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The reproductive morphology and physiological age-grading of female *Cyrtobagous salviniae*, the salvinia weevil

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THE REPRODUCTIVE MORPHOLOGY AND PHYSIOLOGICAL AGE-GRADING OF FEMALE
CYRTOBAGOUS SALVINIAE, THE SALVINIA WEEVIL

A Thesis

Submitted to the Graduate Faculty of
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

the Department of Entomology

by

Lee Jared Eisenberg
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ABSTRACT

Over 1000 dissections were performed on field, greenhouse and lab specimens in order to describe the reproductive system of *Cyrtobagous salviniae*, a biocontrol agent of *Salvinia molesta* (giant salvinia). The reproductive morphology of *C. salviniae* was described, the classes of reproductive development were characterized, and oviposition assessed. The reproductive system of *C. salviniae* consists of two ovaries, each of which is comprised of two membraneous ovarioles. These are each divided into a distal germarium and a proximal vitellarium that is connected to a lateral oviduct. The lateral oviducts unite to form a common oviduct through which eggs must pass for oviposition to take place. A sclerotized spermatheca and accessory glands are also present. There are 5 classes of reproductive development, 2 nonparous (no oviposition) and three parous. These are differentiated primarily by the number and maturity of follicles in the vitellarium, the presence of eggs in the oviducts, and the presence or absence of follicular relics. The number of eggs oviposited was quantified by holding ninety-two *C. salviniae* weevils individually on sprigs of salvinia at 29.5 ° C, with 12:12 daylength, and counting the number of ovipositions per weevil each week. During the course of the five month study, over 12,000 eggs were enumerated. At the end of the study, all of the weevils were dissected, basic statistics calculated, and the data analyzed by ANOVA and Tukey-Kramer procedures.

The mean number of eggs oviposited for each of the parous classes (i.e., P1, P2, and P3) was 22.6, 84.3, and 208.3, respectively. ANOVA indicated that mean egg numbers for each class were significantly different ($F=176.5$, D. F. =2, 44.9, $P<.0001$), and Tukey-Kramer analysis ($P<.0001$) showed that each of the three classes were significantly different from each other

($P < .0001$). Values obtained from the oviposition study were related to the reproductive classes to create a physiological age-grading system, which can be used as a reference to gain a deeper understanding of the population dynamics of this important biocontrol agent.

Introduction and Literature Review

INTRODUCTION

Distribution and Impact of *Salvinia molesta*

The aquatic ferns of the genus *Salviniaceae* are among the world's worst invasive weeds (Wade, 1990). *Salvinia* species are widely distributed, and have caused profound economic, ecological and environmental problems in more than twenty tropical and subtropical countries. By 1996, *Salvinia molesta*, also known as giant salvinia, had been found in Australia, New Zealand, Fiji, the Philippines, Indonesia, Singapore, Malaysia, and Papua New Guinea (Holm *et al.*, 1977; Storrs and Julien, 1996). India has also been plagued with this noxious weed, along with a number of countries in equatorial Africa, including South Africa, Kenya, Zambia, Ghana, Namibia, Botswana, Madagascar, and the Ivory Coast (Storrs and Julien, 1996; Oliver, 1993). More recently, *S. molesta* been reported in Senegal and Mauritania (Pieterse *et al.*, 2003). Infestations have been reported in Colombia and Guyana, as well as the Caribbean countries of Trinidad and Cuba (Storrs and Julien, 1996; Oliver, 1993). *Salvinia molesta* has also become established in the southern U.S. (Chilton *et al.*, 2002; Jacono, 2003).

Giant salvinia's prodigious growth rate enables it to outcompete native plants and form a floating mat (up to 1 meter thick) that can cover slow-moving water bodies (Mitchell, 1978; Room, 1986) (Fig. 1) This can restrict the use of waterways for boat traffic, and clog water supply inlets intended for municipal and agricultural use or electrical generation (Room *et al.*, 1989). Irrigation and drainage are adversely affected (Thomas and Room, 1986; Room *et al.*, 1989). *Salvinia* mats can inhibit the rate of gas exchange that takes place between the atmosphere and a water body, which leads to anoxic conditions. Sunlight is also restricted, which harms submerged plants and other aquatic life (Mitchell, 1978), and further degrades



Figure 1. A 2.4 hectare pond infested with *Salvinia molesta* located in Gheens, LA, in June, 2009.

