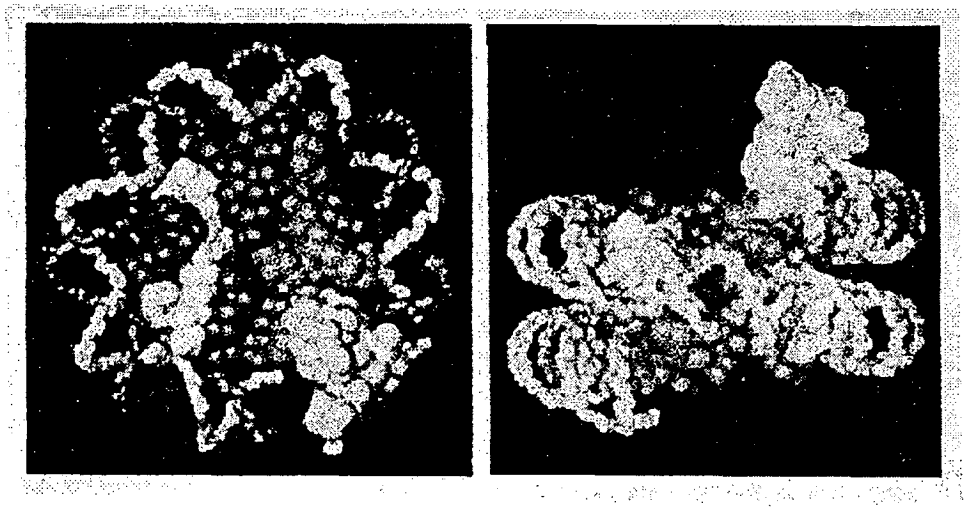
introduced into different strains of *E. coli* by transformation in order to amplify the plasmid and express the mutant protein, which is purified by chromatography (figure 3).

The 112c mutant is compared with the wild type by Electrophoretic Mobility Shift Assays (figure 4). These assays should indicate differences in the DNA binding affinities of each protein and affirm if the tail has an effect on complex formation. From the results, conclusions can be drawn about the location of the tail within the protein-DNA complex and allow additional theories to be composed about the precise function of HMG-D in nucleosome organization.

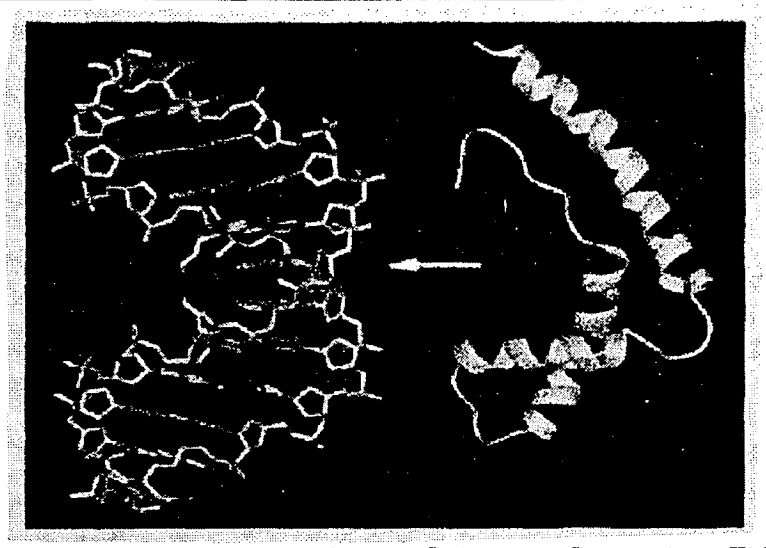
FIGURE 1. PUTATIVE HMG-D INTERACTION IN CHROMATIN



