EXAMINATION OF PROTIST COMMUNITIES IN THREE SPECIES OF *RETICULITERMES* SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE)

by

JENNIFER LYNN LEWIS

(Under the Direction of Brian T. Forschler)

ABSTRACT

Termite hindgut symbionts serve an important role in meeting the nutritional requirements of termites. Studies quantifying *Reticulitermes* protist communities have been conducted using a variety of methods, thus making comparisons between studies difficult. Protist species are involved in separate stages of metabolism and, because termite life stages are fed different diets, their protist communities should reflect that. We compared methods used to estimate termite hindgut protist populations and propose a single technique for ease and accuracy. We looked at protist communities from four life stages of *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), and *Reticulitermes hageni* Banks, and found workers and nymphs have the largest population of protists, followed by soldiers, with the smallest populations in alates. In addition we showed these communities can be used to identify termites using the most common life stage, workers. We showed protist communities are not a good indication of termite colony health.

INDEX WORDS: anaerobic protists, cellulose digestion, life stage/caste comparison, protist communities, sentinel species, techniques.

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THIS THESIS IS DEDICATED

TO MY FAMILY,

PAMELA AND DANIEL RAMIREZ,

WHO TAUGHT ME PERSEVERANCE, COURTESY, AND INTEGRITY

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CHAPTER 1

A REVIEW OF THE LITERATURE ON PROTIST COMMUNITIES IN *RETICULITERMES FLAVIPES*, *RETICULITERMES VIRGINICUS*, AND *RETICULITERMES HAGENI* (ISOPTERA: RHINOTERMITIDAE).[1]

[1] Lewis, J.L. and B.T. Forschler. To be submitted to *Sociobiology*.

Introduction

Termites impact ecosystems by influencing physical, chemical, and structural attributes of soils (Lee & Wood 1971, Wood & Sands 1978). Termite populations can exceed 6000/m^2 in the tropics, but their numbers and diversity decline with distance from the equator (Eggleton et al. 1996, Eggleton 2000). Termites are most noted for their capacity to cause considerable structural damage to buildings and homes. It has been estimated that termites annually cause a billion dollars in damage in the United States alone (Thorne 1998).

Termites are eusocial insects that display distinct castes and life stages including the reproductive, soldier, and worker forms (Noirot & Noirot-Timothee 1969). Each caste performs a role within the colony. Reproductives maintain colony populations through egg production and the soldier caste guards and protect the colony from invaders. Workers, the most numerous life stage in a colony, perform many tasks such as building and maintaining extensive galleries, caring for brood, foraging for and consuming food, and feeding other colony members (McMahan 1969, Honigberg 1970). Workers transfer food to dependent nest mates in the form of stomodeal food (regurgitated) and/or proctodeal food (hindgut contents) (Grassé & Noirot 1945, McMahan 1969, Noirot & Noirot-Timothee 1969).

Live plants, dead wood, and leaf litter are the major food resources of lower termites: Hodotermitidae, Termopsidae, Mastotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae (Lee & Wood 1971, Waller & LaFage 1987). Termites ingest wood fragments, macerating and mixing them with salivary gland secretions that include cellulases (Watanabe et al. 1997, Nakashima et al. 2002) that hydrolyze cellulose polymers to glucose (Inoue et al. 1997). There is limited degradation of xylan (Azuma et al. 1993, Inoue et al. 1997) and lignin in termites (Kuhnick et al. 1994, Brune et al. 1995). Yet, studies have found lignin contributes little

to termite nutrition partly because it requires oxygen to be broken down (Esenther & Kirk 1974, Cookson 1987). Soluble nutrients such as glucose are absorbed from the midgut; however, it has been estimated that about 70 percent of cellulose digestion and absorption takes place in the dilated portion of the termite hindgut, or paunch (Inoue et al. 1997). The paunch, filled with bacteria and protists, accounts for up to 61 percent of a termite's hindgut weight (Odelson & Breznak 1983). These obligate symbionts aid in cellulose digestion, creating a unique system important for termite health (Cleveland 1924, 1925a-c, 1928, Trager 1934, Hungate 1938, 1939).

Importance of Symbionts to Termite Nutritional Requirements

Despite having endogenous cellulases (Tokuda et al. 1999), termites are unable to support their own energy requirements on a strict cellulose diet (Nakashima et al. 2002). The hindgut symbionts assist by breaking down partially digested cellulose and glucose to acetate, carbon dioxide, and hydrogen (Yamin 1980, 1981, Odelson & Breznak 1985a, 1985b, Breznak & Switzer 1986). Termite hindgut symbionts include fungi, bacteria, and protists (Honigberg 1970).

Metabolites within the termite hindgut are radially distributed in lower termites (Brune et al. 1995, Ebert & Brune 1997, Brune 1998, Schmitt-Wagner & Brune 1999). Along the gut periphery is a steep oxygen gradient caused by the large surface-to-volume ratio (Brune 1998). Oxygen is rapidly consumed around the periphery of the paunch (Brune et al. 1995) by aerobic and aerotolerant microflora, creating an anoxic center (Ebert & Brune 1997, Brune 1998). This anaerobic center, less than 40 percent of the total hindgut volume (Brune et al. 1995), is where bacteria and protists produce acetate, carbon dioxide, and hydrogen by fermentation.

Acetate from symbiotic protists is the dominant energy source used by termites (Odelson & Breznak 1983, O'Brien & Breznak 1984) for production of protein bound amino acids (Mauldin et al. 1978) and cuticular hydrocarbons (Blomquist et al. 1979, Mauldin 1982). Without hindgut protists, the host would starve even with a gut filled with wood (Cleveland 1925a). In addition to the protists, acetogenic bacteria within the hindgut use carbon dioxide and hydrogen from glucose digestion to produce acetate and water (Breznak & Switzer 1986).

Wood contains 0.03-0.1% nitrogen and a carbon to nitrogen ratio (~400:1) (Cowling & Merrill 1966) too low for termite nutritional needs (Breznak & Brune 1994). For termites to compensate, they have symbiotic bacteria that can recycle nitrogenous waste (Potrikus & Breznak 1981) or fix atmospheric nitrogen within the termite hindgut for protein biosynthesis (Hungate 1941). Little is know about the role of fungi in the microfloral community within the termite hindgut (Breznak 1982).

Microbial Symbionts

There is a diverse community of known microbes found in lower termites (Breznak & Pankratz 1977), but the majority of prokaryotes in termites have yet to be identified (Ohkuma & Kudo 1996, Brune & Freidrich 2000). Microbial populations can reach 6.3×10^6 cells per gut (Tholen et al. 1997) and include methanogenic Archaea (Leadbetter & Breznak 1996, Tholen et al. 1997, Leadbetter et al. 1998), acetogenic bacteria (Odelson & Breznak 1983), spirochetes (Breznak & Brune 1994, Lilburn et al. 1999), nitrogen-fixing bacteria (Breznak et al. 1973, Potrikus & Breznak 1977, Lilburn et al. 1999, Ohkuma et al. 1999), and lactic acid bacteria (Tholen et al. 1997, Bauer et al. 2000). These range from strict to facultative anaerobes and aerobes in the digestive tract (Schultz & Breznak 1978, Bignell & Anderson 1980, Tholen et. al.

1997). These organisms contribute to the termite's carbon, nitrogen, and energy requirements (Eutik et al. 1978); however most of the cellulolytic decomposition is from protists found almost exclusively in the hindgut of lower termites (Honigberg 1970).

Protist Symbionts

Protists from the orders Trichomonadida, Oxymonadida, and Hypermastigida are specific to certain lower termite families, while only the last two are found in the wood-feeding cockroach, *Cryptocercus* species (Koidzumi 1921, Cleveland et al. 1934, Kirby 1937, Honigberg 1970). The fact that several of these symbionts are present only in lower termites and *Cryptocercus* has fueled debate as to the evolutionary origin of these symbionts. Some studies hypothesize symbionts in either termites or *Cryptocercus* were transferred to the other taxon by consumption (Thorne 1990, 1991), while others believe the symbionts were inherited from a common ancestor of the wood-feeding cockroach and termites (Cleveland et al. 1934, Kirby 1937, Nalepa 1991, 1994, Hahn 1995, Nalepa et al. 2001) and, combined with colony formation and xylophagy, contributed to eusociality in insects (Cleveland et al. 1934, Nalepa 1984, 1994, Noirot 1992, Nalepa et al. 2001). However, this controversy remains unresolved awaiting further study.

Protist communities are qualitatively similar within each termite and wood-feeding cockroach species (Cleveland et al. 1934, Kirby 1937, Cook 1996), creating the possibility that these specific and distinctive protist communities (Kirby 1937, Honigberg 1970) could be used for host species identification. The protist communities found within the subterranean termite genus *Reticulitermes* have been described as species specific (Kirby 1932, 1937).

Protists in *Reticulitermes flavipes*

There have been eleven protist species described in *Reticulitermes flavipes* (Kollar) (Yamin 1979), with populations exceeding 35,000 cells per individual gut (Howard 1984). They are *Dinenympha gracilis* Leidy 1877, *Pyrsonympha vertens* Leidy 1877, *Trichonympha agilis* Leidy 1877, *Spironympha kofoidi* Koidzumi 1917, *Holomastigotes elongatum* Grassi 1917, *Monocercomonas* sp. Grassi 1917, *Dinenympha fimbriata* Kirby 1924, *Trichomonas trypanoides* Dubosq and Grassé 1924, *Microjoenia fallax* (Dubosq and Grassé 1928), *Spirotrichonympha flagellata* (Dubosq and Grassé 1928), *Pyrsonympha major* Powell 1928, *Spirotrichonympha* sp. (Mannesmann 1974 mentioned but not described). Figure 1.1 shows photographs taken using a Leica microscope at 100X magnification and digital images acquired using an AxioCam digital camera. We were able to find all eleven species described.

Lespes (1856) was the first to mention living cells in the hindgut of termites. Leidy described these anaerobic protists in 1877 and 1881 from the eastern subterranean termite, *Termes flavipes* (now *Reticulitermes flavipes)*. He established the genera *Trichonympha*, *Pyrsonympha*, and *Dinenympha* with type species *Trichonympha agilis*, *Pyrsonympha vertens*, and *Dinenympha gracilis*. Leidy (1881) mentioned that further study might reveal additional species because there is considerable variation in size. Several smaller flagellates were described as separate species by Grassi (1892), but these were later listed by Porter (1897) and Dubosq & Grassé (1928) as smaller forms of described species. Within ten years these were again designated separate species (Kirby 1924, Brown 1930a, Brown 1931).

In 1892, Grassi established the genus *Holomastigotes*; however he wrongly referred to some *Holomastigotes* cells as *Pyrsonympha* in 1917 because of the numerous flagella covering some *Pyrsonympha* cells. *Dinenympha fimbriata* was originally described as a young form of

Pyrsonympha vertens (Leidy 1881, Porter 1897), however in 1924, based on cell size and shape, the presence of flagellar cords, and axostyle attachment to the posterior end, Kirby (1924) established it as a separate species. One of the smallest protist species in *R. flavipes, Trichomonas trypanoides,* is distinguished by having 2 to 4 anterior flagella with an undulating membrane, as described by Dubosq & Grassé (1924). In 1928, Dubosq & Grassé described a new species, *Spirotrichonympha flagellata,* and called *Microjoenia fallax* and *Spironympha kofoidi* younger forms of that species (Dubosq & Grassé 1928). *Spironympha* was first described by Koidzumi (1916); this genus used to be included in *Trichonympha* (Leidy 1881) but was later referred to as *Microspironympha* by Koidzumi (1921). In 1928, Dubosq & Grassé placed *Spironympha* in the genus *Spirotrichonympha*. However, based on the rules of nomenclature, it is now known as *Spironympha kofoidi* (Koidzumi 1916, 1917). *Pyrsonympha major* was established in 1928 by Powell examining protists from *Reticulitermes hesperus*. Powell compared these cells with original species descriptions of *P. vertens* and in later publications concluded these species were not the same because of the shape and size differences (Powell 1928). Based on arrangement of flagellar bands, *Microjoenia fallax* and *Spironympha kofoidi* were finally placed in their current genera, as valid species, *Microjoenia* and *Spironympha*, respectively (Brown 1930b).

Protists in *Reticulitermes virginicus*

Looking at the dark southern subterranean termite, *Reticulitermes virginicus* (Banks), Mannesmann (1972) mentioned seven protist species, but described only four. His descriptions were based on morphological comparisons using original species descriptions from other termites. These are *Trichonympha agilis* Leidy 1877, *Dinenympha fimbriata* Kirby 1924,

Pyrsonympha minor Powell 1928, *Spirotrichonympha flagellata* (Dubosq and Grassé 1928). Figure 1.2 shows photographs of the six described protist species taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera.

Powell (1928) described *Pyrsonympha minor* as separate from *P. vertens* and *P. major* based on morphology. Mannesmann estimated *Reticulitermes virginicus* protist populations around 5,000 per termite worker. Cook & Gold (2000) found the four species listed by Mannesmann, as well as *Holomastigotes elongatum* Grassi 1917, *Spironympha kofoidi* (Dubosq and Grassé 1928). Cook & Gold (2000) found that protist populations from *R. virginicus* numbered approximately 12,500.

Protists in *Reticulitermes hageni*

There are only four protist species described from the light southern subterranean termite, *Reticulitermes hageni* Banks (Yamin 1979), *Spironympha kofoidi* (Dubosq and Grassé 1928), *Spirotrichonympha gracilis* Brown 1930, *S. pulchella* Brown 1930, *Microjoenia pyriformis* Brown 1930b. Figure 1.3 shows the protists as described from *R. hageni*, however we could not distinguish *Spirotrichonympha* species apart.

In 1930, Brown studied protists from *Reticulitermes hesperus* and *R. hageni*. Using original protist species descriptions, she listed *Spironympha kofoidi* (Dubosq and Grassé 1928), *Spirotrichonympha gracilis* and *S. pulchella* as found in *R. hageni* (Brown 1930a). She described *Microjoenia pyriformis* (Brown 1930b) and 2 new species of *Spirotrichonympha* based on their morphology. Brown did not believe *Spirotrichonympha flagellata* was in *R. hageni* because the flagellar bands were not as deep or as closely wound around the anterior part of the body, the axostyle was not thick and fibrous, and the shape of the centroblepharoplast (*Spirotrichonympha*

have a combined centrosome and blepharoplast) was different from *S. gracilis* and *S. pulchella*. The axostyle in *Spirotrichonympha gracilis* is large and attached to the posterior body, ending in a point, while in *S. pulchella* it is fine and fibrous, and so Brown believed that there were two different species present in *R. hageni*, naming them *S. gracilis* and *S. pulchella* (Brown 1930a).

Abiotic Factors Influencing Termite Protist Community Structure

There are numerous factors that can influence the protist composition in lower termites. However, because protists are anaerobic and most cannot be cultured, information about specific roles of the various protists has been inferred by altering either the host or the protist community. Those factors that have been studied include: temperature (Cleveland 1924, 1925b, Mannesmann 1969, 1970, Grosovsky & Margulis 1982), changes in oxygen levels (Cleveland 1925b, c, 1928), geographical location (Mannesmann 1974, Grosovsky & Margulis 1982), and season (Mannesmann 1969, 1970, Howard & Haverty 1981, Cook 1996).

Cleveland was the first to use temperature to affect the protist community in insects. He determined that high temperatures eliminated *Trichonympha agilis* and *Pyrsonympha vertens* from the host. He was able to correlate loss of certain protist species with the hosts' inability to digest wood, inferred by comparing termite survival (Cleveland 1924, 1925b). When refaunated with these protists, termites survived, leading him to assume that the termites were again capable of digesting wood. Using the same techniques, Grosovsky & Margulis (1982) found similar results in that high temperatures affected the protist community but the protist species lost were different. Numbers of *Holomastigotes elongatum*, *Microjoenia fallax*, and *Spirotrichonympha* species decreased when termites were exposed to 36° C for 12 hours, but *Trichonympha agilis*, *Dinenympha gracilis*, and *Pyrsonympha vertens* were unaffected and 60 days after treatment the

termite host was dead, suggesting the importance of protists in termite health but illuminating different roles for the various protists (Grosovsky & Margulis 1982).

Increased oxygen tensions have been used to defaunate termites (Cleveland 1925b, c, 1928). When termites from the genus *Zootermopsis* were exposed to 99% oxygen at a pressure of one atmosphere for 24 hours, *Trichomonas* were eliminated (Cleveland 1925b). After 24 hours, the protist species, *Trichonympha*, *Leidyopsis*, and *Streblomastix* started dying and by 72 hours all protists were eliminated (Cleveland 1925b). Cleveland (1925c) found similar results with *Reticulitermes flavipes*.

Protist populations are identical within a termite species (Kirby 1937); however the composition can vary between geographic locations (Mannesmann 1974, Grosovsky & Margulis 1982). Lai et al. (1983) found no differences in colonies collected from different locations on the University of Hawaii campus. Cook & Gold (1999) did find differences in total protist numbers and relative species composition between sites that were separated by at least 70km.

Smaller protist populations are found in spring compared to the summer months (Mannesmann 1969, 1970, Cook 1996). Reasons for this could include reduced termite activity during the winter months or changes related to a shift in caste proportions due to the springtime flight of the alate caste members.

Inference of Protists' Role in Cellulose Digestion

Different protist species are believed to be involved in separate steps of cellulose digestion because these communities respond to various host nutritional sources differently (Cleveland 1924, Hungate 1943, Mannesmann 1972, Mauldin et al. 1972, Smythe & Mauldin 1972, Mauldin et al. 1981, Lai et al. 1983, Yoshimura et al. 1993a, b, 1996, Cook & Gold 2000).

Active ingestion of wood fragments has been shown in *Pyrsonympha vertens* and *Trichonympha agilis* (Yamaoka 1979, Grosovsky & Margulis 1982). In forced feeding experiments, numbers of *Trichonympha agilis* and *Pyrsonympha minor* increase when termites are fed wood compared to filter paper (Cook & Gold 2000). Protist species that have not been observed ingesting wood are *Dinenympha gracilis* (Kirby 1924, Grosovsky & Margulis 1982) and *Spirotrichonympha*, which are believed to be involved in the later stages of cellulose degradation because they do not affect host vitality either when present in small populations or when absent (Grosovsky & Margulis 1982, Smythe & Mauldin 1972, Yoshimura et al. 1993b). Cook & Gold (2000) found an increase in *Pyrsonympha vertens* when termites were force-fed filter paper, while numbers of *Dinenympha fimbriata*, *Pyrsonympha minor*, and *Trichonympha agilis* decreased, implying that *D. fimbriata*, *P. minor*, and *T. agilis* requires more than just cellulose for survival. In starvation experiments *Trichonympha agilis* and *Spirotrichonympha* spp. are the first to disappear, followed by *Pyrsonympha vertens* and *Dinenympha fimbriata* (Cleveland 1925a). However, *Holomastigotes elongatum*, *Trichomonas trypanoides*, and *Microjoenia fallax* populations are not affected when the host is starved (Cleveland 1925a). Grosovsky & Margulis (1982) concur that *Holomastigotes elongatum*, *Trichomonas trypanoides*, and *Microjoenia fallax* are not important for host vitality and, therefore, probably not cellulolytic.