water quality (Mitchell, 1978). Afflicted water bodies tend to have a lowered pH; excessive levels of decaying organic matter can result in higher water temperatures (Storrs and Julien, 1996). Native plants, fish, and gamebirds are adversely affected (Sculthorpe, 1985; Oliver, 1993), and may suffer significant mortality due to habitat degradation (Kannan, 1979; Murphy and Barrett, 1990a). The mat may harbor disease carrying mosquitoes (Creagh, 1991). Once established, salvinia can be very difficult to ameliorate. These problems are often exacerbated in the third world. Some villages in the Sepik River Valley in Papua New Guinea were abandoned (circa 1980) (Jacono and Pittman, 2001) due to a severe salvinia infestation that made fishing or boat travel nearly impossible (Thomas and Room, 1986). Salvinia infestations can also interfere with recreational activities (such as duck hunting), that provide important

economic benefits. For example, duck hunting in Louisiana brings in \$150 million dollars a year in fees, leases, and related expenses (Southwick Associates, 2005).

Two salvinia species, *Salvinia molesta* D. S. Mitchell and *Salvinia minima* Baker, have become established in the southern United States (Jacono, 2003). These are known as giant salvinia (the more serious of the two pests) and common salvinia, respectively. *Salvinia minima* has been present in the U.S for at least seven decades as a horticultural plant (Jacono, 2003), but is far less invasive than giant salvinia. Small to medium size infestations of *S. molesta* (giant salvinia) were noted in natural systems throughout the southern U.S. in 1995 (Johnson, 1995; Jacono and Richerson, 2003). An infestation at Toledo Bend Reservoir, LA, in 1998 covered 485 hectares (1200 acres) (Chilton *et al.*, 1999). By 2010, *S. molesta's* range in Louisiana had grown to an estimated 70,000 acres (Johnson *et al.*, 2010). In 2009, it was estimated that there were 17,292 acres of giant salvinia in state managed water bodies, but a hard freeze in the winter of 2009-2010 reduced that figure to 2,706 acres (Pers. Com., A. Perret, Louisiana Department of Wildlife and Fisheries). However, the surviving salvinia has been proliferating rapidly (Pers. Obs.), and control efforts continue at Lake Bistineau, Caddo Lake, Spanish Lake, the Atchafalaya Basin, and numerous locations on private land (Pers. Comm., S. Johnson). *Salvinia molesta* is found primarily in the Deep South, but it has also been reported in California, Virginia, and Hawaii (Jacono, 2003). Infestations can spread rapidly, due to salvinia's explosive growth rate. For instance, a 200 acre mat of *S. molesta* discovered in May, 2006, in the Jeems Bayou area of Caddo Lake (on the Louisiana-Texas state line), subsequently grew to more than 1000 acres in two years, despite frequent applications of herbicides (Bonds, 2010). By October of 2009, *Salvinia molesta* had spread to eleven nearby reservoirs in Texas (Alford, 2009). Unfortunately,

S. molesta is easily spread by people traveling between water bodies with boats and trailers (McFarland *et al.*, 2004). It has also been introduced unintentionally by the aquarium and water gardening industries, and surprisingly, is available for purchase on the internet (Kay and Hoyle, 2001).

LITERATURE REVIEW

Biological Control with *Cyrtobagous salviniae*

The salvinia weevil, *Cyrtobagous salviniae* (Fig. 2), is the only biocontrol agent that has demonstrated significant activity against *Salvinia molesta* (Thomas and Room, 1986). A compilation of biological control efforts by Tasker (2001) noted that *C. salviniae* reduced major salvinia infestations to 1% or less of their former size in India, South Africa, Botswana, and the Sepik River Valley in Papua New Guinea. "Complete and rapid control" was cited at four other locations in Australia, Papua New Guinea, and Namibia. Failure of the weevils to provide control at two sites in Australia was attributed to high water temperatures in the Northern Territories, while an unfavorably cool climate contributed to variable control in New South Wales. At other locations in Sri Lanka and Papua New Guinea, large infestations of *S. molesta* were greatly reduced by the weevil after addition of nitrogen-based fertilizer improved host plant quality. An estimated \$150 million dollars of economic benefits result from worldwide biocontrol efforts related to giant salvinia on an annual basis (Room and Julien, 1995). This estimate is based on research done by Doeleman (1989) on the impact of salvinia in Sri Lanka. The study considered economic losses, health costs (from increased vector habitat), and abatement costs over a 25 year period. Environmental costs were acknowledged, but not included in the cost/ benefit analysis, due to a lack of data. These estimates were then extrapolated to countries throughout

the range of giant salvinia by Room and Julien (1995). Invasive salvinia is under control throughout most of its introduced range, thanks in large part to biological control programs that utilize the specialist herbivorous weevil *Cyrtobagous salviniae* (Fig. 2). However,