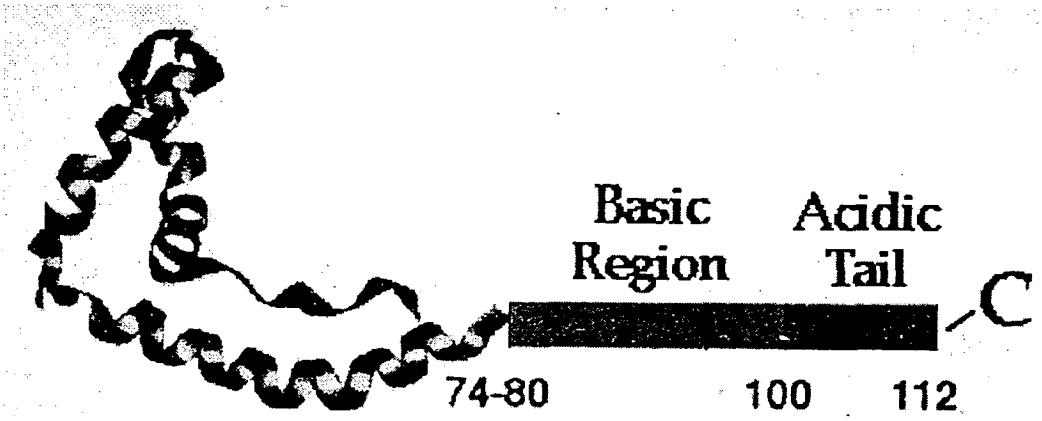
A. This hypothetical model of nucleosome complexes shows a possible role of HMG-type proteins in condensing chromatin (from Alberts *et al.*, 1994).



B. This is the cross section and side of a nucleosome, DNA coiled around a histone octamer. HMG proteins are thought to help form this conformation.



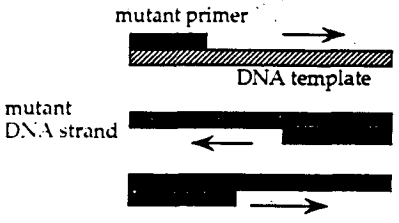
C. This figure shows a conceivable model of the DNA HMG-D complex. This HMG-D protein is bound to a disulfide crosslinked DNA.



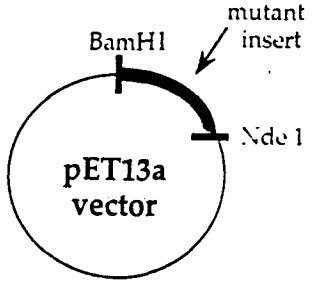
D. This is a structural model of the HMG-D protein. The alpha helices of the HMG-box are followed by the basic region and acidic tail residues. The 112c mutant has a C-terminal cysteine.

FIGURE 2. MUTAGENESIS FLOWCHART

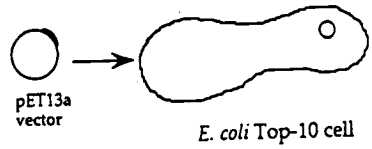
1.PCR-POLYMERASE CHAIN REACTION
This procedure is used to amplify a specific mutant sequence of DNA. The synthetic oligonucleotide mutant primer binds to a template plasmid, which is replicated unidirectionally with Taq polymerase. Cycles of heating, cooling, and annealing of templates and primers result in high levels of amplification of the mutant strands in preparation for plasmid ligation.



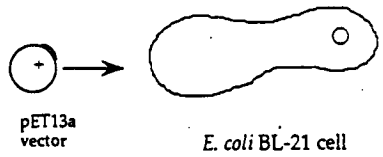
2. DOUBLE DIGEST AND LIGATION
After the mutant sequence is amplified, it is inserted into the pET13a bacterial plasmid, which contains multiple synthetic cloning sites. Restriction enzymes BamH1 and Nde1 cleave the insert and the vector at specific sites so they have compatible sticky ends. T4 DNA ligase ligates the mutant DNA into the vector, forming a recombinant mutant plasmid.



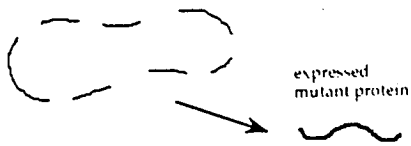
3. PLASMID AMPLIFICATION
The plasmid is introduced into the Top 10 strain of *E. coli* by transformation. This strain harbors extremely high copy numbers of the mutant plasmid.



4. PROTEIN SYNTHESIS
The plasmid is harvested from the *E. coli* Top-10 strain by sonication and freezing. The plasmids are purifed from the culture and positive clones are identified through double digest. The *E. coli* BL-21 strain is induced with the mutant plasmid. This strain exhibits a high level of protein expression.



5. PROTEIN HARVEST
The BL-21 cells are broken open, and the protein is harvested in preparation for purification.



Purification is necessary to separate the mutant protein from its oxidized forms and protein fragments. Four different chromatography columns are used to separate the pure 112c protein from this undesired material. Each column possesses a different basis of separation in order achieve the highest level of purification.