Inference of Protists' Roles Based on Life Stage/Caste Food Source

Protist populations are relatively similar within a caste of a given termite species (Dropkin 1944, Mannesmann 1970, 1972, To et al. 1980) but there are differences between life stages (Cleveland 1925a, Cook & Gold 1998). Cleveland (1925a) found some stages were deficient in certain protist species and hypothesized this was related to the amount and degree of

wood consumed per termite life stage. He hypothesized that because workers are responsible for the initial wood consumption, they should have more cellulolytic protists. Soldiers have a diet similar to workers and therefore should have a similar protist community (Cleveland 1925a). Early stage nymphs ingest wood but during later stages rely on stomodeal food from workers (Cleveland 1925a); hence they should have less cellulose digesting protists. The reproductive caste consumes stomodeal food from workers, but will also ingest some wood. Hence, the termite reproductives' protist community should have some cellulolytic representatives as well as other species involved in the later stages of cellulose degradation (Cleveland 1925a).

Early instar larvae are dependent on workers for proctodeal food until they become inoculated with flagellates (Andrew 1930, Grassé & Noirot 1945, Yamaoka et al. 1986, Kitade et al. 1997). This usually occurs around the 3rd instar (Cleveland 1925a) when the paunch develops epithelial and cuticle cups (Yamaoka et al. 1986), aiding in micro-organisms' attachment (Breznak & Pankratz 1977). Termite workers as well as the other caste members are also dependent on fellow workers because they lose their flagellates during molting and need to be refaunated (Cleveland 1925a, Andrew 1930, Grassé & Noirot 1945, McMahan 1969, Yamaoka et al. 1986, Kitade et al. 1997). Three to four days are required following a molt to re-acquire the protist community (Andrew 1930).

Techniques for the Study of Termite Protists

Studies quantifying *Reticulitermes* species protist communities have been conducted using a variety of cell counting techniques. They include different physiological saline solutions and counting methods (Mannesmann 1969, 1970, 1972, Mauldin et al. 1981, Yamaoka et al.

1983, Howard 1984, Azuma et al. 1993, Yoshimura et al. 1994, 1995, Inoue et al. 1997, Cook & Gold 1998, 2000).

There are several thousand anaerobic protists in each subterranean termite hindgut (Mannesmann 1969). Observing them outside their insect host requires a physiological saline solution that is osmotically balanced and well buffered to assist in keeping cells alive. In the literature, three salt solutions have been used in the study of *Reticulitermes* protist communities and one in the wood-feeding cockroach, *Cryptocercus* spp. These solutions vary in the proportions of sodium, potassium, calcium, and magnesium as well as resulting pH (Appendix A).

There are two common methods for quantifying termite protist populations and several different ways termite colonies are sampled. They vary in the number of termites sampled and the volume of saline solution in which the hindgut contents are suspended.

One counting method involves removing the hindgut content and suspending the cells in a known volume of Trager U saline solution, preparing a wet mount, and then sealing the cover slip (Yoshimura 1994, 1995, Cook & Gold 1998, 2000). Yoshimura (1994, 1995) extracted one termite worker from a colony of *Coptotermes formosanus* and homogenized it in 50 µl Trager U saline solution. He then counted all the protists from a 2 µl subsample. Cook & Gold (1998, 2000) removed 1 *Reticulitermes* gut and placed it in either 40, 80, or 120 µl Trager U, homogenized, and counted a 4 µl subsample.

The other method for counting protists involves the use of a hemocytometer (Mannesmann 1969, 1970, 1972, Howard 1984, Mauldin et al. 1981, Azuma et al. 1993, Inoue et al. 1997). A hemocytometer is a cell counting chamber manufactured for quick and easy cell counts. It consists of a chamber that holds a known volume of solution and upon which is etched

a standardized grid of nine 1.0-mm squares. These 'large squares' are divided and subdivided into smaller squares (9 square millimeters with the central square divided into $1/400^{th}$ $millimeter^2$ areas). Counts are taken in a systematic way with all the cells counted from within a square and only cells touching the line on the top and left side are included. The resulting counts are easily transformed to number of cells per one termite using the following formula: Number of cells counted * volume saline solution / volume counted * number of termites

Looking at the protist community in several different lower termites, Mannesmann (1969, 1970, 1972, 1974) homogenized 3 termite guts in 60 µl of Mannesmann solution, a small amount was loaded onto a hemocytometer, and cells counted. However, he did not mention the volume from which counts were made. Mauldin (1981) and Howard (1984) modified Mannesmann's technique by suspending 1 homogenized *Reticulitermes flavipes* worker in 40 µl Mannesmann solution and counting the cells from 0.40 µl. Azuma (1993) suspended the hindgut content from one *Reticulitermes speratus* worker in 20 µl of Trager U saline solution and counted species like *Pyrsonympha* and *Dinenympha* from 0.10 µl and used a 0.40 µl aliquot for larger species. Also looking at *R. speratus*, Inoue (1997) removed 1 gut, placed it in 15 µl Trager U solution and counted all large cells on the grid using a 0.90 µl sample and smaller species were counted from 0.025-0.10 µl aliquots.

Conclusion and Future Research

There is a diverse community of microbes (Breznak & Pankratz 1977) and protists (Yamin 1979) found in lower termites, and because termites cannot support their own energy requirements (Nakashima et al. 2002), it is important to understand this protist/termite relationship. Many methods have been used to observe hindgut symbionts outside the host.

However, there has been no comparison of techniques in regard to protist survivorship. This is noteworthy because to recognize relationships between protists and termites, techniques used to observe and quantify the protist community must be repeatable. These scientific methods need to be standardized to better understand this important symbiotic relationship and to compare between results. This is further complicated by the lack of dichotomous keys for termite hindgut protists, and these protist species have been described and revised numerous times in the Rhinotermitid host (Dubosq & Grassé 1928, Kirby 1924, Brown 1930a, b, Brown 1931), yet there are no published keys to encourage further inquiry.

A number of factors have been shown to influence protist communities including temperature (Cleveland 1924, 1925b, Mannesmann 1969, 1970, Grosovsky & Margulis 1982), season (Mannesmann 1969, 1970, Howard & Haverty 1981, Cook 1996), oxygen (Cleveland, 1925b, c, 1928), geographical location (Mannesmann 1974, Grosovsky & Margulis 1982), protist roles in cellulose digestion (Mannesmann 1972, Yamaoka 1979, Mauldin et al. 1981, Grosovsky & Margulis 1982, Yoshimura et al. 1993b, Cook & Gold 2000), and caste food source (Cleveland 1925a, Cook & Gold 1998). The majority of termite protists have not been successfully cultured outside the host; therefore the information we have on the specific protist roles has been inferred from *in situ* experiments. Although it is evident that the protist community is involved in various stages of cellulose digestion (Cleveland 1924, Hungate 1943, Mauldin et al. 1972, Smythe & Mauldin 1972, Lai et al. 1983, Yoshimura et al. 1993a, b, 1996), further studies are needed to elucidate how each of the protist species contributes to termite health and how this in turn affects the protists.

Many of the symbiotic protists found in lower termites are specific to certain termite species and therefore represent distinct protist communities (Kirby 1937, Honigberg 1970).

These communities are believed to differ between castes and have been compared from *Reticulitermes flavipes* (Cleveland 1925a, Cook and Gold 1998); however there are no studies examining the protist communities between castes for *R. virginicus* (Banks) and *R. hageni* Banks. Further research is needed to look at roles played by the various protists in termite nutrition and the potential of using these communities as a measure of termite vigor. Protist species proportions might be used as an indicator of termite vigor, in addition to percent termite survival commonly used in bioassays (Mauldin et al. 1981). These studies could potentially determine how termite laboratory colonies are established and maintained, as well as how they are bioassayed and collected from the field. Additional research examining communities in subterranean termites will assist in understanding the social and physiological aspects of this unique symbiosis, and may potentially be used for termite identification (Brown 1930a, Dropkin 1944).

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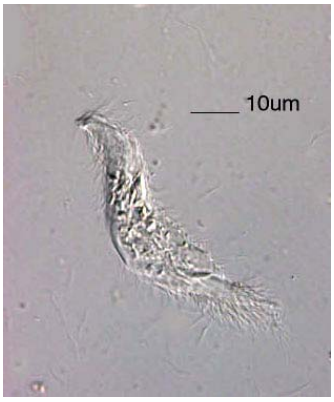
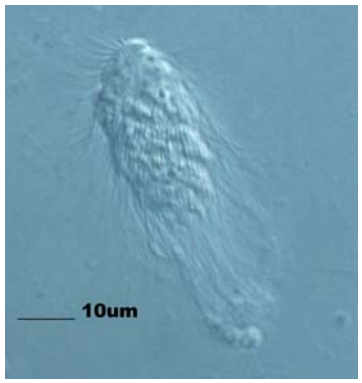
Dinenympha fimbriata



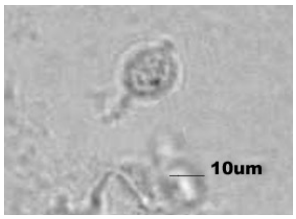
Microjoenia fallax

Dinenympha gracilis

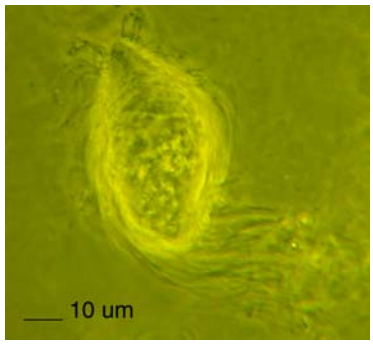
Holomastigotes elongatum



Monocercomonas sp.

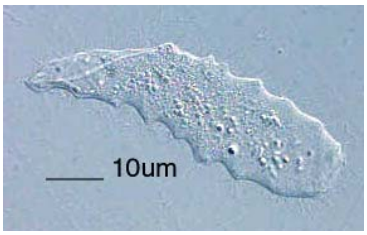


Spirotrichonympha flagellata



Trichomonas trypanoides

Pyrsonympha major



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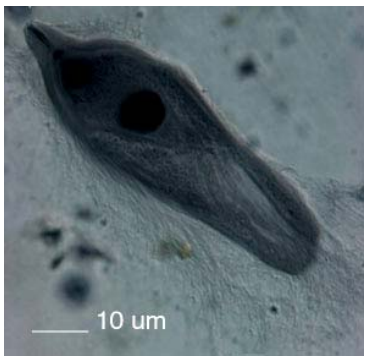


Spironympha kofoidi

Pyrsonympha vertens

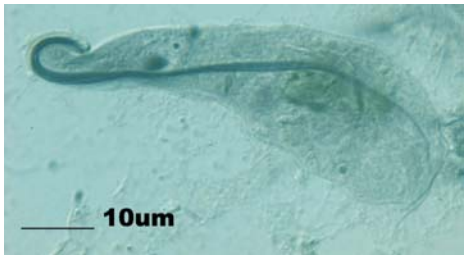
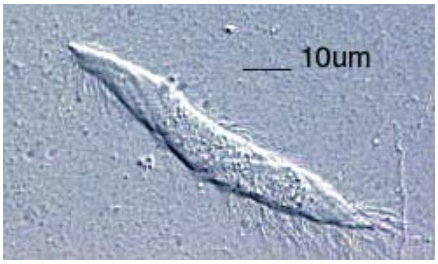
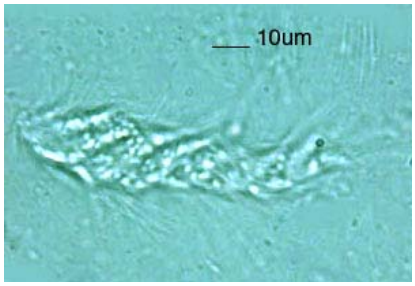


Figure 1.1. Protist species in *Reticulitermes flavipes* (Kollar). (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera).

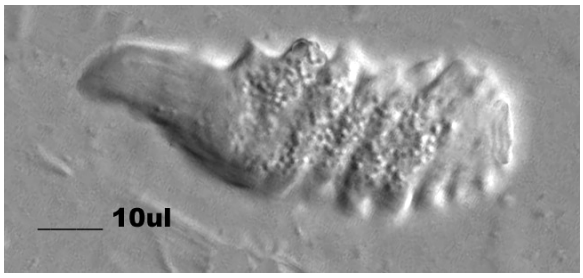
Dinenympha fimbriata



Holomastigotes elongatum

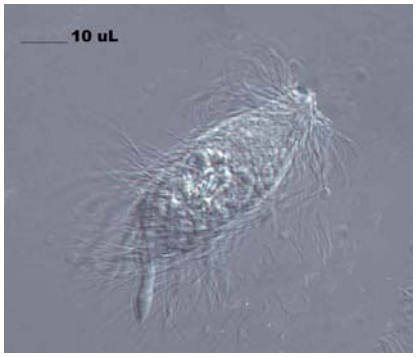


Pyrsonympha minor



Spironympha kofoidi

Spirotrichonympha flagellata



Trichonympha agilis

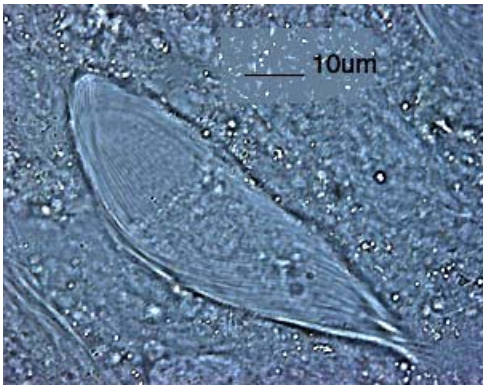
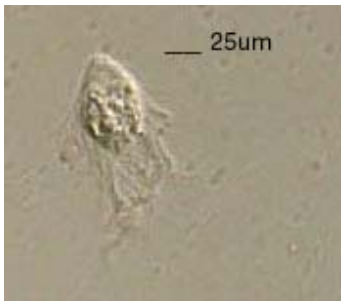


Figure 1.2. Protist species in *Reticulitermes virginicus* (Banks). (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera).

Microjoenia pyriformis

Spironympha kofoidi



Spirotrichonympha sp.

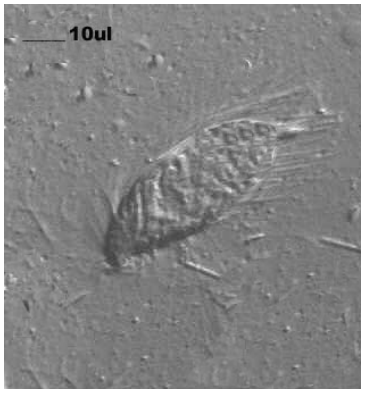


Figure 1.3. Protist species in *Reticulitermes hageni* Banks. (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera).

CHAPTER 2

A COMPARISON OF TECHNIQUES USED FOR COUNTING ANAEROBIC PROTISTS FROM TERMITES[1]

[1]Lewis J.L. and B.T. Forschler. To be submitted to*Environmental Entomology*.

Introduction

Lespes (1856) was the first to mention living cells in the hindgut of termites. The anaerobic protists observed by Lespes are now recognized to represent three orders: Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà (Yamin 1979). These symbiotic gut protists are found only in lower termites and the wood-feeding cockroach, *Cryptocercus* spp., and some protists appear to be specific to certain insect families (Koidzumi 1921, Kirby 1937, 1941, Honigberg 1970). Observation and study of protist communities in *Reticulitermes* spp. subterranean termites requires a physiological solution and a counting/observation platform that can accommodate the time frame needed to complete the observation before cell death occurs.

In the genus *Reticulitermes*, there are several thousand anaerobic protists in each individual subterranean termite hindgut (Mannesmann 1969). Observing them outside their insect host requires a physiological saline solution that should be osmotically balanced and well buffered to assist in keeping the cells alive. In the literature, there are three salt solutions that have been used in the study of *Reticulitermes* and one for the wood-feeding cockroach, *Cryptocercus* spp. looking at hindgut protist communities. These solutions vary in the proportions of sodium, potassium, calcium, and magnesium, as well as in resulting pH (Appendix A).

There are two accepted methods for quantifying termite protist populations. One counting method involves suspending the contents of a termite hindgut in a known volume of saline solution and preparing a wet mount by sealing the cover slip as used by Yoshimura (1994, 1995), and Cook and Gold (1998, 2000). The other method uses a hemocytometer cell counting chamber (Mannesmann 1969, 1970, 1972, Howard 1984, Mauldin et al. 1981, Azuma et al. 1993, Inoue et al. 1997).

In this manuscript we describe our work taking hemocytometer counts to compare different physiological saline solutions using the protist community in the hindgut of *Reticulitermes flavipes* (Kollar). Our objective was to determine if any of the previously described saline solutions affect protist survivorship over time. We hope these studies will stimulate discussion on the standardization of methods used to quantify termite protists toward a better understanding of protist/termite symbiosis.

Materials and Methods

Insects

Logs infested with the eastern subterranean termite, *Reticulitermes flavipes*, were collected from Whitehall Forest in Athens, Georgia, and brought back intact into the laboratory. Termites were identified to species using published keys to the soldier caste (Scheffrahn and Su 1994) and extracted as described by Forschler and Townsend (1996). Once collected, termites were kept in plastic boxes (26.99 X 19.37 X 9.52 cm) with pine slats (approx. 12.5 X 2.54 X 0.2 cm) placed inside an environmental chamber in complete darkness maintained at 24^{o} C. Only worker termites, fourth instar or older, with dark brown abdomens were used for protist counts.

Method of Counting Protists

A hemocytometer cell counting chamber was used for all protist counts. Hemocytometers are manufactured for quick and easy cell counts and consist of a counting chamber holding a known volume of solution upon which is etched a standardized grid of nine 1.0-mm squares. These 'large squares' are divided and subdivided into smaller squares (9 square millimeters with the central square divided into $1/400^{th}$ square millimeter area). Counts are taken in a systematic way, with all the cells counted from within a square and only cells touching the line on the top and left side included. The resulting counts are easily transformed to cell number per termite using the following formula: Number of cells counted * volume saline solution / volume counted * number of termites.

All cells were counted from 5 small squares (0.0125 square millimeters) replicated over time using the same mount, which constituted one replicated sample. Five replicates were used per experiment to determine total protist population estimates and percent live cells over time.

Preparation of Sample Unit

Forceps were used to remove the last two abdominal segments from the worker termite, resulting in the removal of the whole alimentary canal, which was placed in a microcentrifuge tube with 80 µl saline solution. This was homogenized for 10 seconds, and 10 µl was loaded onto a hemocytometer counting chamber.

Saline Solution Comparison

Total protist population counts from an individual worker termite from one colony of *Reticulitermes flavipes* were compared using four different saline solutions replicated five times.

The saline solutions tested were 0.60% NaCl (Kirby 1932), Mannesmann (1969), Trager (1934), and Ritter et al. (1978). Each solution was prepared in 200 milliliter glass sample bottles (Wheaton) as described in Appendix A, sterilized, and all subsamples taken from that reservoir. Kirby (1932) used 0.60% NaCl solution for comparative studies and descriptions of *Trichonympha* from several species of termites. This solution contains sodium chloride salt dissolved in distilled water. Trager developed two media, A and U, in 1934 based on preliminary experiments using protists from *Zootermopsis angusticollis* and *Reticulitermes flavipes*. He determined a solution with 0.30% to 0.40% NaCl, with a ratio of 97:3 sodium to potassium, and pH 6.8 - 7.2 was optimal for culturing protists from *Zootermopsis angusticollis* (Trager 1934). In solution A, the protist *Trichonympha sphaerica* survived for 6 weeks, however in Trager U populations increased during the same period (Trager 1934). Therefore we chose to use the Trager U solution in our comparison. Mannesmann designed 0.90% NaCl based on a comparison of six different salt solutions he designed, to demonstrate the effects of season and temperature on termite protist communities (Mannesmann 1969, 1970). Ritter's solution (1959) has a higher ratio of potassium to sodium than the previous three solutions. This medium was designed using flame-spectrometry to determine the potassium concentration in *Cryptocercus punctulatus*, the wood-eating cockroach, hindguts (Ritter 2002). *Cryptocercus* have hypermastigid and oxymonad symbionts that are closely related to those found in termites (Nalepa et al. 2001). Ritter et al. (1978) was able to double certain protist populations in 6-8 days using this medium in an anaerobic chamber he designed and tested.