Figure 2. *Cyrtobagous salviniae*, the salvinia weevil on a salvinia frond.

giant salvinia in the United States is not considered to be under control (Julien *et al.*, 2002).

Once established, populations of *C. salviniae* can sustain a rapid reproductive rate if *S. molesta* nitrogen content is between 2.0 and 3.0 % (dry weight), providing other conditions are favorable (Sands *et al.*, 1986). Both adults and larvae feed on *S. molesta*. Larvae browse on roots before burrowing in buds and rhizomes; adults feed on buds and leaves. According to Forno *et al.* (1983), mature females can deposit one egg every two-five days for at least 60 days, or 0.42 eggs/day. This is the most cited source, but Sands *et al.* (1986) reported fecundity values of 374 (3.70 eggs/day) for lab-reared weevils, over a lifespan of 14.5 weeks, and Jayanth

(1989) found that fecundity was 290 (1.15 eggs/day) over a 36 week life span under greenhouse conditions.

Although effective throughout most of its introduced range (Tasker, 2001; Cilliers, 1991; Room *et al.*, 1981; Room and Thomas, 1985) introduced *C. salviniae* populations may fail to establish, or fail to provide a significant level of control for reasons that are not readily apparent (Hennecke and Postle, 2006; Skeat, 1990). Research that helps to bring about a deeper understanding of the ecology of *C. salviniae* would be of great utility in understanding these occasional failures. Forno and Semple (1987) investigated the role of host plant quality in *C. salviniae* population dynamics. Sands *et al.*, (1986) looked at intrinsic population growth rates under different conditions, and Cilliers (1991) studied the role of temperature. However, there has been no research that characterized the reproductive system of *C. salviniae*, or sought to develop a physiological age-grading system. Investigations of population dynamics, especially age structure, could benefit greatly from physiological age-grading research, which is discussed below.

The Benefits of Physiological Age-Grading

An age-grading system can be used to gain insight into the factors that drive population dynamics, such as nutrition, temperature or other environmental variables (Tyndale-Biscoe, 1984). Intensive sampling and dissection can provide an index of morphological characters that can be used to determine an individual's physiological age and investigate the age structure of a population, as well as other important metrics of a population (Tyndale-Biscoe, 1984). The efficacy of *C. salviniae* as a biocontrol agent of *S. molesta* is a function of its reproductive rate (Sands *et al.*, 1986; Room and Thomas, 1985). *Salvinia molesta* is, at once, food, shelter and

oviposition substrate for the cryptic weevil-and yet results can vary widely. Spectacular results, such as those obtained in early biocontrol efforts at Lake Moondarra, Australia, where weevils destroyed an estimated 18,000 tons of salvinia in three months (Room *et al.*, 1981), are puzzling in light of an average of 20-25 eggs per female (or 0.42 eggs/ day) oviposited over a sixty day period (Forno *et al.*, 1983). Sex ratio is another important factor that influences reproductive rates, and it also varies widely. Based on a sample size of 200, Sands *et al.* (1986) found that healthy field populations of *C. salviniae* in Australia and Papua New Guinea had a healthy (nearly 1:1) sex ratio. However, badly skewed sex ratios have been observed in a field population from Toledo Bend Reservoir (Sabine Parish, LA) that was introduced at a Gheens, LA, field site (Johnson *et al.*, 2010), and in our LSU greenhouse colony in 2007 (Pers. Obs.). Neither the Toledo Bend population nor the greenhouse population established, and it was necessary to re-introduce the weevils. Both of these questions– oviposition rates and sex ratios–can be investigated using age-grading techniques, along with many other questions regarding the ecology of a species (Tyndale-Biscoe, 1984).