The first step following the generation of the mutant protein is *Ion Exchange Chromatography*. This cation exchange column fractionates the protein according to charge. The matrix is filled with negatively charged sulphopropyl functional groups. As the positively charged protein enters the column, it sticks to the negative charges in the column. As a gradient of NaCl is introduced, the sodium ions compete for the column and eventually displace the protein. This column is also utilized as a final purification step to separate the protein from acetonitrile, the elution chemical used in the reverse phase column.

The second step is *Covalent Chromatography*. This column is a thiopropyl sepharose filtration column, filled with porous beads. This unique column can separate the cysteinated mutant protein on the basis of a covalent interaction. The beads of the matrix are covered with sulfhydryl groups. This causes the C-terminal cysteine to form a disulfide bond with the matrix, and thus retaining the protein in the column. ß-mercaptoethanol is a chemical that breaks disulfide bonds. The protein is detached and eluted when a gradient of ß-mercaptoethanol is introduced.

The third step is *Reverse Phase Chromatography*. The protein is retained in this column, which is based on hydrophobic interaction, in order to achieve high degrees of purification. Within the column, the protein unfolds itself and reveals its hydrophobic regions because the pH of the matrix is 2. The hydrophobic areas of the protein stick to the walls of the column, which are comprised of carbon chains and silicon. As acetonitrile, a hydrophobic solution, is introduced into the column, it competes for the hydrophobic regions of the column and elutes the protein.

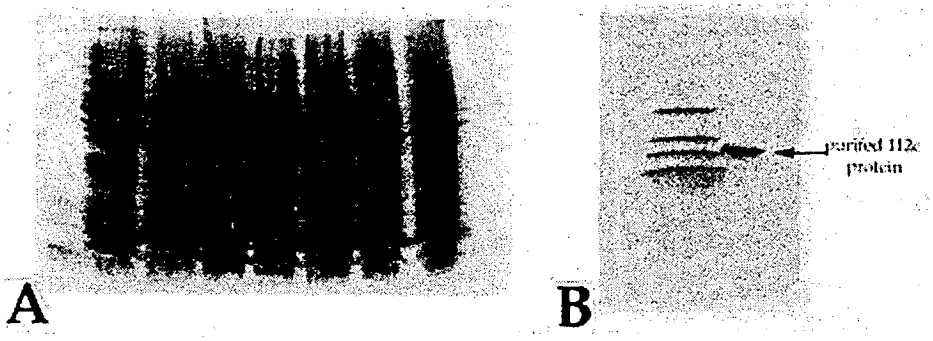
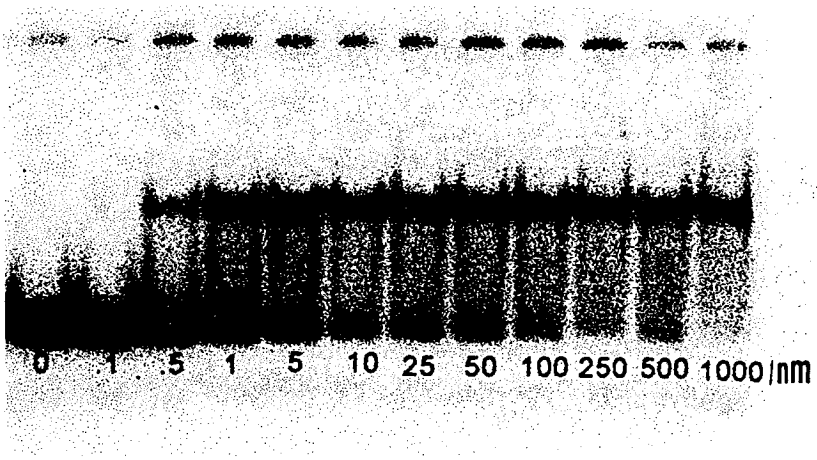