In our experiments, protist samples were prepared as described above and initial cell counts were made within three minutes and taken every five minutes for one hour. The four saline solutions were compared using percent-remaining live cell data of total protist numbers over time.

Anaerobic Saline Solution

Trager U saline solution was compared to an anaerobically prepared medium. Trager U was used because it is the most often used saline solution (Azuma et al. 1993, Yoshimura et al. 1994, 1995, Inoue et al. 1997, Cook and Gold 1998, 2000), has a stable pH, and does not precipitate out of solution when sterilized, as did the Ritter solution.

Six milliliters of each solution were sterilized and bubbled with a nitrogen gas mixture (92.5% N, 5.0% CO_2, and 2.5% H) at 1 liter per minute for 5 minutes prior to protist sample preparation. For the sake of discussion the nitrogen-sparged solutions will be termed anaerobic solutions. Percent-remaining protist counts from anaerobic and aerobic solutions were compared over time (every five minutes) using a hemocytometer.

Statistical Analysis

Data were analyzed by Mann Whitney U nonparametric test ($P \leq 0.05$) using Statistica for Windows Package (Statistica 6.0, 2000 edition, StatSoft Inc., Tulsa, OK). Response variables were total protist counts and percent protist presence over time per saline solution.

Results

Saline Solution Comparison

Numbers of live cells decreased rapidly and after 5 minutes $\leq$ 72.17% ± 12.52 of the protists counted in the first survey were present, regardless of saline solution (Figure 2.1). There were no differences between saline solutions for up to 10 minutes. However, Ritter's solution maintained cells longer than 0.60% NaCl (U=0.0, Z=2.611, p=0.009) after 15 minutes but was not different from Trager U solution (table 2.1). At 20 minutes, significantly more living protists were present in Ritter's solution than in 0.60% NaCl (U=1.0, Z=2.402, p=0.016), Mannesmann (U=2.0, Z=2.193, p=0.028), or Trager U solution (U=3.0, Z=1.984, p=0.047). All cells were dead after 40 minutes on the microscope regardless of the salt medium tested.

There was no difference between total protist populations taken from Trager U and Ritter saline solution (Table 2.2), however counts from 0.60% NaCl had fewer cells at time 0 (U=0.0, Z=2.611, p=0.009). There were also fewer cells in Mannesmann saline solution than Trager U (U=2.0, Z=2.193, p=0.028) at time 0 and in Ritter at 15 (U=0.0, Z=2.611, p=0.009), 20 (U=0.0, Z=2.611, p=0.009), and 30 minutes (U=3.0, Z=1.984, p=0.047). Counts showed Mannesmann solution kept the hindgut protists alive longer than 0.60% NaCl at counts taken from 0 (U=1.0, Z=2.402, p=0.016), 20 (U=1.5, Z=2.299, p=0.022), 25 (U=2.0, Z=2.193, p=0.028), and 30 (U=3.0, Z=1.984, p=0.047) minutes, yet there was no difference from counts made at 5, 15, 35, and 40 minutes. There were no living cells present after 40 minutes in any of the four solutions saline solution used.

Anaerobic Saline Solution

Protists survived longer in the anaerobically prepared media (Figure 2.3). Percent total protist population in Trager U decreased significantly after 15 minutes, with only 55.84 ± 15.49% cells present (U=0.0, Z=2.611, p=0.009). In the anaerobic Trager U percent cell presence decreased significantly after 25 minutes, with 70.42 ± 15.18% cells alive (U=0.0, Z=2.61, p=0.009). Cells in anaerobic Trager U saline solution at 10 minutes were not different from those in anaerobic Trager U at 25 minutes. All living cells were eliminated after 30 minutes in Trager U; however in anaerobic Trager U 54.62 ± 20.75% of the cells were still alive.

Discussion

We have attempted to synthesize from the literature a method for estimating termite hindgut protist populations and propose a single technique for ease and accuracy. Estimates of protist populations were greater in our study than previously reported in the literature. We counted an average protist population of 72,000 ± 18,444.8 per individual in *Reticulitermes flavipes* across all solutions tested. When an anaerobic saline solution was used, however, populations averaged 96,800 ± 12,457.93. Previous counts from *Reticulitermes flavipes* found protist populations at 40,083.33 ± 3,642.91 by Mannesmann (1969), 32,320 by Mauldin et al. (1981), 31,120 ± 8,404.57 by Howard (1984), 21,043.5 ± 8,293.5 by Cook and Gold (1998), and 14,641.67 ± 5,879.66 from Cook and Gold (2000).

Total protist population numbers are but one reason to optimize the counting methods used. Identification of species and counts of protist species per termite require additional time. The decrease in numbers of live protists counted per square over time is most likely due to cell death. Extending cell life by optimizing a counting technique would theoretically provide more

accurate counts over time. This point is critical because up to 15 minutes on the microscope might be needed to make an accurate assessment. To make accurate counts, either the time spent needs to be decreased or cell life needs to be extended.

The increase in time needed to complete a protist count results from at least two factors. First is the need to count an increased number of hemocytometer squares to increase the likelihood that rare species (<10% of the total population) are accounted for in the survey. Second is the need to identify the protists to species, which takes longer and is largely influenced by familiarity with species characteristics. Species identification is simplified by observation of living cells because movement patterns can quickly distinguish similar species – forgoing examination of less obvious and therefore harder (and longer) to find characters.

These studies have demonstrated that by using nitrogen sparged Trager U physiological saline the time frame available for making accurate protist counts increases from 5 to 20 minutes. Also, further studies are needed to elucidate how each of the protist species contributes to termite health, which in turn affects the protists. This is significant, because recognizing the important relationships between protists and termites, one must understand their biology and how they interact.

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Table 2.1. Average percent total protist population over time in various saline solutions.

M[1]	Trager	Ritter	Mannesmann	0.60% NaCl
0	100 ± 0a[2]	100 ± 0a	100 ± 0a	100 ± 0a
5	70.46 ± 11.62a	82.13 ± 14.25a	72.43 ± 6.98a	63.67 ± 11.81a
10	60.97 ± 14.41a	66.50 ± 18.58a	52.34 ± 17.45a	48.22 ± 11.01a
15	50.62 ± 13.99ab	61.37 ± 11.01b	44.35 ± 11.59ab	39.91 ± 10.45a
20	36.53 ± 9.82a	52.69 ± 8.56b	36.68 ± 10.23a	18.40 ± 18.16a
25	8.36 ± 10.49a	34.28 ± 9.71b	22.84 ± 12.00b	8.36 ± 10.22a
30	15.27 ± 8.77a	20.42 ± 3.35a	12.37 ± 9.27a	3.33 ± 7.45b
35	11.01 ± 5.04a	11.29 ± 5.95a	2.60 ± 3.59ab	0.00 ± 0.00b
40	0.00 ± 0.0a	0.00 ± 0.0a	0.00 ± 0.0a	0.00 ± 0.0a

1= Time in minutes. 2=Mean ± SD with the same letter were not significantly different at each time interval between treatments using Mann Whitney U test ($p \leq 0.05$).

Table 2.2. Average total protist population over time in various saline solutions.

M[1]	Trager	Ritter	Mannesmann	0.60% NaCl
0	90,400 ± 13,440a[2]	79,200 ± 14,240ab	68,000 ± 10,200b	50,400 ± 6,080c
5	64,000 ± 16,000ab	64,000 ± 10,200a	48,800 ± 4,360b	32,000 ± 6,320c
10	55,200 ± 14,800ab	51,200 ± 12,120ab	35,200 ± 10,720bc	24,000 ± 4,880c
15	45,600 ± 13,160ab	48,000 ± 9,360a	29,600 ± 6,080b	17,600 ± 3,560c
20	33,600 ± 11,880ab	41,600 ± 10,440a	24,800 ± 7,160b	8,800 ± 8,680c
25	24,800 ± 12,120a	27,200 ± 9,960a	15,200 ± 7,680a	4,000 ± 4,880b
30	14,400 ± 9,200ab	16,000 ± 2,840a	8,000 ± 5,640b	1,600 ± 3,560c
35	10,400 ± 7,280a	8,800 ± 4,360a	1,600 ± 2,200b	0.00 ± 0.0b
40	0.00 ± 0.0a	0.00 ± 0.0a	0.00 ± 0.0a	0.00 ± 0.0a

1= Time in minutes. 2=Mean ± SD with the same letter were not significantly different at each time interval between treatments using Mann Whitney U test ($p \leq 0.05$).

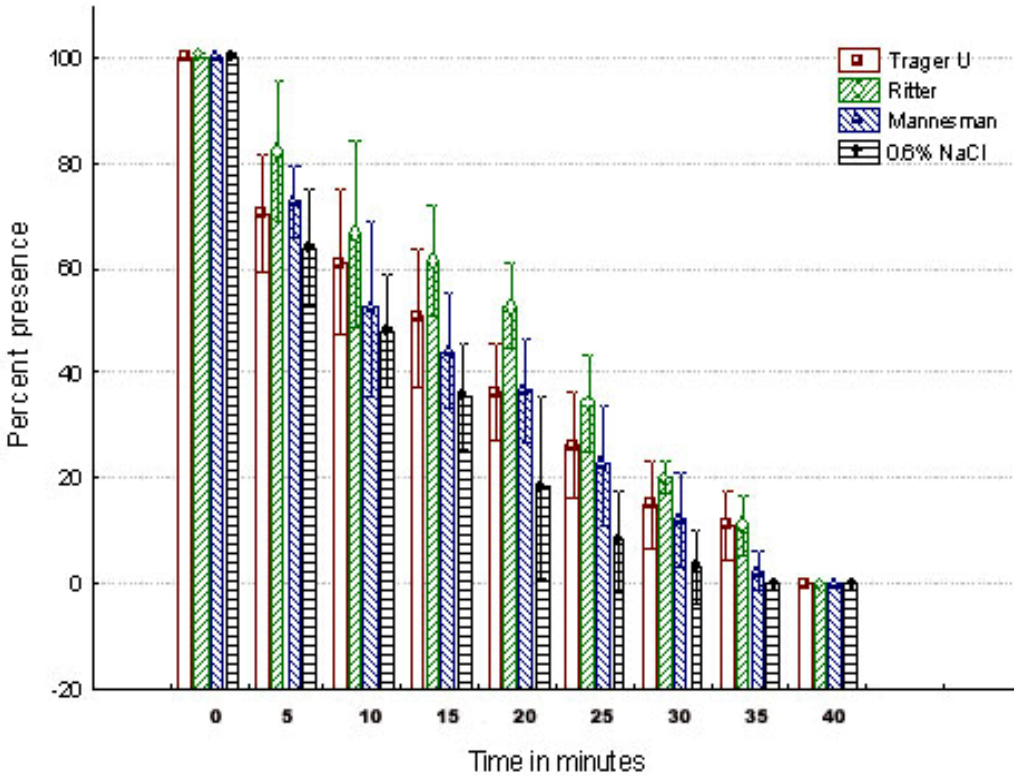

Figure 2.1. Average percent total protist population per termite individual in 80 µl saline solution over time using Mann Whitney U test ($p \leq 0.05$).

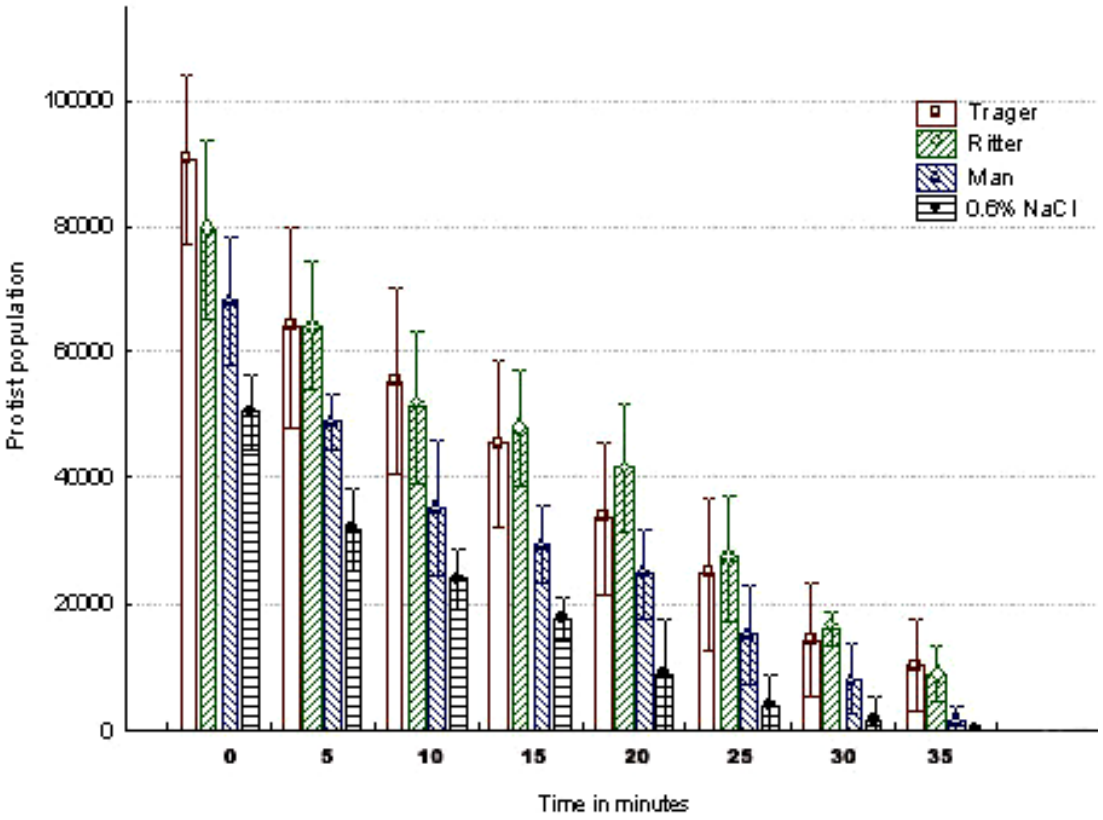

Figure 2.2. Average total protist population per termite individual in 80 µl saline solution over time using Mann Whitney U test ($p \leq 0.05$).

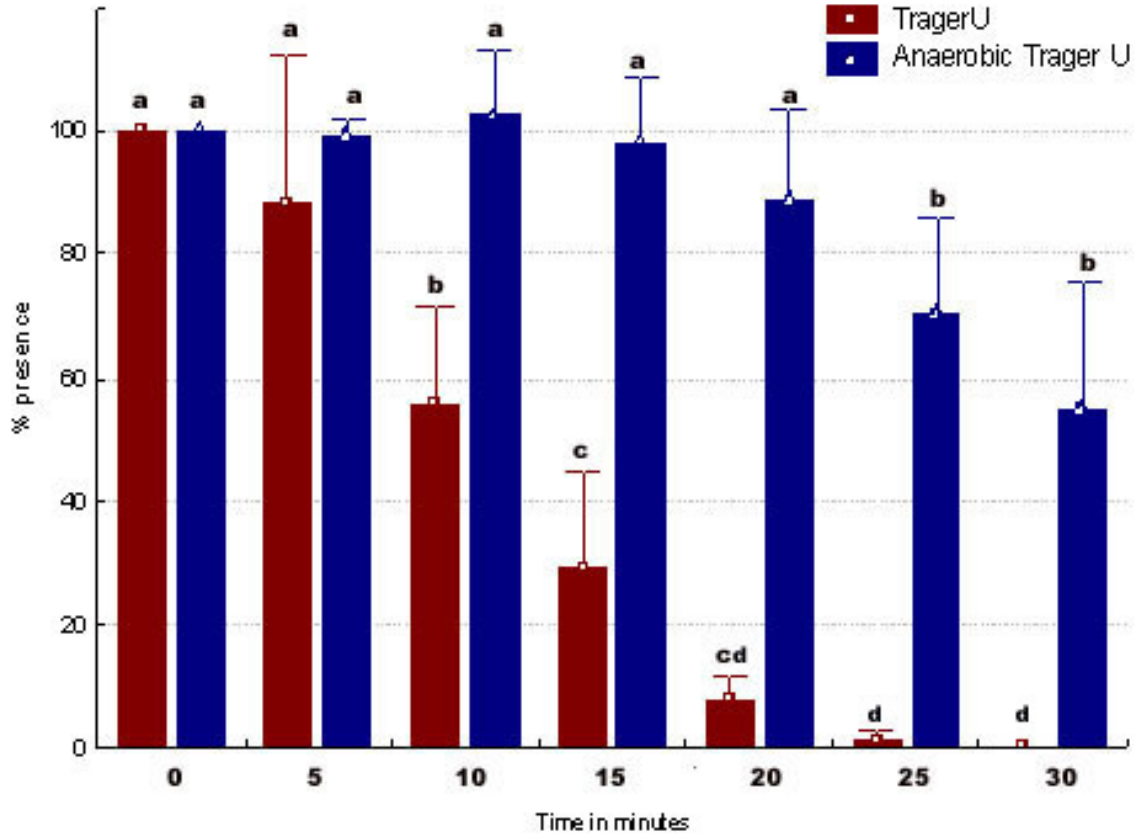

Figure 2.3. Average percent total protist population per termite individual in 80 µl Trager U and anaerobic Trager U saline solution over time. Means ± SD with the same letter were not significantly different over time using Mann Whitney U test ($p \leq 0.05$).

CHAPTER 3

A COMPARISON OF PROTIST COMMUNITIES FROM FOUR LIFE STAGES AND THREE SPECIES OF SUBTERRANEAN TERMITES (ISOPTERA: RHINOTERMITIDAE)[1]

[1] Lewis J.L. and B.T. Forschler. To be submitted to *Environmental Entomology*.

Introduction

Lower termites from the family *Rhinotermitidae* have obligate symbiotic protists (Oxymonadida, Trichomonadida, and Hypermastigida) that are important for proper digestion of cellulose and therefore termite health (Cleveland 1924, 1925a-c, 1928, Trager 1934). It is believed that the different protist species are involved in separate steps of cellulose digestion (Cleveland 1924, Hungate 1943, Mauldin et al. 1972, Smythe and Mauldin 1972, Lai et al. 1983, Yoshimura et al. 1993a, b, 1996), and that, without these protists, termite survival is compromised (Cleveland 1924, 1925a-c).

Hindgut protists from the eastern subterranean termite, *Termes flavipes* (now *Reticulitermes flavipes* Kollar) were first described by Leidy in 1877. In the past 70 years the taxonomic status of protist species from Rhinotermitid hosts has been described and revised several times, yet there are no published keys (Dubosq and Grassé 1928, Kirby 1924, Brown 1930a, b, Brown 1931). The taxonomic work has been driven by the belief that protist species are specific to certain subterranean termite species (Koidzumi 1921, Cleveland et al. 1934, Kirby 1937, Honigberg 1970) with each termite species having a characteristic flagellate community (Yamin 1979).

There are several thousand anaerobic protists in one *Reticulitermes* hindgut and several techniques have been used to observe them outside their insect host (Mannesmann 1969, 1970, Mauldin et al. 1981, Howard 1984, Cook and Gold 1998, 2000). The past use of disparate techniques to enumerate protist populations makes comparisons between studies difficult, and the lack of dichotomous keys for protist species discourages further inquiry.