Physiological age-grading is based on irreversible changes that occur in an organism over time (Tyndale-Biscoe, 1984). Chronological age is a simple measurement of the time that has passed since easily recognizable events such as emergence or pupation (Tyndale-Biscoe, 1984). However, when chronological measurements are taken of physiological processes, they do not reflect the interaction between an organism and the environment. Consider how environmental challenges such as drought or extreme temperatures (Hayes and Wall, 1999) can affect population growth rate. Unusually cool weather may retard reproductive development, due to the reduced metabolic activity of poikilotherms at low temperatures (Forno *et al.*, 1983).

Host plant quality may decline due to extremes of weather (Room, 1986), and cause oviposition rates to decline (Room and Thomas, 1985). Insect behavior is greatly influenced by physiological development (Tyndale-Biscoe, 1984).

Common methods of age-grading (in insects) include examining somatic changes such as cuticular growth rings (Neville, 1963), the concentration of fluorescent pigments in the eyes (Mail *et al.*, 1983), pigmentation of the abdomen (Dyce, 1969), or fat body characteristics (Tyndale-Biscoe and Hughes, 1969; Vogt *et al.*, 1974). Mechanical damage like wing fray (Jackson, 1946) or mandibular wear (Tyndale-Biscoe, 1984) can also be used to help establish relative age. Most important are techniques that address physiological age, such as the irreversible changes that take place during reproductive development. These are the most accurate (Hayes and Wall, 1999), and are particularly relevant to an herbivorous biocontrol agent like *C. salviniae*, whose reproductive rate and impact on *S. molesta* is related to host plant quality (Room and Thomas, 1985; Calder and Sands, 1985).

When the physiological age of a weevil is known (based on their reproductive characters), and the number of eggs they have oviposited is known, this provides information that can be used to explore the ecology of a species (Tyndale-Biscoe, 1984). For example, if weevils from field samples are dissected, their physiological age class can be determined, and, by extension, the approximate number of times they have oviposited. The condition and distribution of individuals in different physiological age classes can be used to evaluate the reproductive health of a population (Hayes and Wall, 1999). Most of the physiological age-grading research has been done on insects of medical or veterinary importance (e.g., vectors), which are primarily dipteran (Hayes and Wall, 1999). However, physiological age-grading

research has been conducted on the reproductive systems of a number of economically important curculionids such as the boll weevil, *Anthonomus grandis* Boheman (Spurgeon *et al.*, 2003; Grodowitz and Brewer, 1987), the mottled water hyacinth weevil, *Neochetina eichorniae* (Warner) (Grodowitz *et al.*, 1997), and the rice weevil, *Sitophilus oryzae* (L.), a pest of stored grain (Perez-Mendoza *et al.*, 2004). Yet little work has been done on the reproductive system of *C. salviniae*, whose efficacy with regard to invasive salvinia is linked to its population dynamics. Basic research that expands the knowledge base will permit a more refined assessment of the effects of nutrition and other drivers of the reproductive development and oviposition of *C. salviniae*. Such information can provide insight into constraints on population growth, or suggest alternative strategies with which to address problems, and potentially augment the successful deployment of this important biocontrol agent.

Biology of *Salvinia molesta*

The order Salviniiales contains the family Salviniaceae, which includes the notorious *Salvinia molesta* D.S. Mitchell (giant salvinia), and *Salvinia minima* Baker (common salvinia) (Room, 1983). These are free-floating aquatic ferns with submersed rhizomes connecting ramets (or nodes) that have buds, a pair of floating leaves (fronds), and a submerged root formed from an adapted leaf (Fig. 3a). Ramets, though joined by rhizomes, are capable of independent survival and vegetative growth if detached from the parent plant (Room, 1983). *Salvinia molesta* has three growth forms associated with intra-specific competition (e.g., crowding), with leaves ranging from 15 mm wide (1° growth form) to 60 mm wide (3° growth form). As plants move into the 3° stage, the leaf folds upward from the rachis (midrib), and presents a keeled appearance. An intermediate growth form (2°) has leaves up to 25 mm in