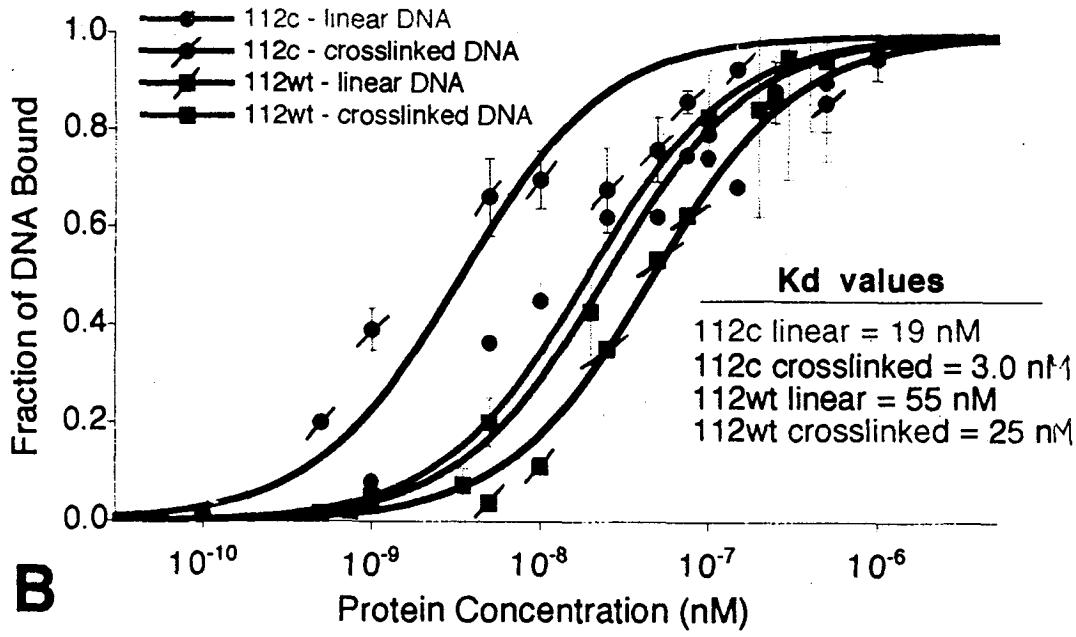
FIGURE 3 Samples of the protein were run on SDS-PAGE gels before (A) and after (B) purification.

FIGURE 4 COMPARISON OF BINDING AFFINITY



A. BAND SHIFT ASSAY

This figure shows a phosphorimage of an electrophoretic mobility shift assay (EMSA) or band shift assay. Band shift gels separate DNA according to size. In this experiment, the HMG-D protein binds to DNA, forming a complex that will migrate slower than free DNA. The fraction of DNA bound to the protein is a function of increasing protein concentration. Therefore, the ratio of DNA-protein complexes to free DNA is equal to the equilibrium binding constant (Kd). Band shift gels performed on the mutant are compared with preliminary data of the wild type protein in order show any affinity differences due to the acidic tail. For these assays, two types of 32P-labled DNA are utilized. The binding ability of linear DNA to the protein is compared to that of prebent disulfide crosslinked DNA. Analysis of the mutant protein and the wild type protein with both types of DNA should provide adequate data to determine if the acidic tail is altering the binding. This 8% acrylamide band shift gel shows how the 112 cysteinated mutant binds with disulfide crosslinked DNA as its concentration is raised from 0.1nm to 1000nm.



B. HMG-D DNA BINDING CURVES

This figure shows the quantative relationship between the binding affinity of 112c mutant and the 112 wild type HMG-D protein with two types of DNA, linear and crosslinked. The Kd values shown indicate the protein concentration at which approximately half the DNA is bound. The Kd value of the 112c mutant is slightly lower than the 112wt protein. Therefore, the concentration at which the mutant can initially bind DNA is smaller, which signifies a higher binding affinity. The cysteinated mutant appears to have a higher binding affinity than the wild type to both DNAs, and binds approximately 7 times better to the crosslinked DNA.

CONCLUSIONS

The electrophoretic mobility shift assays in this study agree with preliminary data from previous experiments which show that HMG-D prefers to bind prebent DNA. The cysteinated mutant, however, exhibits a binding affinity slightly higher than the wild type for both the linear and the crosslinked DNA, leading to the conclusion that the cysteinated acidic tail increases binding affinity of HMG-D to the DNA. A possible explanation is that the cysteine facilitates binding by contributing to a greater "hydrophobic effect." This idea can be further investigated with calorimetric experiments. Since the 112c mutant is now purified, performance of site-specific DNA cleavage studies may help to indicate the location at which the cysteine is bound to the DNA. As the knowledge from this and future studies increases, the understanding of the precise conformation of HMG-D and of nucleoprotein complexes will continue to improve.

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