The species composition of protist communities is similar within a caste of a particular termite species (Dropkin 1944, Mannesmann 1970, 1972, To et al. 1980) but can differ in

proportions between castes (Cleveland 1925a, Cook and Gold 1998). Two studies have compared protist communities within castes of *Reticulitermes flavipes* (Cleveland 1925a, Cook and Gold 1998); however no study has examined the protist communities between castes for *R. virginicus* (Banks) and *R. hageni* Banks. Further examination of the protist communities in subterranean termites will aid in illuminating the social and physiological aspects of this unique symbiosis. In this study we compared protist populations in the worker, soldier, nymph, and alate life stages of *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni*.

Materials and Methods

Insects

Reticulitermes flavipes, *R. virginicus*, and *R. hageni* were collected from Clarke County and Sapelo Island, Georgia. All were garnered from field sites with the exception of one *R. hageni* laboratory colony that was initiated in 1996 using alates. We sampled a total of six *R. flavipes*, five *R. virginicus*, and three *R. hageni* colonies. Once collected, termites were brought back to the laboratory and maintained in an environmental chamber at 24^{o} C in complete darkness. *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni* alates were collected following swarms in March, June, and August 2003, respectively. Termites were identified using dichotomous keys to the soldier and alate castes (Scheffrahn and Su 1994). The termite life stages examined in this study included alate, early stage nymphs (6-7 instar), workers, and soldiers (as identified in Buchli 1958). All termites included in the study were used within 24 hours of collection. The termites chosen for protist counts were randomly selected with the exception of worker termites where only those with dark brown, distended abdomens were used

to ensure they had not recently molted. The number of individuals examined within each termite caste varied according to availability per collection site.

Method of Protist Identification

Original species descriptions were used to identify the eleven protists described from *Reticulitermes flavipes* (Yamin 1979), six in *R. virginicus* (Cook and Gold 2000), and four in *R. hageni* (Yamin 1979). Original species descriptions were used to identify protists because there are no published keys for termite protists.

The protist species described from *R. flavipes* are *Dinenympha fimbriata* Kirby 1924, *D. gracilis* Leidy 1877, *Holomastigotes elongatum* Grassi 1917, *Microjoenia fallax* (Dubosq and Grassé 1928), *Monocercomonas* sp. Grassi 1917, *Pyrsonympha major* Powell 1928, *P. vertens* Leidy 1877, *Spironympha kofoidi* (Dubosq and Grassé 1928), *Spirotrichonympha flagellata* (Dubosq and Grassé 1928), *Spirotrichonympha* sp. (Mannesmann 1974 mentioned from *R. flavipes* but did not describe), *Trichomonas trypanoides* Dubosq and Grassé 1924, and *Trichonympha agilis* Leidy 1877.

Protists described from *R. virginicus* include: *Dinenympha fimbriata* Kirby 1924, *Holomastigotes elongatum* Grassi 1917, *Pyrsonympha minor* Powell 1928, *Spironympha kofoidi* (Dubosq and Grassé 1928), *Spirotrichonympha flagellata* (Dubosq and Grassé 1928), and *Trichonympha agilis* Leidy 1877.

Protist species have been described from the light southern subterranean termite, *R. hageni* are *Microjoenia pyriformis* Brown 1930, *Spironympha kofoidi* (Dubosq and Grassé 1928), *Spirotrichonympha gracilis* Brown 1930, and *S. pulchella* Brown 1930 (Brown 1930a, b).

Method of Counting Protists

Termite hindguts were removed from all castes by gently holding the termite by the thorax, and removing the last two abdominal segments using fine-tipped forceps and a gentle pulling motion. The contents from five individuals were pooled from each life stage of *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni*. Samples were homogenized for 15 seconds in a 1.5 milliliter microcentrifuge tube with Trager U saline solution (Trager 1934) pH 6.8-7.2 bubbled with a nitrogen gas mixture (N_2 92.5%, H_2 2.5%, and CO_2 5%) at 1 liter per minute for five minutes (Lewis and Forschler submitted for publication). Workers, nymphs, and soldiers were placed in 250 µl saline solution and alates placed in 60 µl saline solution. When the number of soldiers was less than five, we used one termite in 100 µl. Then 10 µl was loaded onto a cell counting chamber (Levy Counting Chamber) and the protists identified and counted from 4 µl for workers, nymphs, and soldiers, and 5 µl for alates. The time needed to make one count (identify and count the protist community from one termite sample) was less than 20 minutes. The resulting counts are then transformed to number of cells in one termite using the following formula: Number of cells counted * volume saline solution / volume counted cells from * number of termites

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA, $P \leq 0.05$). Variables analyzed were total protist population number and protist species proportions between life stage from each termite species and within workers between termite species. In case of significant differences, means were separated by Tukey's Honestly Significant Difference (HSD) test

($P \leq 0.05$). Statistical analyses were conducted using Statistica for Windows Package (StatSoft, version 6.0).

Results

Protist Species Identification

The flagellate species identified in *Reticulitermes flavipes* were *Dinenympha fimbriata*, *D. gracilis*, *Holomastigotes elongatum*, *Microjoenia fallax*, *Monocercomonas* sp., *Pyrsonympha major*, *P. vertens*, *Spironympha kofoidi*, *Spirotrichonympha flagellata*, *Trichomonas trypanoides*, and *Trichonympha agilis* (Figure 3.1). We could not find any unidentifiable *Spirotrichonympha* species as mentioned by Mannesmann (1974). All protist species described in *Reticulitermes flavipes* were present in workers, nymphs, and soldiers; however, we did not observe *Trichomonas trypanoides* in alates.

Protist species recognized from *Reticulitermes virginicus* were *Dinenympha fimbriata*, *Holomastigotes elongatum*, *Microjoenia* sp., *Monocercomonas* sp., *Pyrsonympha minor*, *Spironympha kofoidi*, *Spirotrichonympha flagellata*, and *Trichonympha agilis* (Figure 3.2). Each of these protist species were found in all termite life stages sampled. This is the first mention of the genera *Microjoenia* and *Monocercomonas* in *R. virginicus*.

Protist species in *Reticulitermes hageni* workers, nymphs, soldiers, and alates appeared qualitatively similar to those found in *R. virginicus* (Figure 3.3). We found *Spironympha kofoidi*, *Spirotrichonympha* sp., and *Microjoenia pyriformis* as described from *R hageni*, but we also report for the first time: *Dinenympha fimbriata*, *Holomastigotes elongatum*, *Pyrsonympha minor*, and *Trichonympha agilis* as described from *R.virginicus* and *Monocercomonas* sp. *Spironympha kofoidi* and *Monocercomonas* sp. were not found in alates.

Total Protist Population

Protist population estimates were greatest across all termite species in worker and nymphal termites, followed by the soldier and alate castes (figure 3.4). Workers and nymphs of *Reticulitermes flavipes* averaged three times more protists than in the other two termite species, with 58,369 ± 16,021 symbionts per individual. *R. flavipes* soldiers had 21,668 ± 6,394 which were equivalent to the numbers found in *R. hageni* workers, 20,779 ± 3,582 cells per termite and nymphs 18,875 ± 2,973. *R. hageni* soldiers had 12,771 ± 3,176 protists per termite. Total protist populations in *R. virginicus* averaged 13.970 ± 3,738 cells per termite workers and nymphs, while soldiers had 10,167 ± 6,056. Alate protist populations were significantly less than in the other termite life stages ($F=50.85$, $df=3, 215$; $p=0.000$), but not significantly different between species, with *Reticulitermes flavipes* averaging 1,854 ± 1,643, 1,505 ± 635 in *R. virginicus*, and 2,052 ± 532 in *R. hageni.*

Protist Proportions Between Termite Castes

Reticulitermes flavipes protist proportions between workers, nymphs, and soldiers were similar (Table 3.1), with 55.23 ± 12.93% of the protists being *Dinenympha gracilis*. There were smaller proportions in alates than the other life stages. Alates had 1.12 ± 2.18% D. *fimbriata* ($F=11.11$, $df=3, 95$; $p \leq 0.005$), 1.19 ± 1.60% *Holomastigotes elongatum* ($F=8.42$, $df=3, 95$; $p \leq 0.026$), 1.85 ± 6.01% *Microjoenia fallax* ($F=7.76$, df 3, 95; $p \leq 0.033$), and 1.49 ± 2.32% *Spirotrichonympha flagellata* ($F=8.02$, $df=3, 95$; $p=0.024$). Proportions of *Trichonympha agilis* were 33.47 ± 35.02% in the alate caste, which was higher than 3.67± 2.66% counted from workers, nymphs, and soldiers ($F=19.23$, $df=3, 95$; $p=0.000$). There were no differences in

Dinenympha gracilis, Monocercomonas sp., *Pyrsonympha major, Spironympha kofoidi,* and *Trichomonas trypanoides* between castes.

Counts from *Reticulitermes virginicus* were more variable between castes than recorded in the other two termite species (Table 3.2). *R. virginicus* had 6.06 ± 8.34% *Dinenympha fimbriata* in soldiers which was higher than the 1.63 ± 2.14% found in workers (F=8.47, df=3, 86; p=0.008) and the 0.11 ± 0.43% in alates (F=8.47, df=3, 86; p=0.000). There were no differences between caste percentages of *Holomastigotes elongatum*. The highest proportions of *Spirotrichonympha flagellata* were 31.15 ± 7.66% in workers and 25.43 ± 9.49% in soldiers, compared to 13.24 ± 5.18% in alates (F=34.01, df=3, 86; p=0.000). Alates had significantly greater proportions of *Microjoenia* sp. (27.34 ± 11.57%) than the 14.94 ± 3.84% in workers and 16.23 ± 8.75% in soldiers (F=13.69, df=3, 86; p=0.000). Alate proportions with 24.47 ± 11.01% *Monocercomonas* sp. (F=16.47, df=3, 86; p≤0.015) and 10.07 ± 4.41% *Spironympha kofoidi* (F=9.93, df=3, 86; p=0.000) were higher than the other life stages examined. Smaller proportions of 0.31 ± 1.00% *Pyrsonympha minor* were counted in alates than the 6.15 ± 6.95% in workers, with the largest proportion found in nymphs (16.16 ± 6.95%) and in soldiers (12.05 ± 5.29) (F=24.01, df=3, 86; p=0.000). *Trichonympha agilis* proportions of 19.43 ± 5.71% in workers were greater compared with 14.87 ± 6.36% in alates (F=4.65, df=3, 86; p=0.028) and 13.62 ± 6.27% in soldiers (F=4.65, df=3, 86; p=0.031).

Two *Spirotrichonympha* species, *S. gracilis* and *S. pulchella,* have been described in *R. hageni* and they differ in morphology of the axostyle and centroblepharoplast (Brown 1930b). Brown did not believe *Spirotrichonympha flagellata* was in *R. hageni* because the flagellar bands were not as deep or as closely wound around the anterior part of the body, the axostyle was not thick and fibrous, and the shape of the centroblepharoplast (*Spirotrichonympha* have a combined

centrosome and blepharoplast) was different from *S. gracilis* and *S. pulchella* (Brown 1930b). However, we could not tell the *Spirotrichonympha* species found in *R. hageni* apart from those in *R. flavipes* and *R. virginicus*.

Species proportions in *R. hageni* were similar between the worker, nymph, and soldier life stages, although there was a difference between the proportions in nymphs and soldiers of *Monocercomonas* sp. (Table 3.3). *Monocercomonas* sp. proportions were greater at 8.20 ± 2.38% in nymphs than the 1.53 ± 2.17% in workers (F=10.03, df=3, 26; p=0.003) and they were not found in alates. The percent presence of 28.38 ± 7.48% *Trichonympha agilis* was greater in alates than the other termite life stages examined (F=22.54, df=3, 26; p=0.000). *Dinenympha fimbriata* proportions of 40.24 ± 10.71% were greater than 20.47± 4.74% in nymphs (p=0.001) and 16.41 ± 12.06% in soldiers (F=7.07, df=3, 26; p=0.015). Percent presence of *Pyrsonympha minor* was 26.29 ± 14.79% and significantly greater than 4.64 ± 2.25% in alates (F=3.45, df=3, 26; p=0.050). *Spironympha kofoidi* were not present in alates and were significantly less than the 11.48 ± 5.89% found in soldiers (F=4.92, df=3, 26; p=0.005). *Holomastigotes elongatum*, *Microjoenia pyriformis*, and *Spirotrichonympha* sp. protist proportions were not different between termite life stages in *R. hageni.*

Protist species proportions in workers of *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni* were different between *Dinenympha gracilis* (F=7.07, df=3, 26; p=0.000), *D. fimbriata* (F=101.34, df=3, 26; p=0.000), *Spirotrichonympha* species (F=191.31, df=3, 26; p=0.000), and *Trichonympha agilis* (Table 3.4; F=114.01, df=3, 26; p=0.000). In *Reticulitermes flavipes*, *Dinenympha fimbriata* proportions were greatest in *R. hageni*, at 27.20 ± 9.03% but were significantly less (F=101.34, df=2, 84; p=0.000) at 7.91 ± 6.26% in *R. flavipes* and 1.63 ± 2.14% in *R. virginicus*. *Dinenympha gracilis* were 59.35 ± 12.11% of the total protist population but

they were not found in *R. virginicus* and *R. hageni*. The greatest proportions of *Spirotrichonympha* spp. were 31.15 ± 7.66% in *R. virginicus*, followed by 20.45 ± 6.02% in *R. hageni*, and 4.94 ± 2.93% in *R. flavipes* ($F=191.31$, $df=2, 84$; $p=0.000$). Workers of *Reticulitermes virginicus* had the highest percentage of *Trichonympha agilis* at 19.43 ± 5.71% compared to 4.15 ± 2.27% in *R. flavipes*, and 6.69 ± 5.43% in *R. hageni* ($F=114.01$, $df=2, 84$; $p \leq 0.000$).

Discussion

This was the first study to compare the protist communities found in subterranean termites from the southeastern United States. These studies illustrate the need for standardizing the taxonomy of termite protists and their potential in describing subterranean termite species using the most numerous life stage-workers.

The lack of a dichotomous key to the protist symbionts in subterranean termites requires reliance on protist species descriptions that were last revised over 60 years ago (Brown 1930a). Our attempts at protist species identification were hampered by the lack of a morphometric guide. For example, we could not distinguish *Spirotrichonympha flagellata* found in *Reticulitermes flavipes* and *R. virginicus* from *Spirotrichonympha gracilis* or *S. pulchella* in *R. hageni*. We also found that Cook and Gold (1999) identified their protist species based on Leidy (1877, 1881), Grassi and Sandias (1894), Koidzumi (1916, 1917), Kirby (1924), Dubosq and Grassé (1928), not the revision of Brown (1930a) who separated *Spirotrichonympha*, *Spironympha*, and *Microjoenia* into distinct genera. In addition, we found two protist species in *R. virginicus* and five in *R. hageni* that have never been described from those termites. Molecular

markers may aid in future studies of termite protists yet a visual, morphometric key would, invariably, encourage more enquiries into this important symbiotic relationship.

The study of subterranean termites is likewise hindered by the lack of morphometric dichotomous keys to the worker life stage. There are keys to the alate and soldier castes but the first is ephemeral and the second manifested in less than 2% of a subterranean termite population. The most numerous life stage, the worker (>80%), cannot be identified by published keys and using sentinel protist species could offer an alternative to identifying termite samples collected from field sites that do not contain either soldiers or alates.

Many of the symbiotic protists found in lower termites are specific to certain termite species and therefore represent distinct protist communities (Kirby 1937, Honigberg 1970). It has been hypothesized that this community might be used for termite identification (Brown 1930a, Dropkin 1944). There have been several studies looking at the protists in *Reticulitermes flavipes* (Cleveland 1924, 1925a-c, 1928, Mannesmann 1970, 1972, Cook and Gold 1998) and two in *R. virginicus* (Mannesmann 1972, Cook and Gold 2000). However no one has looked at *R. hageni* (Brown 1930) and no one has compared all three using a single, standardized, sample preparation and counting technique. Our results describe the potential that protist community proportions present for identification of termite species using the worker (Table 3.4). *Dinenympha gracilis* represented 59.35 ± 12.11% of the protist population in *Reticulitermes flavipes*. This protist species was not found in *R. hageni* and *R. virginicus*. Proportions of *Dinenympha fimbriata* in *R. flavipes* and *R. virginicus* were less than 10%, however in *R. hageni* they were 27.20 ± 9.03%. *R. virginicus* has a larger percentage of 31.15 ± 7.66% *Spirotrichonympha flagellata* followed by 19.43 ± 5.71% *Trichonympha agilis*. The protist proportions of *Dinenympha gracilis*, *Dinenympha fimbriata*, *Spirotrichonympha* spp, and *Trichonympha agilis* found in termite

workers could be used for separating the termite species *R. flavipes*, *R. hageni*, and *R. virginicus*, respectively.

Caste trends presented in this work concur and disagree with the published literature. Lai et al. (1983) compared protist numbers from *Coptotermes formosanus* life stages and found populations greatest in workers and nymphs, followed by alates, then soldiers. However, when examining total protist population estimates, Cook and Gold (1998) found workers had the highest protist population, followed by soldiers and alates – they did not examine nymphs. We found the same trend with protist populations in workers and nymphs being greater than soldiers, followed by alates (Figure 3.4). *Reticulitermes flavipes* generally had larger protist populations than *R. hageni* and *R. virginicus* (Figure 3.4). *Reticulitermes flavipes* workers had 58,369 ± 16,021 flagellates compared to 20,779 ± 3,582 in *R. hageni* and 14,017 ± 3,705 in *R. virginicus*. Our *Reticulitermes flavipes* symbionts population estimates were greater than protist populations previously reported in the literature. Previous counts from *R. flavipes* found protist populations ranging from 21,000 to 40,000 cells per termites with 40,083.33 ± 3,642.91 from Mannesmann (1969), 32,320 by Mauldin et al. (1981), 31,120 ± 8,404.57 by Howard (1984), and 14, 642 ± 3.395 in Cook and Gold (1999). The one study comparing protist populations in castes of *Reticulitermes flavipes* had 21,043.5 ± 8,293.5 in workers and 2,022.5 ± 869.5 in alates (Cook and Gold 1998), which was similar to our estimates. Yet, soldiers from their counts averaged 6,219 ± 2,004 while we found 21,668 ± 6,394 protists per termite (Figure 4). In *Reticulitermes virginicus*, protist populations reported from workers were 14,641.67 ± 5,879.66 from Cook and Gold (2000) and comparable to 14,017 ± 3,482 from our counts.

This study raises questions about the role played by the various protists in termite nutrition and how the protist communities carried by the alate caste are used to re-faunate the

emerging colony. Cleveland (1925a) hypothesized that the amount of raw wood eaten by the different castes should indicate which protists were most involved in cellulose digestion. Workers are the initial consumers of wood (McMahan 1969, Honigberg 1970) and feed the other life stages (Grassé and Noirot 1945, McMahan 1969, Noirot and Noirot-Timothee 1969). Early stage nymphs ingest raw wood (Cleveland 1925a) and soldiers rely on workers for proctodeal food (Cleveland 1925a). Using this rationale, early stage nymphs and workers should have similar protist communities. Soldiers rely on pre-digested wood supplied by workers and should have similar protist proportions compared to workers. The reproductive caste is fed stomodeal food from workers but can still ingest raw wood (Cleveland 1925a) suggesting a different protist community. Termite reproductives should have hindgut symbionts that include some cellulolytic representatives as well as other species involved in the later stages of cellulose degradation (Cleveland 1925a). We found that the protist populations of early stage nymphs and workers were not different nor did they differ in proportions of most protist species (Table 1, 2, and 3). Our findings did not agree with Cleveland (1925a) because protist proportions in soldiers were similar to those in workers and nymphs, although they had significantly fewer. The reproductive caste of *Reticulitermes flavipes* had a different protist community, with larger proportions of *Trichonympha agilis* and less *Dinenympha fimbriata* than the other termite life stages. *Reticulitermes hageni* alates also had a higher proportion of *Trichonympha agilis* than the other termite life stages we examined (Table 3.3). These trends were also seen between life stages of *Reticulitermes flavipes* by Cook and Gold (1998), with nymphs, workers, and soldiers having similar protist proportions, yet soldiers had smaller total protist populations. In addition, alates had significantly more *Trichonympha agilis* and less *Dinenympha fimbriata* than other castes (Cook and Gold 1998).