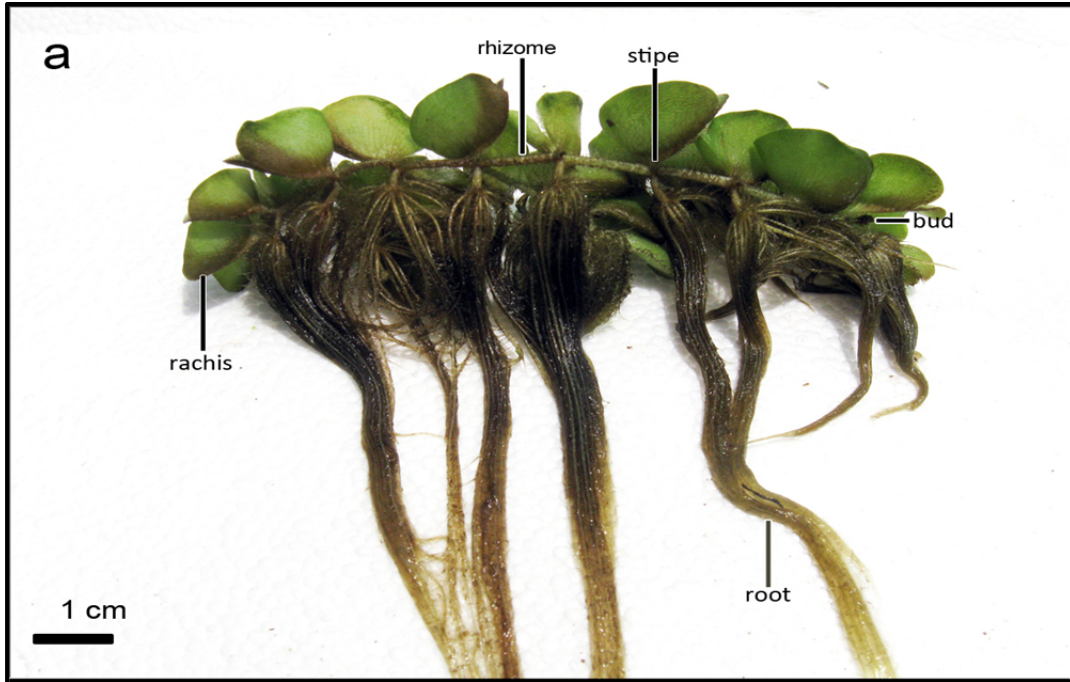


Figure 3. (a) The morphology of *Salvinia molesta*. Rhizome (stem), stipe (petiole), and rachis (midrib) are all terms specific to ferns. The root is a dissected leaf. (b) The sporocarps of *Salvinia molesta*. The roots have been cut away to reveal the structures, which would normally hang apex downward among the roots. The sporocarps appear during the tertiary stage, although the pentaploid plant cannot produce viable spores.

width, and can also be keeled, but to a lesser degree. The 1° growth form of *S. molesta* is usually the invading form, with small leaves on isolated ramets. The 2° growth form, also known as the open water colonizing form, grows at the edge of incomplete mats (Mitchell and Tur, 1975). Growth of the 3° form takes place in mature infestations, like a mat that has covered a lake or slow-moving river, where competition for sunlight, space, and available nutrients can be intense (Mitchell and Tur, 1975). Although the two salvinia species known to be present in North America, *S. molesta* and *S. minima*, are very similar in appearance (Jacono and Pittman, 2001), they can be differentiated by the appearance of the leaf hairs. In *S. molesta*, groups of four hairs join to form an eggbeater-like structure (Fig. 2). In *S. minima*, the hairs are all free (McFarland *et al.*, 2004).

Vegetative Reproduction

Salvinia has a pentaploid genome and is incapable of sexual reproduction; mature plants may have a vestigial sporocarp (Croxdale, 1981) (Figure 3b). Cytological studies by Loyal and Grewal (1966) indicated that meiosis was greatly disrupted, and the plant cannot produce viable spores. Despite this, its productivity is formidably high; it spreads by means of vegetative growth and fragmentation (Loyal and Grewal, 1966). A stand of salvinia may be a genet; a colony of plants (ramets or nodes) that appear to be individuals, but are in fact joined in some way. In this case, they may be linked by a continuous submersed rhizome (Room, 1983). A mat of salvinia covering a water body may therefore be one clonal plant, with each individual ramet sharing the same genome (Room, 1983). A mat may also be formed from a great number of distinct, i.e., unjoined, ramets. Salvinia spreads by the growth of apical or axillary buds, which become new ramets (Croxdale, 1981). New mats may form due to fragmentation initiated by

wind, wave action or senescence (Room, 1983). Each intact node includes one primary and three axillary buds. As many as six buds can regenerate from a node that is severely damaged if dissolved nitrogen is abundant (Julien and Bourne, 1986).

Productivity

Salvinia adapts well to water with low nutrient levels. However, it is capable of achieving a prolific growth rate when temperature, nutrients, and sunlight are optimal. Salvinia in the 1° growth form has been shown to double its leaf area in 2-4 days under ideal (laboratory) conditions (Mitchell and Tur, 1975; Cary and Weerts, 1983). Finlayson (1984) and Room (1986) reported leaf doubling time under field conditions to be 1-8 days. Fresh (wet) biomass estimates range from 49.5 to 109.5 tons/ha/yr (Mitchell and Tur, 1975). The growth rate is correlated with dissolved nitrogen levels (Room and Thomas, 1986), and this appears to be the limiting factor under field conditions (Mitchell and Tur, 1975). Plants growing at the edge of a mat, which have access to sunlight and a rich nitrogen source (from decomposing 3° plants), have the highest productivity (Mitchell and Tur, 1975). The nitrogen content of (dry weight) salvinia biomass ranges from 0.6 to 4 % (Room and Thomas, 1986). In the lab, the maximum rate of nitrogen uptake is 8 mg of nitrogen per gram of dry weight biomass/per day, which corresponds to 6000 kg/ha/yr (Room, 1986). Actual values obtained under field conditions (taken at a sewage plant) indicated that nitrogen uptake was 1580 kg N/ha/yr (Finlayson and Mitchell, 1982).

Temperature and pH

Room (1986) noted that optimal temperature for *S. molesta* growth was 30° C, and that no growth took place below 10°C or above 40°C in laboratory experiments. The effects of

extreme ambient temperature are mitigated to some degree by cooler water temperature in natural systems. Apparently, the water acts as a heat sink, even at ambient temperatures of 50° C (Storrs and Julien, 1996). Temperatures below - 3°C or above 43°C for two hours killed *S. molesta* in the laboratory (Whiteman and Room, 1991). *Salvinia* is capable of growth at pH values between 5.2 and 9.5 (Storrs and Julien, 1996). Cary and Weerts (1984) found that growth at pH 6 is optimal for *salvinia*, and that productivity at pH 8 is reduced by 59%. Owens *et al.* (2005) reported that *S. molesta* grown at low pH in ponds and tanks at the Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (LAERF) achieved greater surface coverage and higher biomass values than *salvinia* grown at higher pH. Owens *et al.* (2005) also noted that lowered pH values enhanced the availability of nutrients present in sediment.

Salinity and Conductivity

S. molesta is not salt tolerant, and colonies are not found in brackish water (Oliver, 1993). Salinities over 7% (70 ppt) inhibit the growth of *salvinia*, but salinity of 11% (110 ppt) or greater was required for desiccation to occur, and the time required varied greatly. Salinity of 25% (250 ppt) killed *S. molesta* within an hour (Divarkaran *et al.*, 1980). In the field, *salvinia* infests water bodies with conductivities between 239.3 (\pm 77.9) and 503.5 (\pm 446.2) μ S/cm (Room and Gill, 1985). Standard deviations are in parentheses. A Sieman (S) is the standard international unit of electrical conductance, equal to 1 ampere per volt. *Salvinia* has been observed achieving high growth rates in sewage lagoon water with conductivity values up to 1375 (\pm 149.5) μ S/cm (Room and Gill, 1985). *Salvinia* productivity was reduced by conductivity values of approximately 2000 μ S/cm (Storrs and Julien, 1996). A water sample obtained in July, 2008, at an infestation at Toledo Bend Reservoir (Sabine Parish, LA), and analyzed by the Soils

Lab of the LSU School of Plant, Environmental, and Soil Sciences, had a conductivity value in excess of 1400 μS (Pers. Obs.)