In conclusion these studies illustrate the need for a revision of the taxonomic status of termite protists. It suggests the likelihood of using protist species proportions for identifying termite species using the worker caste and calls for a re-evaluation of the role played by the various protist symbionts found in subterranean termites.

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Table 3.1. Average percent protist species presence between life stages of *Reticulitermes flavipes* (Kollar).

	R. flavipes worker	*R. flavipes* nymph	*R. flavipes* soldier	*R. flavipes* alate
DF[1]	7.91± 6.26a[2]	12.00±7.80a	9.91±6.71a	1.12±2.18b
DG	59.35±12.11a	52.08±12.30a	51.09±13.09a	51.4±26.14a
HE	3.18±1.21a	2.68±1.34a	3.20±1.94a	1.19±1.60b
MG	9.18±6.8a	12.62±9.68a	12.77±10.05a	1.85±6.01b
Mono	0.87±1.12a	0.12±0.27a	0.56±1.12a	0.35±1.26a
PMA	2.01±1.74a	2.33±1.96a	3.13±2.72a	1.71±2.38a
PMI	0a	0a	0a	0a
PV	4.95±2.34a	5.25±2.48ab	8.72±4.14b	6.73±6.41ab
SK	2.99±1.68a	2.03±1.38a	2.46±1.46a	1.90±2.96a
SF	4.94±2.9.a	5.69±2.36a	5.10±3.78a	1.49±2.23b
TA	4.15±2.27a	4.70±3.26a	2.26±a2.26	33.47±35.02b
TT	0.47±0.88a	0.58±0.64a	0.80±1.81a	0a

[1] is DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PMA= *Pyrsonympha major*, PMI= *P. minor*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*.

[2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 3.2. Average percent protist species presence between life stages of *Reticulitermes virginicus* (Banks).

	R. virginicus worker	*R. virginicus* nymph	*R. virginicus* soldier	*R. virginicus* alate
DF[1]	1.63±2.14a[2]	3.30±3.67ab	6.06±b	0.11±0.43a
DG	0a	0a	0a	0a
HE	9.65±3.78a	5.15±1.93a	6.40±4.41a	9.59±5.10a
MG	14.94±3.84a	18.13±ab	16.23±8.75a	27.34±11.56b
Mono	11.095.23±a	3.39±2.95a	15.28±11.91a	24.46±11.01b
PMA	0a	0a	0a	0a
PMI	6.15±6.95a	16.16±9.88b	12.05±5.29b	0.31±1.00c
PV	0a	0a	0a	0a
SK	5.96±3.62a	5.24±1.95a	4.93±3.04a	10.07±4.41b
SF	31.15±7.66a	22.03±5.52ab	25.43±9.49a	13.24±5.18b
TA	19.43±5.71a	16.98±8.16ab	13.62±6.27b	14.87±6.36b
TT	0a	0a	0a	0a

[1] is DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PMA= *Pyrsonympha major*, PMI= *P. minor*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*.

[2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 3.3. Average percent protist species proportions between life stages of *Reticulitermes hageni* Banks.

	R. hageni worker	*R. hageni* nymph	*R. hageni* soldier	*R. hageni* alate
DF[1]	27.20±9.03ab[2]	20.47±4.74a	16.41±12.06a	40.24±10.71b
DG	0a	0a	0a	0a
HE	1.93±2.97a	4.14±1.99a	3.41±3.45a	0.16±0.40a
MG	10.38±3.77a	7.30±3.32a	17.20±8.59a	9.55±7.27a
Mono	1.53±2.17a	8.20±2.38b	4.23±4.69ab	0a
PMA	0a	0a	0a	0a
PMI	26.29±14.79a	19.37±4.27ab	19.72±4.27ab	4.65±2.25b
PV	0.00a	0.00a	0.00a	0.00a
SK	5.54±3.29ab	8.04±7.77ab	11.48±8.59a	0b
SF	20.45±6.02a	28.14±8.22a	20.75±12.35a	17.02±10.71a
TA	6.69±5.43a	4.34±2.86a	6.78±7.04a	28.38±7.48b
TT	0a	0a	0a	0a

[1] is DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PMA= *Pyrsonympha major*, PMI= *P. minor*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*.

[2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 3.4. Protist species proportions from the worker life stage for use in termite identification.

	R. flavipes	***R. virginicus***	***R. hageni***
Dinenympha fimbriata	7.91±6.26a[1]	1.63±2.14b	**27.20±9.03c**
Dinenympha gracilis	**59.35±12.11a**	0.0±0b	0.0±0b
Spirotrichonympha species	4.94±2.93a	31.15±7.66c	20.45±6.02b
Trichonympha agilis	4.15±2.27a	**19.43±5.71b**	6.69±5.43a

[1] Mean ± SD within a row followed by the same letter are not significantly different at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test. Bold type represents potential sentinel species.

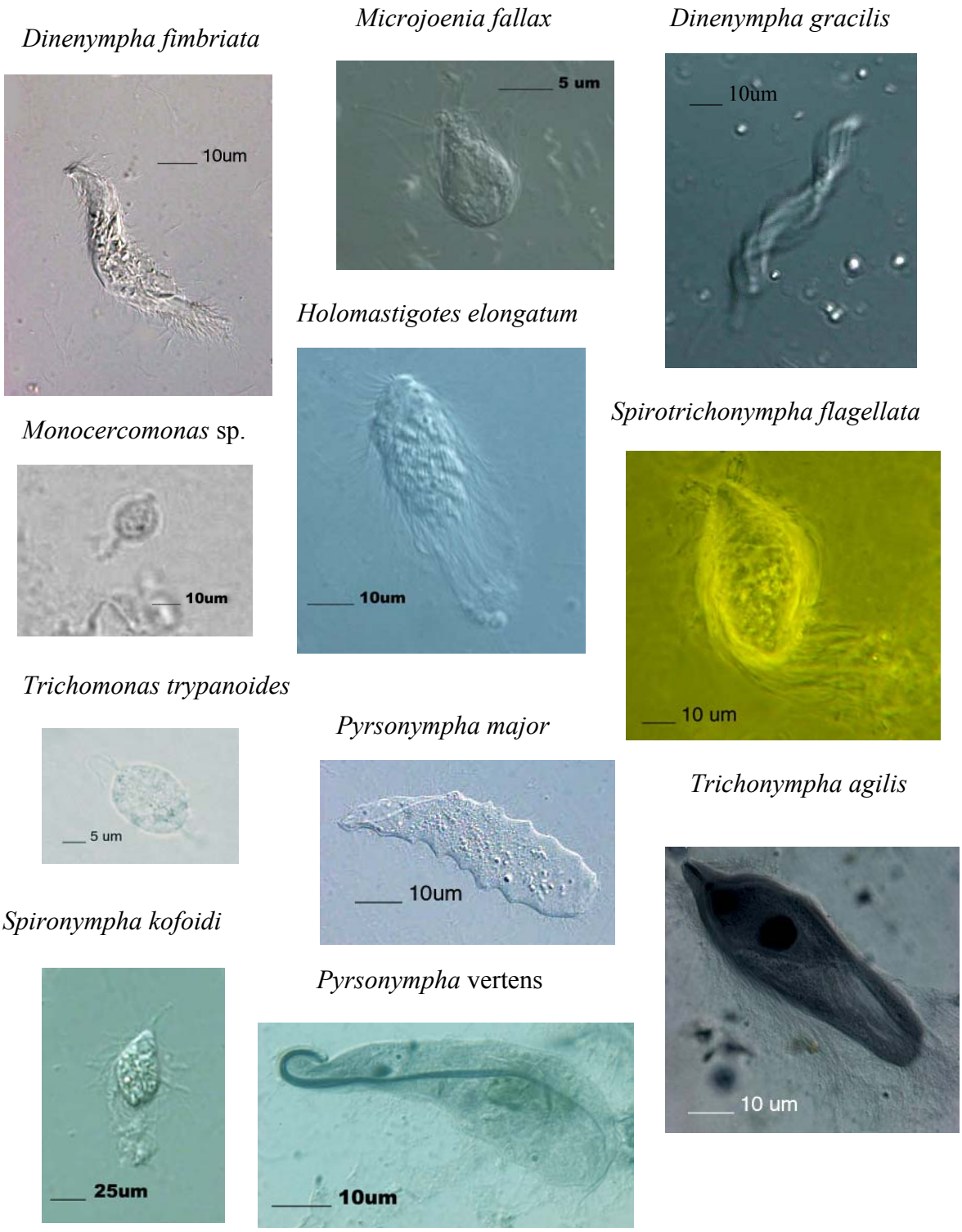


Figure 3.1. Protist Species in *Reticulitermes flavipes* (Kollar). (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera).

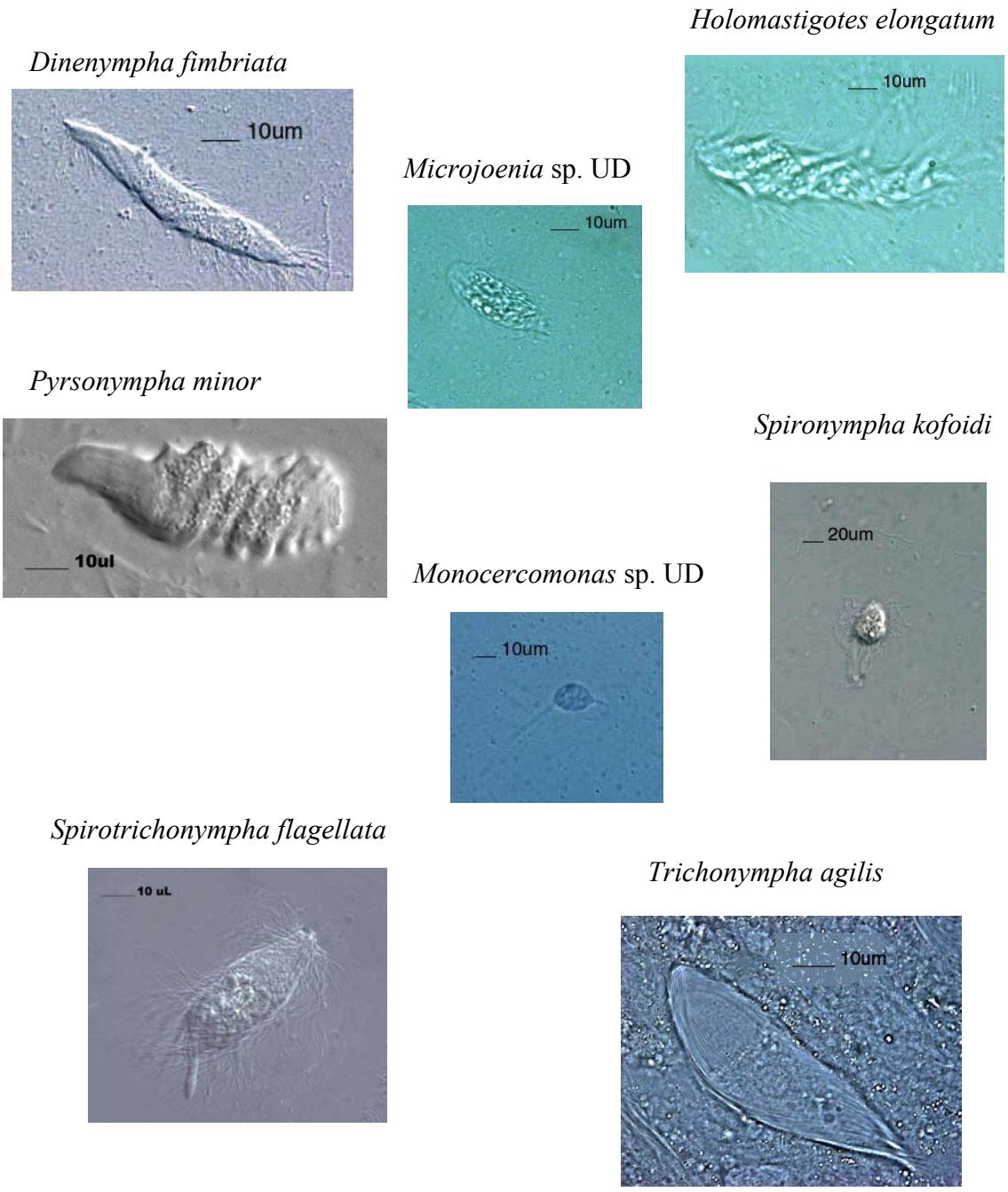
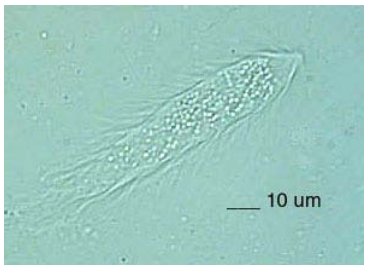


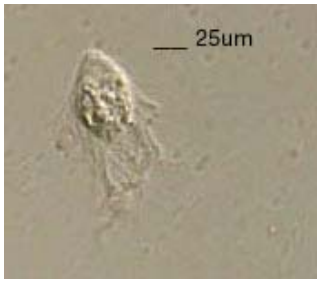
Figure 3.2. Protist species in *Reticulitermes virginicus* (Banks). (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera). UD are those protists previously undescribed from this termite species.

Microjoenia pyriformis

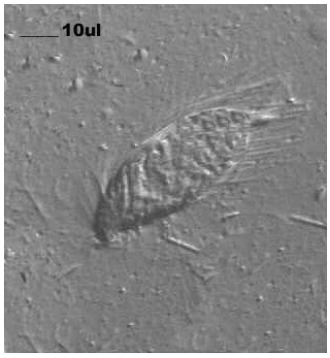
Holomastigotes elongatum UD



Spironympha kofoidi



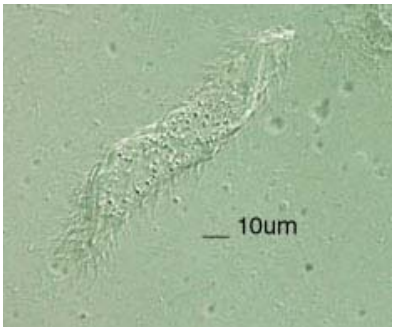
Spirotrichonympha sp.



Monocercomonas sp. UD

Pyrsonympha minor UD

Dinenympha fimbriata UD



Trichonympha agilis UD

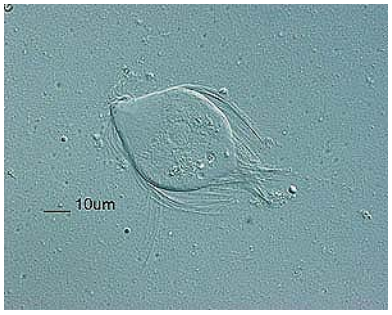


Figure 3.3. Protist species in *Reticulitermes hageni* Banks. (Photos taken using a Leica microscope at 100X magnification and images acquired using an AxioCam digital camera).

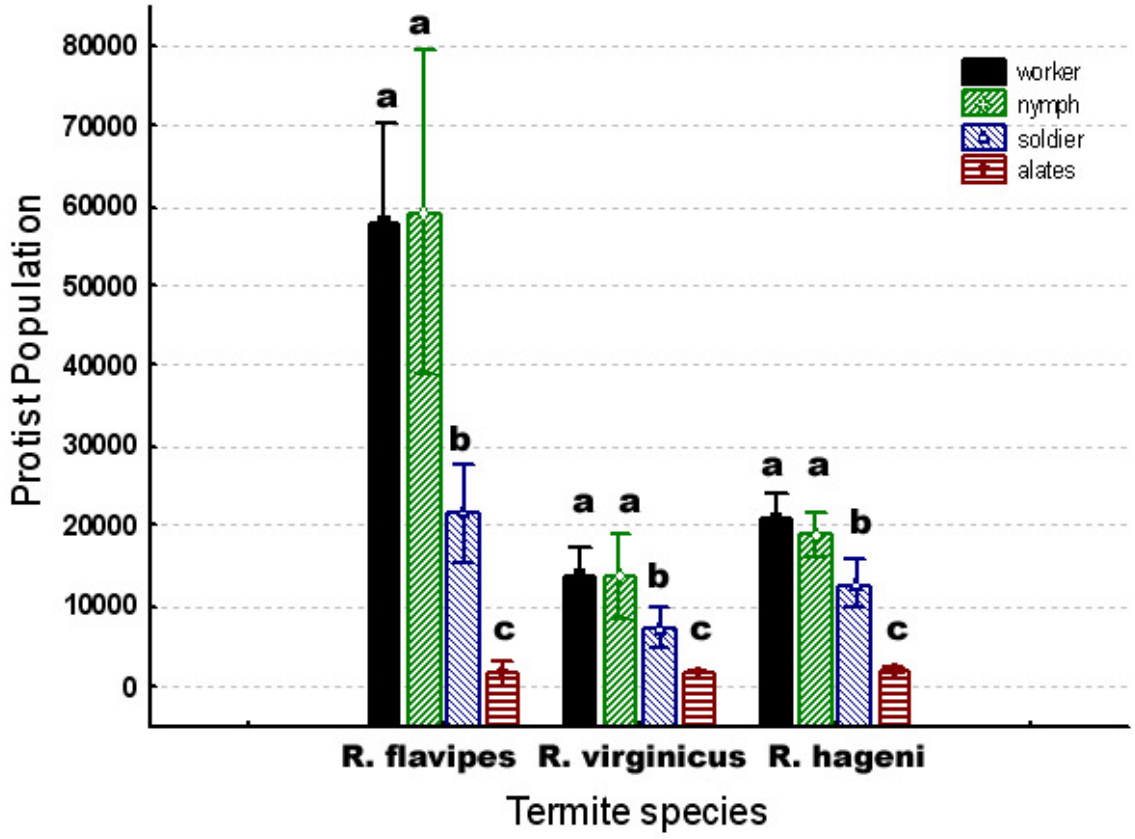


Figure 3.4. Average total protist population in life stages of *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni*. Means followed by the same letter are not significantly different from each other within termite species at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

CHAPTER 4

THE EFFECTS OF DIFFERENT FOOD SUBSTRATES ON HINDGUT PROTIST COMMUNITIES IN *RETICULITERMES FLAVIPES* (ISOPTERA: RHINOTERMITIDAE)[1]

[1] Lewis J.L. and B.T. Forschler. To be submitted to *Environmental Entomology*.

Introduction

Measures of vigor commonly used to determine the health of a termite colony include percent survival and consumption rates obtained during bioassay (Su and LaFage 1984, Delaplane and LaFage 1987, Lenz 1994, Cornelius and Osbrink 2001, Morales-Ramos and Rojas 2001). However, termites placed in laboratory experiments can, on occasion, exhibit low survivorship and variable consumption rates (Smythe and Mauldin 1972, Thorne 1998, Morales-Ramos and Rojas 2001). A reliable measure of termite vigor would be useful in determining which termite colonies are fit enough to survive experimental manipulation prior to, rather than during or after, bioassay.

Termites from the families Hodotermitidae, Termopsidae, Mastotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae ingest wood fragments, assimilating nutrients critical to the health of the individual termite, and by extension, the nestmates of these social insects (Cleveland 1925a, Andrew 1930, Grassé and Noirot 1945, Yamaoka et al. 1986). There is limited xylan (Azuma et al. 1993, Inoue et al. 1997) and lignin digestion in termites (Kuhnick et al. 1994, Brune et al. 1995) and these constituents of wood do not seem to contribute to termite nutrition (Esenther and Kirk 1974, Cookson 1987). Termites macerate wood, mixing it with salivary gland secretions that include cellulases (Watanabe et al. 1997, Nakashima et al. 2002) that begin to hydrolyze cellulose (Inoue et al. 1997). Soluble nutrients such as glucose are absorbed through the midgut, however it has been estimated that about 70 percent of cellulose digestion and absorption takes place in the dilated portion of the termite hindgut, or paunch (Inoue et al. 1997). The paunch is filled with anaerobic protists, which aid in digestion and create a unique system important for termite health (Cleveland 1924, 1925a-c, 1928, Trager 1934,

Hungate 1938, 1939). The obligate protists present in the hindgut are represented from three orders: Trichomonadida, Oxymonadida, and Hypermastigida.

The protists described from the eastern subterranean termite, *Reticultermes flavipes* (Kollar) include eleven species (Yamin 1979) and many of these protists are believed to be involved in different stages of cellulose metabolism (Cleveland 1924, Hungate 1943, Mauldin et al. 1972, Smythe and Mauldin 1972, Lai et al. 1983, Yoshimura et al. 1993a, b, 1996). Previous research has indicated that several protist species are important in termite cellulose digestion, while other species may play a minimum role in digestion (Cleveland 1925a, Smythe and Mauldin 1972, Grosovsky and Margulis 1982). It has been hypothesized, although never tested experimentally, that termite protist populations could be used as an indicator of termite colony vigor if the health of the termite were indicated by the composition of the protist community.

We were interested in determining if counts of protist communities found in subterranean termites could be used as a predictive measurement of termite colony vigor. Towards this end, we describe a series of experiments using a standardized protist counting protocol and several "traditional" measures of colony vigor. We hypothesized there is a correlation between protist populations and termite worker live weights and feeding and survival rates.

Materials and Methods

Termites

Three colonies of *Reticulitermes flavipes* were collected from Whitehall Forest, Clarke County, Georgia and brought back to the laboratory intact within their food source. Termites were identified to species using published keys to the soldier caste (Scheffrahn and Su. 1994). Termites were extracted from their food source and used in bioassay within 24 hours of

collection from the field. Colonies 1, 2, and 3 were collected 25 March 2003, 29 April 2003, and 3 June 2003, respectively.

Force Feeding Experiment

Groups of 100 worker termites, fourth instar or older, were kept in plastic Petri dishes (100X25mm) with 10 grams of sand moistened with 2-ml of distilled water and placed inside an environmental chamber in complete darkness maintained at 24°C. There were four treatments that included: starvation - no food, cellulose powder - α-cellulose (Whitmire-Microgen Research Labs), pine blocks - blocks of weathered pine (1-cm^3), or sawdust/pine - weathered, mixed pine and hardwood sawdust with weathered pine blocks (1-cm^3). In those treatments using cellulose powder and sawdust/pine, two grams of the appropriate substrate were packed into Tygon® tubing (5/8 ID X 20mm) with one end covered by Parafilm® that served as a base for the vessel. Placement of the loose substrate into the Tygon® vessel served to aid in recovery of the food substrate for determination of amounts consumed. The pine blocks treatments included 6 pine blocks (1-cm^3) as the food substrate. In the sawdust/pine treatment, three pine wood blocks (1-cm^3) were added to a Petri dish containing the aforementioned sawdust filled Tygon® vessel. Termites placed in the starvation treatment were provided with two empty Tygon® tubes. Pine blocks and sawdust/pine were oven-dried for 48 hours at 80°C and cellulose powder air dried for 5 days and weighed before and after inclusion in bioassay.

From colony 1, there were three replicates with termites and three replicates without termites, which served to provide a correction factor for the handling, drying, and weighing of food treatments including cellulose powder, sawdust/pine, and pine blocks. There were a total of 36 Petri dishes per food treatment and 18 Petri dishes for starved termites. Because low numbers

of termites were collected from colonies 2 and 3, each colony was provided a single replicate for a total of 12 Petri dishes per treatment.

Food substrates were weighed before being placed inside the Petri dish, and then removed every sample period, cleaned, dried, and reweighed. Fresh substrates, including empty Tygon® tubes in the starvation treatment, were replaced every sampling period from all replicates to ensure that food was not a limiting factor.

Total protist populations and protist species proportions per termite individual were examined within 24 hours of termite collections. Termite survivorship, termite food removal rates, mean termite worker weights, termite running speeds, and total protist populations and protist species proportions per termite individual from each treatments were taken at one, two, four, six, nine, and twelve weeks after initiation of the study.

Measures of Termite Vigor

Termite survivorship was determined by counting the number of live termites on each respective sample date from the Petri dishes being sampled. In addition, food removal rates were determined by obtaining a mean weight change for the food substrates held in bioassay without termites and adding or subtracting, as appropriate, the average weights obtained from each replicate with termites. The difference in weight without and with termites was considered the amount of food removed. Average termite weights were obtained by weighing live termites randomly selected in five groups of ten individuals for a given sample date. Termites were also timed using a stopwatch to see how long it took for one individual to run along a 6-centimeter line using a Papermate® ballpoint pen. This was replicated ten times with a fresh ink line drawn each time (as described by Arquette and Forschler, unpublished). Termites use a trail pheromone

to foraging for food, and it has been shown that ink solvent in ballpoint pens is very similar to this pheromone and termites will follow it (Chen and Henderson 1997).

Method of Protist Identification

Original and revised species descriptions were used to identify the eleven protists described from *Reticulitermes flavipes* (Leidy in 1877, 1881 Koidzumi 1916, 1917, Grassi 1917, Kirby 1924, Dubosq and Grassé 1924, 1928, Powell 1928, Brown 1930a, 1930b, Brown 1931). They are *Dinenympha fimbriata* Kirby, *D. gracilis* Leidy, *Holomastigotes elongatum* Grassi, *Microjoenia fallax* (Dubosq and Grassé), *Monocercomonas* sp. Grassi, *Pyrsonympha major* Powell, *P. vertens* Leidy, *Spironympha kofoidi* (Dubosq and Grassé), *Spirotrichonympha flagellata* (Dubosq and Grassé), *Trichomonas trypanoides* Dubosq and Grassé, and *Trichonympha agilis* Leidy.

Measurements of Protist Populations

Forceps were used to remove the last two abdominal segments from individual worker termites, extracting the whole alimentary canal, which was placed in a microcentrifuge tube with 250 µl Trager U saline solution (pH 7.1) previously bubbled with a nitrogen gas mixture (N_2 92.5%, H_2 2.5%, and CO_2 5%) at 1 liter per minute for 5 minutes. Only worker termites with dark brown, distended abdomens were used to ensure they had not recently molted. The contents of five termite guts were homogenized for 10 seconds and 10 µl were removed and loaded onto a hemocytometer counting chamber. All protists were counted and identified from 0.4 µl (4 large squares) using a phase-contrast microscope. Protist counts were replicated five times from each sample period/food substrate combination and these were always conducted within 24 hours of

the sample date, for a total of seventy-five termites sampled per treatment per sampling date. Resulting counts were transformed to the number of cells per termite using the following formula: Number of cells counted * volume saline solution / volume counted * number of termites.

Statistical Analysis

To determine if the protist community can be used as a measure for health in termite colonies, data analyzed were average termite weights, percent termite survivorship, food removal rates, termite running speeds, total protist populations, and protist species proportions within and between treatments analyzed using one-way analysis of variance (ANOVA, $P \leq 0.05$). In case of significant differences, means were separated by Tukey's Honestly Significant Difference (HSD) test ($P \leq 0.05$). Statistical analyses were conducted using Statistica for Windows Package (StatSoft's, version 6.0).

Results

Percent Termite Survivorship

Percent termite survivorships decreased in sawdust/pine ($F=4.92$, $df=6, 23$, $p=0.002$), pine blocks ($F=4.18$, $df=7, 24$, $p=0.004$), cellulose powder ($F=10.01$, $df=6, 24$, $p=0.000$), and starved ($F=15.56$, $df=3, 15$, $p=0.000$) treatments over time (Table 4.1). Sawdust/pine treatments had significantly fewer survivors at 12 weeks, with 40.00 ± 8.48% of the termites still living ($p=0.006$). Termites in the pine block treatments decreased to 83.20 ± 10.06%termites alive after 6 weeks ($p=0.023$). Cellulose powder treatments decreased termite survivorship to 82.00 ±

15.26% after 4 weeks (p=0.040). Starved termites had significantly lower survival rates, with 64.40 ± 30.10% (p=0.027) after two weeks.

Termites assumed to be healthy enough for manipulation in bioassay, (≥80% survivorships) were taken at for 2 weeks in starved termites, 4 weeks for sawdust/pine and cellulose powder, and 9 weeks for pine block treatments (Table 4.1). Greater termite survivorships were observed in sawdust/pine, pine blocks, and cellulose powder treatments, averaging 86.87 ± 9.55% as compared to only 24.25 ± 15.69% survival in starved termites following 4 weeks.

Food Removal Rate

The amount of food removed between termites in pine block and cellulose powder treatments were not significantly different over time, and had an average of 32.22 ± 15.88 milligrams of substrate removed per gram of termite per day (mg/g/day) (Table 4.2). Termites in sawdust/pine treatments had an average food removal rate of 142.18 ± 26.26 mg/g/day after week 1 and decreased to 87.43 ± 12.53 mg/g/day at 4 weeks (p=0.014)

The amount of food removed by termites in sawdust/pine treatments averaged 105.75 ± 33.67 mg/g/day and were greater than 35.30 ± 16.32 mg/g/day in pine blocks and 30.04 ± 17.03 mg/g/day in cellulose powder over time (p≤0.045), except at week 9 when there were no differences observed between food removal rate in sawdust/pine and pine blocks treatments (Table 4.2).

Average Termite Weight

Field collected termites weighed an average of 3.04 ± 0.25 milligrams (mg) per individual (Table 4.3), and sawdust/pine ($F=11.55$, $df= 6, 127$; $p=0.000$), pine block ($F=9.76$, $df= 6, 143$; $p=0.000$) and cellulose powder ($F=10.58$, $df= 6, 139$; $p=0.000$) treatments showed a trend of increasing weight over time. The average termite weight increased after one week in sawdust/pine and cellulose powder groups from 3.04 ± 0.25 mg to 3.56 ± 0.45 mg ($p=0.000$) and 3.59 ± 0.30 mg ($p=0.006$), respectively. Termites fed pine block increased weight from 3.04 ± 0.25 mg to 3.48 ± 0.58 mg after 4 weeks ($p=0.020$). Starved termites decreased in weight after 4 weeks to 2.45 ± 0.52 mg ($p\leq0.010$). Average termite weight was 3.77 ± 0.26 mg from termites fed sawdust/pine and tended to weigh more than 2.69 ± 0.43 mg in starved termites for 4 weeks ($p=0.000$).

Termite Running Speeds

Termites collected from the field ran along a fresh 6 centimeter ink line in an average of 4.22 ± 1.18 seconds (s) (Table 4.4). Running speeds of termites from sawdust/pine and pine block treatments tended not to be different between sampling dates. Termites fed cellulose powder had significantly slower running speeds of 5.93 ± 2.72 s after 9 weeks ($p=0.025$). Starved termites ran 5.74 ± 2.62 s/6cm after 2 weeks and were significantly slower than 4.22 ± 1.18 s on day 1 ($p=0.045$), but were not different from 5.43 ± 2.66 s on week 1 and 5.59 ± 2.26 s on week 4.

Termites fed with pine blocks had an average running speed of 4.05 ± 1.15 s throughout the sampling period and were faster than 5.69 ± 2.56 s in cellulose powder at each sampling date ($p\leq0.005$), except for week 2.

Total Protist Populations

All protist species described from the eastern subterranean termite, *Reticulitermes flavipes* (Yamin 1979) were found from the three termite colonies sampled, except *Trichomonas trypanoides*, which was found only in one of the colonies. The average hindgut protist population decreased significantly per termite in those fed sawdust/pine ($F=10.01$, $df=6, 143$; $p=0.000$), pine blocks ($F=32.00$, $df=7, 165$; $p=0.000$), or cellulose powder ($F=31.18$, $df=6, 143$; $p=0.000$), and starved termites ($F=379.54$, $df=3, 91$; $p=0.000$) over time (Table 4.5).

Protist populations in termites collected from the field averaged 72,635.00 ± 17,279.60 per termite and populations decreased to 48,155.00 ± 8,425.24 in termites fed sawdust/pine, 27,830.00 ± 15,207.54 in those fed on pine blocks, and 3,990.00 ± 3,456.56 in starved termite treatments following 1 week in captivity (Table 4.5). Protist populations from termites in the sawdust/pine treatments varied over time, but following 12 weeks in bioassay, were not different from estimates from field collected termite protist counts. Protist populations from termites fed only cellulose powder were somewhat similar for 2 weeks, then decreased by 70 percent to 19,190.00 ± 20,401.64 after 4 weeks ($p=0.000$) and remained low for the other sampling dates. Protist populations in starved termites decreased by over 90 percent, to 3,990.00 ± 3,456.56 after 1 week, and after 4 weeks all protists were eliminated.

Over time, protist counts from termites in the sawdust/pine treatments were significantly greater ($p\leq0.001$), than those from termites in pine block treatments. Termites in sawdust/pine treatments also showed significantly larger protist populations than those termites with cellulose powder, but only at weeks 4, 6, and 12 (Table 4.5; $p\leq0.001$).

Termite Protist Species Proportions Within Treatments

Protist communities in termites fed sawdust/pine, pine blocks, and cellulose powder treatments had some fluctuations in proportions over time, but tended not to change after 12 weeks (Tables 4.6, 4.7, and 4.8). In contrast, starved termites had several protist species proportions change after 1 week (Table 4.9).

Protist species from termites fed sawdust/pine did not change in proportions over time were *H. elongatum*, *Monocercomonas* sp, and *Trichomonas trypanoides* (Table 4.6). *Dinenympha fimbriata* proportions were 9.86 ± 4.50% at week 1 and decreased to 7.17 ± 2.41% at week 4 ($p= 0.046$), but were not different the following sampling dates. *Dinenympha gracilis* proportions were 64.46 ± 3.87% on day 1 ($p=0.039$) and 66.67 ± 5.15% at week 2 ($p=0.000$) and decreased to 59.2 ± 9.05% after 6 weeks. *Microjoenia fallax* proportions were 5.60 ± 2.87% on week 1 ($p=0.005$) and increased to 8.67 ± 2.66% after 4 weeks, then decreased to 4.09 ± 2.42% on week 12 ($p=0.010$). *Pyrsonympha major* proportions increased from 1.26 ± 0.94% on day 1 to an average of 2.98 ± 1.39% during weeks 1 through 6 ($p< 0.017$). Proportions on day 1 of *P. vertens* were 5.68 ± 2.00%, and decreased to 4.14 ± 1.53% during week 2 ($p=0.019$) and 4.21 ± 1.36% by week 4 ($p=0.031$) and were not different at weeks 6, 9, and 12. Protist proportions of *S. kofoidi* were 2.11 ± 1.45% at week 1, and decreased to 1.26 ± 0.60% at week 2 ($p=0.019$). *Spirotrichonympha flagellata* proportions increased from an average of 4.88 ± 2.04% during day 1 through week 6, to 8.47 ± 2.79% on weeks 9 and 12 ($p< 0.020$). *Trichonympha agilis* proportions decreased from 3.48 ± 1.59% at week 1 to 1.37 ± 1.22% after 12 weeks ($p=0.024$), but were not different from other sample dates.

Microjoenia fallax, Spironympha kofoidi, *S. flagellata*, and *Trichomonas trypanoides*, did not change in proportions over time in pine block fed termites (Table 4.7). *Dinenympha*

fimbriata averaged 9.14 ± 4.04% on day 1 through week 6, and increased by 40 percent to 14.76 ± 3.34% at 9 weeks ($p < 0.014$), but were not different at week 12 (8.18 ± 2.08%). *Dinenympha gracilis* proportions decreased from 64.46 ± 3.87% on day 1 to 55.75 ± 9.16% after 1 week ($p=0.003$). *Holomastigotes elongatum* proportions increased from 3.20 ± 1.17% on day 1 to 5.08 ± 2.01% after 1 week ($p=0.004$), but were not different thereafter. Proportions of *Monocercomonas* sp. on day 1 were 0.69 ± 1.1% and did not change over time for 9 weeks, then increased to 3.00 ± 2.60% at 12 weeks ($p \leq 0.000$). Proportions of *P. major* were 1.26 ± 0.94% on day 1 ($p=0.007$) and 1.33 ± 1.20% at week 2 ($p=0.014$) and increased to 2.61 ±1.80% at 4 weeks. *Pyrsonympha vertens* increased in proportion from 5.22 ± 2.32% at week 4 to 7.49 ± 4.20% at week 6 ($p=0.037$). There were no differences in proportions of *T. agilis* over time except that percent presence increased from 2.29 ± 1.57% on day 1 to 4.57 ± 2.40% at week 1 ($p=0.003$).

Termites fed cellulose powder did not change proportions of *P. vertens, S. flagellata*, and *Trichomonas trypanoides* over time (Table 4.8). The percent presence of *D. fimbriata* was 8.48 ± 3.28% at day 1and decreased by 60 percent (to 3.43 ± 2.28%) at week 4 ($p=0.000$). *Dinenympha gracilis* proportions averaged 65.83 ± 4.76% on day 1 and weeks 1 and 2, which was significantly greater than 53.02 ± 21.41% at week 4 ($p < 0.001$), but were not different from later sampling dates. *Holomastigotes elongatum* proportions decreased from 3.2 ±1.17% at day 1 to 1.88 ± 0.72% after 1 week ($p \leq 0.033$). *Microjoenia fallax* were 7.26 ± 4.60% on day 1 and increased by about 50 percent (to 15.04 ± 10.67%) at week 4 ($p=0.000$) and (15.48 ± 4.35%) at week 6 ($p=0.000$). Proportion of *Monocercomonas* sp. averaged 0.63 ± 0.91% on day 1 through week 2 and increased to 9.35 ± 22.18% at week 4 ($p \leq 0.013$), then decreased to an average of 0.66 ± 0.81% during weeks 6 and 9 ($p \leq 0.016$). *Pyrsonympha major* increased in proportion from 1.26 ± 0.94% on day 1 to 0.42 ± 0.35% at week 9 ($p=0.048$). Proportions of *S. kofoidi* at day 1

were 1.59 ± 1.00%, and were greater than an average of 0.67 ± 0.85% from weeks 2 through 9. *Trichonympha agilis* had similar proportions throughout the sampling period, averaging 2.19 ± 1.61%.

Termite starvation had the greatest effect on protist populations, compared to sawdust/pine, pine blocks, and cellulose powder treatments (Table 4.6-4.9). Proportions of *D. gracilis*, *P. vertens*, *S. flagellata*, and *T. agilis* decreased significantly during the 1st week (Table 4.9; $p<0.000$). *Dinenympha gracilis* proportions were 64.46 ± 3.87% on day 1 and decreased to 9.50 ± 9.89% after 1 week ($p<0.000$). Protist populations on day 1, of *P. vertens* were 5.68 ± 1.95%, *S. flagellata* 4.45 ± 2.15%, and *T. agilis* 2.29 ± 1.60% and were eliminated after 1 week (Table 4.9; $p=0.000$). Proportions of *H. elongatum* increased from 3.20 ± 1.19% on day 1 to 12.94 ± 10.64% 1 week later ($p=0.040$), but were not different thereafter. *Microjoenia fallax* increased from 7.26 ± 4.60% of the protist population on day 1 to 54.16 ± 23.94% at week 1 and 76.07 ± 24.60% at week 2 ($p<0.000$). *Monocercomonas* sp. at day 1 had proportions of 0.69 ± 1.09% and increased to 12.87 ±13.63% by week 1 ($p=0.014$) and 50.00 ± 70.71% on week 4 ($p<0.003$). *Dinenympha fimbriata*, *P. major*, *S. kofoidi,* and *Trichomonas trypanoides* proportions did not change over time.

Termite Protist Species Proportions Between Treatments

Protist species proportions had some significant differences between treatments, but there did not seem to be an overall pattern over time (Table 4.10-4.20). *Trichonympha agilis* constituted a larger proportion of the protist population in termites fed pine blocks (3.56 ± 2.13%), then in termites from the sawdust/pine treatments (2.01 ± 1.25%), and lower proportions

in termites fed cellulose powder (1.99 ± 1.53%) at weeks 2, 6, 9, and 12 ($p \leq 0.038$), and were eliminated in starved termites following day 1 (Table 4.19; $p=0.000$).

Proportions of *D. fimbriata*, *P. major*, and *S. flagellata* tended to be higher in termites from sawdust/pine and pine block treatments, than in the cellulose powder, and starved termite treatments (Table 4.10, 4.15, and 4.18). However, at several sampling dates they were not statistically different among treatments. *Dinenympha fimbriata* proportions in termites from sawdust/pine and pine block treatments at weeks 1, 4, and 12, averaged 9.80 ± 4.22%, and were greater than 4.86 ± 3.18% in termites from cellulose powder and 3.70 ± 6.42% in starved termite treatments (Table 4.10; $p \leq 0.004$). The average percentage of *P. major* in termites from sawdust/pine treatments (3.10 ± 1.45%) and from pine block treatments (2.21 ± 1.45%) at weeks 1, 4, 6, and 9 were larger than 0.82 ± 0.96% in termites from cellulose powder treatments (Table 4.15; $p \leq 0.014$). Termites from sawdust/pine (5.94 ± 2.48%) and pine block treatments (5.47 ± 3.57%) also tended to have greater proportions of *S. flagellate*, compared with 2.39 ± 1.38% in termites from cellulose powder treatments and 0.00 ± 0.0% in the starved termites treatment at weeks 2, 6, and 9 (Table 4.18; $p \leq 0.001$).

Proportions of *H. elongatum* were greater in starved termites, than in termites from sawdust/pine, pine block, and cellulose powder treatments (Table 4.12). *Holomastigotes elongatum* made up 12.48 ± 15.11% of the protist population in starved termites at weeks 1, 2, and 4 and tended to be greater in proportion than 3.04 ± 2.03% in sawdust/pine, pine block, and cellulose powder treatments ($p \leq 0.0019$). Proportions of *M. fallax* tended to be greater in termites from cellulose powder treatments and starved termites, compared with termites from sawdust/pine and pine block treatments (Table 4.13; $p=0.000$). At weeks 1 and 2, *M. fallax* constituted 48.13 ± 34.56% of the protist population in starved termites, compared with termites

from sawdust/pine (7.02 ± 2.86%) and pine blocks (7.13 ± 4.08%) (Table 4.13; $p \leq 0.024$). Protist proportions of *M. fallax* in cellulose powder treatments averaged 13.80 ± 7.10% and were greater than 7.10 ± 3.67% in sawdust/pine and pine block treatments ($p \leq 0.022$) between weeks 4 through 12.

The protist species, *D. gracilis*, *Monocercomonas* sp., *P. vertens*, *S. kofoidi*, and *T. trypanoides* did not seem to vary in proportions between treatments over time (Tables 4.11, 4.14, 4.16-17, 4.20).

Discussion

Measures of termite vigor are important in determining the health of a colony. Termite hindgut protists are obligate symbionts and, without these protists, termites would die (Cleveland 1925a). Protist communities in termites are known to respond to environmental influences such as temperature and oxygen levels (Cleveland 1924, 1925b, Mannesmann 1969, 1970, Howard and Haverty 1981), as well as their host's diet (Mannesmann 1972, Mauldin et al. 1981, Cook and Gold 2000). It is believed that these responses are caused by the specific roles different protist species have in cellulose degradation (Cleveland 1924, Hungate 1943, Mauldin et al. 1972, Lai et al. 1983, Yoshimura et al. 1993a, b, 1996). The protist community found in the termite hindgut might be used to indicate colony vigor if termite health could be predicted by the composition of their protist complement.

Percent survivorship and food consumption are the most common measures of termite colony vigor. In the literature, survivorship can range between 71- 87% after 3 weeks (Su and LaFage 1984, Cornelius and Osbrink 2001). Our findings were similar, with an average percent termite survivorship of 86.89 ± 9.55% after 4 weeks, regardless of the treatment (Table 4.1).

There were differences in percent termite survivorship between food treatments over time, with only 24.25% survival in starved termites at week 4, which was significantly less than the other treatments on those dates, and with starved termites reaching 0% survivors by week 6 (Table 4.1; p=0.000). After 12 weeks, the sawdust/pine treatments had 40% termite survivorship, which could be explained by the handling disturbance required to remove termites from the sawdust-filled Tygon® tube on every sample period. Whereas removal of termites from the food source took seconds with the pine block and cellulose powder treatments, termites tunneled into and inhabited the sawdust/pine treatment substrate which required 10-15 minutes per sample, at each sample period, to gently separate termites from the debris and place them back into the Petri dish along with a new tube of sawdust. It has been hypothesized that termites are stressed by vibration and exposure to oxygen (Grosovsky and Margulis 1982) and the added handling time needed for the sawdust/pine treatments may have contributed to the low survivorship in that regime (Table 4.1). Survivorship rates in the low 80's are generally considered unacceptable for control groups placed in bioassay. In our experiment that point was reached after 4 weeks, despite starting with termites used within 24 hours of collection from the field (Table 4.1). Therefore the termites examined in our experiment, using percent survivorship as the measure of vigor, would be considered unsuitable for use in bioassay by week 4, and this sample date could be used to search for a predicative indicator within the protist community.

Food consumption rates of termites reported in the literature range from 29-79 milligrams of food per gram of termites per day in *Reticulitermes flavipes* (Lenz 1994, Cornelius and Osbrink 2001). The termite food removal rates we recorded were within that range with sawdust/pine removal highest at 142.18 ± 26.26 mg/g/d and lowest at 27.68 ± 8.47 mg/g/d in the cellulose powder treatment by week 4 (Table 4.2). The sawdust/pine consumption was typically

three to four times greater than cellulose powder or pine block consumption for 6 weeks (Table 4.2; p=0.000). Consumption in the pine block treatment remained equivalent throughout the trial, while termites in the sawdust/pine treatments showed a trend toward diminished consumption and the cellulose powder treatments provided a steady decline in termite consumption (Table 4.2). Declining consumption rates, viewed in combination with the survivorship data, could be used as an indication of reduced termite vigor because termites in the sawdust/pine and pine block treatments did not survive beyond 12 weeks; in addition termites in the two treatments generally ingested less substrate on each successive sampling date (Tables 4.1 & 4.2).

We also examined average termite weight and running speed. Average termite weight increased an average of 0.66 mg in sawdust/pine (F=11.55, df=6, 127; p=0.000), 0.39 mg in pine block (F=9.76, df=6, 143; p=0.000), and 0.35 mg on the cellulose powder diet (F=10.58, df=6, 139; p=0.000), while average weight decreased by about 0.20 mg in the starved treatment (Table 4.3; F=6.83, df=3, 66; p=0.000). Termites' running speeds tended to be faster in the pine block and sawdust/pine treatments than in the cellulose powder treatments and starved termites; however they were not statistically different over time (Table 4.4)

Protist population estimates from our field collected *R. flavipes* provided an average of 72,635.00 ± 17,279.6 per termite. We have also counted protist populations from *R. flavipes* within 24 hours of collection from the field averaging 77,584.5 ± 14,239 protists per termite (Lewis and Forschler unpublished data). These estimates are greater than the 21,043 to 40,083 protists per termite previously reported in the literature (Mannesmann 1969, Mauldin et al. 1981, Howard 1984, Cook and Gold 1998), but are within the range of estimates we obtained during the course of our experiment (Table 4.5). We were surprised by the significant decline in estimated protist population obtained from termites maintained for only one week in culture

(Table 4.5). It could be that termites used in previous studies were removed from the field at least one week prior to experimentation and therefore do not represent the protist community of termites in their natural state. In the present study, termite protist populations remained significantly lower in the pine block treatments from the first week through the end of the experiment (Table 4.5), whereas numbers of protists estimated in the sawdust/pine treatments varied from equivalent to less than the field population estimate (Table 4.5). Total protist populations decreased by over 50 percent after one week of termites in the pine block treatments and by at least 30 percent in the other treatment regimes after four weeks (Table 4.5). Protist populations remained similar in termites from the cellulose powder termite treatments for the first two weeks, and decreased at week 4, remaining low for the remainder of the experiment. After 1 week in bioassay, protist populations decreased by over 90 percent in the starvation treatment (Table 4.5; p=0.000). Clearly, total protist population estimates cannot be used to predict termite colony vigor, although changes in proportions of various protists could indicate a decline in termite health (Table 4.6-9).

The assumption underlying termite-protist roles is that starved termites lose cellulolytic protists first, therefore we expected termites in our pine block, sawdust/pine block, and cellulose powder treatments would show no change in proportions of cellulolytic species. The literature reports that *Dinenympha fimbriata*, *Pyrsonympha vertens*, and *Trichonympha agilis* are cellulolytic because they actively ingest wood fragments (Yamaoka 1979, Grosovsky and Margulis 1982) and among one of the first protists to disappear when termites are starved (Grosovsky and Margulis 1982). Our data showed both *P. vertens* and *T. agilis* were gone by week 1 counts, and *D. fimbriata* proportions were half their original estimate during weeks 1 and 2, and eliminated by week 4 (Tables 4.9). *Dinenympha gracilis* and *Spirotrichonympha* spp. have

not been observed ingesting wood fragments. In starvation experiments they are the second group of protists to be eliminated and are believed to be involved in the later stages of cellulose degradation (Grosovsky and Margulis 1982). In our study, *D. gracilis* proportions dropped 85 percent by week 1 in the termite starvation treatment, but were present through the last sample date, week 4, whereas, *S. flagellata* was gone by week 2 (Tables 4.9). *Spironympha kofoidi* and *Pyrsonympha major* are considered facultatively cellulolytic, but there have been no studies on their role in cellulose digestion. The proportions of these two species did not change at week 1 but both species were gone by week 2 (Tables 4.9). The protists *Holomastigotes elongatum*, *Microjoenia fallax*, and *Trichomonas trypanoides* do not appear to be important in host nutrition because their populations are not affected when termites are starved (Cleveland 1925a, Grososky and Margulis 1982), therefore their proportions should increase with the loss of other protist species. Our data showed an increase in all three genera on the first sample date and, with the exception of *T. trypanoides*, they were also present on the last sample date (Tables 4.9). In previous starvation experiments with *Reticulitermes* termites, all protists were eliminated between 3 to 4 weeks (Cleveland 1925a). Mauldin (1981) found mean termite biomass dropped by 0.56 mg per termite when protists populations dropped below 5,000. We did not find this in our experiment; the total protist population per termite individual after 1 week dropped from 72,635 to 3,990 (Table 4.5) and weight decreased by only 0.01 mg (Table 4.3). In our starvation treatments, populations of *T. agilis* and *P. vertens* were the first to disappear, *P. major*, *S. flagellata*, and *S. kofoidi* were gone by week 2, and *T. trypanoides* and *D. fimbriata* were eliminated after 4 weeks. *D. gracilis*, *H. elongatum,* and *Monocercomonas* sp. were still present at week 4, although in very small proportions, and total protist populations were less than 20 cells per termite.

Cook and Gold (2000) tested the hypothesis that cellulolytic protist proportions increased when termites were fed a diet of wood. They found proportions of *T. agilis* increased, while *D. fimbriata* decreased in *Reticulitermes virginicus*. When termites were fed filter paper (alpha cellulose), they found an increase in *P. vertens*, while numbers of *D. fimbriata*, *P. minor*, and *T. agilis* decreased (Cook and Gold 2000). In general, we found those proportions of cellulolytic species, *D. fimbriata* and *T. agilis*, as well as later stage cellulolytic species, *S. flagellate, P. major,* and *S. kofoidi*, increased. However, there were no statistically significant trends.

The proportions of the various termite protist species did not change significantly over time in any of our treatments other than starvation (Tables 4.6-9). There were dates when there was a statistical difference between and within treatments, but no obvious trend indicative of a proportional shift correlated with colony vigor. It would appear that proportions of the various protist species remain relatively consistent in the event that conditions for maintenance of higher populations return. Our data suggest that proportions of the protist community also hold little or no value as a predictive indicator of termite colony vigor.

There are numerous factors that can influence termite colony vigor including diet, temperature, moisture, and previous feeding experience (Lenz and Barrett 1982, Lenz 1985). We were not able to correlate percent survivorship, worker live weights, or food consumption rates of termites with the composition of the protist community from termite hindguts. It appears that changes in the protist community of subterranean termites maintained in laboratory culture are not a good indicator of colony vigor. The rapid decrease in total protist numbers from the field condition (Table 4.5) was a surprising result that offers opportunities for future research. The decrease in protist numbers from the termite starvation treatment was most dramatic, although not unexpected. Interestingly, the low protist numbers in the pine block treatments following

only one week in bioassay and four weeks in the cellulose powder treatments mirrored numbers previously reported from *R. flavipes* (Mauldin et al. 1981, Howard 1984, Cook and Gold 1998, 1999). The sawdust/pine treatments were designed to mimic a field diet containing microorganisms common in decayed wood, a preferred termite food source. The maintenance of higher protist numbers in the sawdust/pine treatments hints at the value of providing decayed wood for termites kept in culture, yet the variability in those counts and the low numbers obtained in the other treatments suggests that other factors are important in keeping termite cultures healthy in the long term. It could be that termite protist populations are adversely affected when termites are manipulated outside of the atmospheres maintained in their galleries and feeding sites. For instance, carbon dioxide concentrations in soil are typically 8-300 times that of aboveground levels (Brady 1974). It could be that placing termites in elevated oxygen levels adversely affects their ability to maintain appropriately anoxic conditions in the hindgut and the high protist numbers required for adequate cellulose digestion. Future research is planned to address this possibility.

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Table 4.1. Termite survivorship in food treatments over time.

	Percent Termite Survivorship (% from initial counts)			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day 1	100.00a[2]	100.00a	100.00	100.00a
Wk 1	84.00±22.08abA	93.60±4.16abA	89.60±3.36abA	87.00±8.37abA
Wk 2	84.00±9.92abA	90.20±5.45abA	90.60±4.16abA	64.40±30.10bA
Wk 4	90.60±4.72aA	88.00±3.94abA	82.00±15.26bA	24.25±15.69bB
Wk 6	72.40±23.85abA	83.20±10.06bA	76.00±7.55bA	
Wk 9	74.00±5.29abAB	85.67±4.04abA	62.80±11.17bB	
Wk 12	40.00±8.48bA	79.00±17.06bA	69.00±0.00bA	

sd[1]=sampling date counts taken. [2] Mean ± SD within columns with the same lower case letter and rows with the same upper case letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.2. Average food removal rate by termites over time.

Food Removal Rate (mg/g of termite/day)			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder
Wk 1	142.18±26.26aA[2]	29.21±6.86aB	45.00±27.85aB
Wk 2	120.12±30.65abA	40.09±14.37aB	38.01±11.52aB
Wk 4	87.43±12.53bA	33.69±18.66aB	27.68±8.47aB
Wk 6	98.77±22.37abA	30.76±13.42aB	23.15±10.71aB
Wk 9	63.28±19.91bA	47.71±30.46aAB	16.88±4.73aB
Wk 12	67.62±10.22bA	31.58±7.18aB	14.72±0.00aB

sd[1]=sampling date counts taken. [2] Mean ± SD within columns with the same lower case letter and rows with the same upper case letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.3. Average termite weight in various food treatments over time.

Average Termite Individual Weight (milligram)				
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day 1	3.04±0.25aA[2]	3.04±0.25aA	3.04±0.25aA	3.04±0.25aA
Wk 1	3.56±0.45bAB	3.30±0.33abAC	3.59±0.30bB	3.03±0.35aC
Wk 2	3.73±0.29bA	3.24±0.41abB	3.70±0.43bA	2.78±0.36abC
Wk 4	3.81±0.23bA	3.48±0.58bcA	2.92±0.62aB	2.45±0.52bB
Wk 6	3.62±0.37bA	3.54±0.41bcA	3.23±0.45aB	
Wk 9	3.82±0.19bA	3.93±0.10cA	3.47±0.38abB	
Wk 12	3.73±0.30bA	3.22±0.17abB	3.54±0.23abA	

sd[1]=sampling date counts taken. [2] Mean ± SD within columns with the same lower case letter and rows with the same upper case letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.4. Time in seconds for a termite to run 6 centimeters.

Termite Running Speeds (sec/6cm)				
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day 1	4.22±1.18a[2]	4.22±1.18ab	4.22±1.18a	4.22±1.18a
Wk 1	4.34±1.54aAB	4.03±0.94aA	5.15±2.15abBC	5.43±2.66abC
Wk 2	4.05±1.84aA	5.15±2.62bA	5.28±3.14abA	5.74±2.62bA
Wk 4	4.75±2.85aAB	4.18±1.01abA	5.78±2.30abcB	5.59±2.26abAB
Wk 6	4.56±1.76aAB	3.75±1.17aA	5.32±1.82abB	
Wk 9	4.14±2.31aAB	3.93±1.15aA	5.93±2.72bcB	
Wk 12	4.80±1.71aA	4.13±1.25abA	8.93±4.86cB	

sd[1]=sampling date counts taken. [2] Mean ± SD within columns with the same lower case letter and rows with the same upper case letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.5. Total protist population over time per treatment.

	Total Protist Populations per Termite			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day 1	72,635.00±17,279.60a[2]	72,635.00±17,279.60a	72,635.00±17,279.60a	72,635.00±17,279.60a
Wk 1	48,155.00±8,425.24bA	27,830.00±15,207.54bB	65,515.00±10,927.34aC	3,990.00±3,456.56bD
Wk 2	61,965.00±12,636.21abA	30,58.000±15,018.21bB	59,905.00±14,750.14abA	340.00±44.18bC
Wk 4	50,460.00±10,117.97bA	34,320.00±16,723.08bB	19,190.00±20,401.64cC	18.75±61.17bD
Wk 6	48,470.00±17,410.46bA	27,445.65±16,921.39bB	31,855.00±14,988.74bcB	
Wk 9	54,333.33±12,289.90bA	41,391.67±6,323.83bA	46,881.25±23,028.92bcA	
Wk 12	64,550.00±22,535.48abA	28,387.50±9,381.00bB	42,850.00±8,055.67abcB	

sd[1]=sampling date counts taken. [2] Mean ± SD within columns with the same lower case letter and rows with the same upper case letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.6. Protist species proportions in termites fed sawdust/pine over time.

sd[1]	DF	DG	HE	MF	Mono	PM	PV	SK	SF	TA	TT
Day 1	8.48±3.2ab[2]	64.46±3.9a	3.20±1.2a	7.26±4.6ab	0.69±1.1a	1.26±0.9a	5.68±2.0a	1.59±1.0ab	4.45±2.2a	2.29±1.6ab	0.64±0.9a
Wk 1	9.86±4.5a	63.33±6.7ab	3.03±1.0a	5.60±2.9a	0.94±1.4a	2.44±1.0b	4.46±2.1ab	2.11±1.45a	4.52±1.8a	3.48±1.6a	0.23±0.5a
Wk 2	7.55±2.3ab	66.67±5.1a	2.37±0.7a	6.95±2.2ab	0.54±0.5a	2.32±1.1b	4.14±1.5b	1.26±0.6b	5.38±1.9a	2.31±1.1ab	0.52±1.1a
Wk 4	7.17±2.4b	62.58±5.9ab	3.14±1.2a	8.67±2.7b	1.23±1.3a	3.37±1.2bc	4.21±1.4b	1.45±0.7ab	4.75±2.3a	2.87±1.6ab	0.56±1.2a
Wk 6	9.87±3.7a	59.20±9.1b	3.16±1.2a	7.35±2.0ab	1.34±1.9a	3.77±1.6c	5.07±1.5ab	1.13± 0.8b	5.32±1.9a	2.62±1.5ab	1.17±2.4a
Wk 9	8.37±2.3ab	61.17±4.8ab	2.25±1.0a	6.25±2.9ab	1.12±1.2a	2.62±1.6abc	5.56±1.5ab	1.45 ±0.6ab	7.95±3.1b	2.42±1.6ab	0.84±1.3a
Wk 12	9.51±2.1ab	63.78±4.5ab	2.23±0.9a	4.09±2.4a	0.28±0.5a	1.92±1.5ab	4.51±1.0ab	1.88±0.6ab	9.25±2.1b	1.37±1.2b	1.18±1.3a

sd[1]=sampling date counts taken. DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PM= *Pyrsonympha major*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*. [2] Mean ± SD within columns with the same letter do not differ significantly at p≤0.05; Tukey's Honestly Significant Difference (HSD) test.

Table 4.7. Protist species proportions in termites fed pine blocks over time.

sd[1]	DF	DG	HE	MF	Mono	PM	PV	SK	SF	TA	TT
Day 1	8.48±3.2a[2]	64.46±3.9a	3.20±1.2a	7.26±4.6a	0.69±1.1a	1.26±0.9a	5.68±2.0ab	1.59±1.0a	4.4 ±2.2a	2.29±1.6a	0.64±0.9a
Wk 1	10.78±4.1a	55.75±9.2b	5.08±2.0b	7.83±4.1a	1.11±1.8ab	2.22±1.4ab	5.31±1.2ab	2.1±3.0a	4.37±3.0a	4.57±2.4b	0.88±2.0a
Wk 2	8.18±2.9a	62.16±8.8a	3.44±2.4a	7.15±4.6a	1.40±2.2ab	1.33±1.2a	5.56±2.8ab	1.29±1.4a	4.51±2.7a	4.00±2.3ab	0.96±2.1a
Wk 4	8.30±3.4a	60.9±9.3ab	3.18±1.5a	6.39±3.5a	1.12±1.4ab	2.61±1.8b	5.22±2.3a	1.73±0.9a	4.76±2.0a	3.76±2.1ab	2.03±4.6a
Wk 6	10.05±5.7a	54.13±11.2b	3.68±2.4ab	8.33±4.5a	1.74±2.3ab	2.04±1.3ab	7.49±4.2b	1.44±1.5a	6.48±4.9a	3.58±2.3ab	1.06±2.3a
Wk 9	14.76±3.3b	54.91±4.8b	2.41±1.1a	6.33±3.1a	1.27±1.4ab	1.88±1.2ab	6.85±2.3ab	1.58±0.8a	5.51±2.5a	3.41±1.8ab	1.09±2.4a
Wk 12	8.81±2.1a	61.93±6.8ab	2.81±1.3a	7.29±2.5a	3.00±2.6b	1.45±1.6ab	4.81±1.2ab	1.66±0.9a	5.26±2.0a	2.97±1.9ab	0.00±0a

sd[1]=sampling date counts taken. DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PM= *Pyrsonympha major*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*. [2] Mean ± SD within columns with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.8. Protist species proportions in termites fed cellulose powder over time.

sd[1]	DF	DG	HE	MF	Mono	PM	PV	SK	SF	TA	TT
Day 1	8.48±3.3a[2]	64.46±3.9a	3.20±1.2ab	7.26 ±4.6a	0.69±1.1a	1.26±0.9a	5.68±2.0a	1.59±1.0a	4.45±2.2a	2.29±1.6ab	0.64±0.9a
Wk 1	6.15±2.3ab	65.34±5.3a	1.88±0.7bc	10.65±4.3ab	0.82±1.0a	0.8±1.0ab	6.46±1.9a	1.05±0.8ab	3.74±1.1a	2.9±1.1a	0.22±0.5a
Wk 2	6.08±2.2ab	67.69±4.6a	1.80±1.2bc	10.35±4.4a	0.38±0.4a	1.14±0.7ab	6.67±2.8a	0.57±0.4b	2.78±1.3a	2.37±1.3ab	0.17±0.4a
Wk 4	3.43±2.3c	53.02±21.4b	3.44±3.7a	15.04±10.7b	9.35±22.2b	1.24±1.4ab	7.67±7.3a	0.85±1.1b	2.98±2.2a	2.39±2.2ab	0.6±1.6a
Wk 6	6.07±3.9ab	62.67±7.9a	1.55±1.4c	15.48±4.4b	0.97±1.0a	0.97±1.3ab	5.64±2.6a	0.61±0.9b	2.84±1.8a	2.3±1.8ab	0.9±2.2a
Wk 9	5.43±2.9bc	70.71±7.4a	0.60±0.5c	11.59±4.4ab	0.36±0.5a	0.42±0.4b	6.50±3.2a	0.67±0.6b	2.17±0.9a	1.25±0.9b	0.3±0.6a
Wk 12	3.32±2.6abc	70.81±4.1ab	1.41±2.2abc	10.33±3.6ab	0.44±0.5ab	1.74±2.0ab	6.86±2.5a	0.67±0.7ab	4.05±0.5a	0.38±0.5ab	0.00±0a

sd[1]=sampling date counts taken. DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PM= *Pyrsonympha major*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*.[2] Mean ± SD within columns with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.9. Protist species proportions in the starved termite treatment over time.

sd[1]	DF	DG	HE	MF	Mono	PM	PV	SK	SF	TA	TT
Day 1	8.48±3.2a[2]	64.46±3.9a	3.20±1.2a	7.26 ±4.6a	0.69±1.1a	1.26±0.9a	5.68±2.0a	1.59±1.0a	4.45±2.2a	2.29±1.6a	0.64±0.9a
Wk 1	4.18±6.7a	9.49±9.9bc	12.94±10.6b	54.16±23.9b	12.87±13.6b	2.11±3.4a	0.08±0.4b	1.74±3.4a	0.30±0.8b	0.00±0b	2.12±4.7a
Wk 2	3.54±9.7a	0.76±2.6b	14.71±21.2ab	76.07±24.6c	2.78±9.6ab	0.76±2.6a	0.00±0b	0.00±0a	0.00±0b	0.00±0b	1.39±4.8a
Wk 4	0.00±0a	25.00±35.3c	25.00±35.3ab	0.00±0a	50.00±70.7c	0.00±0a	0.00±0b	0.00±0a	0.00±0b	0.00±0b	0.00±0a

sd[1]=sampling date counts taken. DF=*Dinenympha fimbriata*, DG= *D. gracilis*, HE= *Holomastigotes elongatum*, MF= *Microjoenia fallax*, Mono= *Monocercomonas* sp., PM= *Pyrsonympha major*, PV= *P. vertens*, SK= *Spironympha kofoidi*, SF= *Spirotrichonympha flagellata*, TA= *Trichonympha agilis*, and TT= *Trichomonas trypanoides*.
[2] Mean ± SD within columns with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.10. *Dinenympha fimbriata* as a proportion of total protist complement by treatment.

	Dinenympha fimbriata			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	8.48±3.2a[2]	8.48±3.2a	8.48±3.2a	8.48±3.2a
Wk1	9.86±4.5a	10.77±4.1a	6.15±2.3b	4.18±6.7b
Wk2	7.55±2.3ab	8.18±2.9a	6.08±2.2ab	3.54±9.7b
Wk4	7.17±2.4a	8.29±3.4a	3.43±2.3b	0.00±0.0b
Wk6	9.87±3.7a	10.05±5.7ab	6.07±3.9b	
Wk9	8.37±2.3a	14.76±3.3b	5.43±2.9a	
Wk12	9.51±2.1a	8.81±2.1a	3.32±2.6b	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.11. *Dinenympha gracilis* as a proportion of total protist complement by treatment.

	Dinenympha gracilis			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	64.46±3.9a[2]	64.46±3.9a	64.46±3.9a	64.46±3.9a
Wk1	63.33±6.7a	55.75±9.2b	65.33±5.3a	9.50±9.9c
Wk2	66.67±5.1a	62.16±8.8b	67.69±4.6a	0.76±2.6c
Wk4	62.58±5.9a	60.9±9.3a	53.02±21.4a	25.00±35.3a
Wk6	59.20±9.0ab	54.13±11.2a	62.67±7.9b	
Wk9	61.17±4.8a	54.91±4.8b	70.71±7.4c	
Wk12	63.78±4.5a	61.93±6.8a	70.81±4.1a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.12. *Holomastigotes elongatum* as a proportion of total protist complement by treatment.

	Holomastigotes elongatum			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	3.20±1.2a[2]	3.20±1.2a	3.20±1.2a	3.20±1.2a
Wk1	3.03±1.0a	5.08±2.0a	1.88±0.7a	12.94±10.6b
Wk2	2.37±0.7a	3.44±2.4ab	1.80±1.2a	14.71±21.2b
Wk4	3.14±1.2a	3.17±1.5a	3.44±3.7a	25.00±35.3b
Wk6	3.16±1.2a	3.67±2.4a	1.55±1.4b	
Wk9	2.25±1.0a	2.41±1.1a	0.60±0.5b	
Wk12	2.23±0.9a	2.81±1.3a	1.41±2.2a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.13. *Microjoenia fallax* as a proportion of total protist complement by treatment.

	Microjoenia fallax			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	7.26±4.6a[2]	7.26±4.6a	7.26±4.6a	7.26±4.6a
Wk1	5.60±2.9a	7.83±4.1a	10.65±a4.3a	54.16±23.9b
Wk2	6.95±2.2a	7.15±4.6a	10.35±4.4a	76.07±24.6b
Wk4	8.67±2.7a	6.39±3.5a	15.04±10.7b	0.00±0.0ab
Wk6	7.35±2.0a	8.33±4.5a	15.48±4.3b	
Wk9	6.25±2.9a	6.33±3.1a	11.59±4.4b	
Wk12	4.09±2.4a	7.29±2.5a	10.33±3.6b	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.14. *Monocercomonas* sp. as a proportion of total protist complement by treatment.

	Monocercomonas sp.			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	0.69±1.1a[2]	0.69±1.1a	0.69±1.1a	0.69±1.1a
Wk1	0.94±1.4a	1.11±1.8a	0.82±1.0a	12.87±13.6b
Wk2	0.54±0.5a	1.40±2.2a	0.38±0.4a	2.78±9.6a
Wk4	1.23±1.3a	1.12±1.4a	9.35±22.2a	50.00±70.7b
Wk6	1.34±1.9a	1.74±2.3a	0.97±1.0a	
Wk9	1.12±1.2ab	1.27±1.4a	0.36±0.5b	
Wk12	0.28±0.5a	3.00±2.6b	0.44±0.5ab	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.15. *Pyrsonympha major* as a proportion of total protist complement by treatment.

	Pyrsonympha major			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	1.26±0.9a[2]	1.26±0.9a	1.26±0.9a	1.26±0.9a
Wk1	2.44±1.0a	2.22±1.4a	0.8±0.5b	1.94±3.4ab
Wk2	2.32±1.1a	1.33±l1.2a	1.14±0.8a	0.45±2.6a
Wk4	3.37±1.2a	2.61±1.8a	1.24±1.4b	0.00±0.0ab
Wk6	3.77±1.6a	2.04±1.3b	0.97±1.3c	
Wk9	2.62±1.6a	1.88±1.2a	0.41±0.3b	
Wk12	1.92±1.5a	1.45±1.6a	1.74±2.0a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.16. *Pyrsonympha vertens* as a proportion of total protist complement by treatment.

Pyrsonympha vertens				
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	5.68±2.0a[2]	5.68±2.0a	5.68±2.0a	5.68±2.0a
Wk1	4.46±2.1a	5.31±a1.2ab	6.46±1.9b	0.08±0.4c
Wk2	4.14±1.5a	5.56±2.9ab	6.67±2.8b	0.00±0.0c
Wk4	4.21±1.4a	5.22±2.3ab	7.67±7.3b	0.00±0.0ab
Wk6	5.07±1.5a	7.48±4.2b	5.64±2.6ab	
Wk9	5.56±1.5a	6.85±2.3ab	6.50±3.2b	
Wk12	4.51±1.0a	4.81±1.2a	6.86±2.5a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.17. *Spironympha kofoidi* as a proportion of total protist complement by treatment.

	Spironympha kofoidi			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	1.59±1.0a[2]	1.59±1.0a	1.59±1.0a	1.59±1.0a
Wk1	2.11±1.4a	2.1±1.2a	1.05±0.8a	1.74±2.4a
Wk2	1.26±0.6a	1.29±1.4a	0.57±0.4b	0.00±0.0b
Wk4	1.45±0.7ab	1.73±0.9a	0.85±1.1b	0.00±0.0ab
Wk6	1.13±0.8ab	1.44±1.5a	0.61±0.9b	
Wk9	1.45±0.6a	1.58±0.8a	0.67±0.6a	
Wk12	1.88±0.6a	1.66±0.9a	0.67±0.8a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.18. *Spirotrichonympha flagellata* as a proportion of total protist complement by treatment.

	Spirotrichonympha flagellata			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	4.45±2.1a[2]	4.45±2.1a	4.45±2.1a	4.45±2.1a
Wk1	4.52±1.8a	4.37±3.0a	3.74±1.4a	0.30±0.8b
Wk2	5.38±1.9a	4.51±2.7a	2.78±1.3b	0.00±0.0c
Wk4	4.75±2.3a	4.76±2.0a	2.97±3.6a	0.00±0.0a
Wk6	5.32±1.9a	6.48±4.8a	2.83±1.2b	
Wk9	7.95±3.1a	5.51±2.5b	2.17±1.6c	
Wk12	9.25±2.0a	5.26±2.0a	4.05±1.4a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.19. *Trichonympha agilis* as a proportion of total protist complement by treatment.

	Trichonympha agilis			
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	2.29±1.6a[2]	2.29±1.6a	2.29±1.6a	2.29±1.6a
Wk1	3.48±1.6ab	4.57±2.4a	2.9±1.1b	0.00±0.0c
Wk2	2.30±1.1a	4.00±2.3b	2.37±1.3a	0.00±0.0c
Wk4	2.87±1.6a	3.76±2.1a	2.39±2.2a	0.00±0.0a
Wk6	2.62±1.5a	3.58±2.3b	2.30±1.8c	
Wk9	2.42±1.6a	3.41±1.8b	1.25±0.9c	
Wk12	1.37±1.2a	2.97±1.9b	0.38±0.5c	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

Table 4.20. *Trichomonas trypanoides* as a proportion of total protist complement by treatment.

Trichomonas trypanoides				
sd[1]	Sawdust/pine	Pine blocks	Cellulose powder	Starved
Day1	0.64±0.9a[2]	0.64±0.9a	0.64±0.9a	0.64±0.9a
Wk1	0.23±0.5a	0.88±2.0a	0.22±0.5a	2.12±4.7a
Wk2	0.52±1.1a	0.96±2.06a	0.17±0.4a	1.39±4.8a
Wk4	0.56±1.2a	2.03±4.6a	0.60±1.6a	0.00±0.0a
Wk6	1.16±2.4a	1.06±2.3a	0.90±2.2a	
Wk9	0.84±1.3a	1.09±2.4a	0.30±0.6a	
Wk12	1.18±1.3a	0.00±0.0a	0.00±0a	

sd[1]= sampling date counts taken. [2] Mean ± SD within rows with the same letter do not differ significantly at $p \leq 0.05$; Tukey's Honestly Significant Difference (HSD) test.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Hindgut protists found only in lower termites and wood-feeding cockroaches serve an important symbiotic role in the health of these insects. Many methods have been used to quantify termite protists, making comparisons between studies difficult. There are several thousand protists per termite individual, and to observe them outside of the host, a physiological saline solution is needed. This solution should be osmotically balanced, stable, and keep the cells alive when outside of the host. We compared some of these techniques to see if there were differential effects on termite survivorship between saline solutions. In our studies we found cell life decreased significantly after five minutes, regardless of the saline solutions compared. However, when an anaerobically prepared medium was used, we were able to extend cell life from 5 to 20 minutes. This is important because cell counts and identification typically take between 15 and 20 minutes and an anaerobic media provides more time to make accurate counts.

It is believed protist communities respond to the host's diet because different protist species are involved in separate stages of cellulose degradation. Cleveland hypothesized (1925) that because castes are fed different diets, their protist communities should reflect these food variations. He hypothesized those castes that ingest wood should have more cellulolytic protists than those castes that are fed salivary secretions. Two studies have compared protist communities within castes of *Reticulitermes flavipes* (Cleveland 1925a, Cook and Gold 1998); however no study has examined the protist communities between castes for *R. virginicus* (Banks) and *R. hageni* Banks. We compared protist populations in the worker, soldier, nymph, and alate life stages of *Reticulitermes flavipes*, *R. virginicus*, and *R. hageni* and found nymphs and workers have the largest population of protists, followed by soldiers, with the smallest populations in alates. We did not find differences between caste protist proportions; however we did find differences within castes between termite species. Our studies show protist proportions of

Dinenympha gracilis, *Dinenympha fimbriata*, *Spirotrichonympha* spp, and *Trichonympha agilis* found in termite workers could potentially be used for separating the termite species *R. flavipes*, *R. hageni*, and *R. virginicus*, respectively.

Several factors can influence termite protist communities including temperature, season, and geographical distribution, in addition to termite host nutritional sources. Measures of termite vigor are important in determining the physical condition of a colony. The protist community found in the termite hindgut are obligate symbionts important in termite digestion and might be used to indicate colony vigor if termite health could be predicted by the composition of their protist complement. We looked at several "traditional" measures of colony vigor as well as well as at total protist community and protist species proportions. The protist community decreased significantly after one week in bioassay, but we were not able to correlate percent survivorship, termite worker live weights, or food consumption rates with the composition of the protist community from termite hindguts.

APPENDIX A. PHYSIOLOGICAL SALINE SOLUTIONS

in grams	Trager U	Mannesmann	Ritter	0.60% NaCl
NaCl	0.1082	0.400		0.600
KCl		0.010	0.1247	
$CaCL_2$	0.0041	0.010	0.041	
$NaHCO_3$	0.0386	0.005		
$NaH_2PO_4 \cdot H_20$			0.0145	
KH_2PO_4	0.0892			
$MgSO_4 \cdot 7H_2O$	0.0024		0.0376	
$Na_3C_6H_5O_7 \cdot 2H_20$	0.0754			
$KHCO_3$			0.0333	
total volume	50mL	50mL	50mL	50mL
pH	7.1	7.8	7.1	7.0