Chemical Control of *Salvinia Molesta*

Early efforts at chemical control of *S. molesta* included the use of pentachlorophenol (circa 1940) in Sri Lanka, as well as a variety of unlikely toxins throughout the 1960's and 1970's (Thomas and Room, 1986). The unsuccessful herbicides tested included monuron, formalin, anhydrous ammonia, niclosamide and thiram (Thomas and Room, 1986). Kam-Wing and Furtado (1977) investigated the effectiveness of paraquat, diquat, nitrophen and dalapon as control measures against *S. molesta* in Malaysia. Of these, only paraquat and diquat provided substantial control-100% mortality in the case of paraquat applied at a rate of 1.1 kg/ha, and 99% mortality when diquat was applied at 4.5 kg/ha. The rate of application for diquat was considered too expensive, so paraquat became the herbicide of choice throughout the late 1970's and 1980's (Oliver, 1993). It was widely used as a control measure throughout the introduced range of *S. molesta*, in Kenya, Botswana, Australia, Sri Lanka, and Papua New Guinea, although lower rates of application were found to allow rapid regrowth (Mitchell, 1979; Thomas and Room, 1986; Oliver, 1993). Paraquat, however, is moderately toxic to many aquatic organisms (Eisler, 1990) and highly toxic to mammals, particularly when inhaled (Ross and Lembi, 1999a). Paraquat must be applied with the aid of adjuvants, some of which are toxic to fish (Way *et al.*, 1971). Paraquat is a restricted use herbicide in the U. S., and is not labeled for aquatic use (EXTOXNET, 1996). A mixture of kerosene, surfactants, and diuron (an herbicide) was found by Diatloff *et al.* (1979) to be effective against *S. molesta*, even when diuron was present at levels as low as 0.15 kg/ha. The surfactants and kerosene helped to wet

the upper surface of salvinia leaves and enhance penetration. This mixture (known as AF-101) was widely used in Australia against giant salvinia and other noxious aquatic weeds, but never certified for aquatic use in the United States because it is moderately toxic to fish, and highly toxic to aquatic invertebrates (EXTOXNET, 1996).

Patnaik (1976) reported that 2,4-dichlorophenoxyacetic acid (2,4-D) was successfully employed to control giant salvinia in India. Thayer and Haller (1985), however, reported that 2,4-D was not effective against *S. molesta*. Phenoxy acid herbicides, including 2,4-D, have a chemical structure similar to auxin, a plant hormone that regulates growth. They are used against a variety of forbs (broad-leaved plants), but some monocots are also susceptible (Ross and Lembi, 1999b). The use of phenoxy acids for aquatic application has declined somewhat because of concern over safety issues (Ross and Lembi, 1999c) but it is still widely used for the control of water hyacinth (Center *et al.*, 1999). Two other phenoxy acid herbicides, Silvex (2,4,5-TP) and 2,4,5-T, were banned in 1985 by the E.P.A. because of dioxin contamination, which has been linked to potential fetotoxic, teratogenic and oncogenic effects (Gintautas *et al.*, 1992). Traces of dioxin, also known as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), a persistent toxin and carcinogen, are produced by a side reaction during the synthesis of Silvex and 2,4,5-T if temperature is not carefully controlled (Gangstad, 1983; Solomons, 1996). Dearden (1984) found that public perception of the danger associated with 2, 4-D was exaggerated, probably because it was a component (along with 2, 4,5-T), in the defoliant “Agent Orange”, which was widely used during the Viet Nam War. The EPA ruled in 1988 that existing data did not support the premise that 2,4-D was a carcinogen (EPA Document 53 FR 9590; FRL-3353-3, 2005).

However, Cochrane *et al.* (1981), using gas chromatography, found levels of dioxin between 5 and 23.8 ppb in 2,4-D esters.

Streams and groundwater, especially in urban areas, often contain detectable levels of 2,4-D, which is still employed for home, garden, and agricultural use (Gilliom *et al.*, 1999), even though aquatic applications accounts for only 3% of the total use (Spectrum Laboratories Chemical Fact Sheet # 94757). It is a widely used herbicide, but should not be applied to water used for crops, livestock, or human consumption. The ester formulation is more active against weeds but also more toxic to fish. It is usually applied as an amine (Ross and Lembi, 1999d). Environmental persistence is also a concern (Hoepfel and Westerdahl, 1983). Estrogenic effects were reported in juvenile rainbow trout exposed to concentrations of 2,4-D as low as 1.6 ppm. These effects were enhanced by the addition of commonly used aquatic surfactants (Xie *et al.*, 2005).

A chelated copper product, Komeen® (SePro Corporation, Carmel, IN), formulated with Cide-kick® (Applied Biochemists, Germantown, WI) as a surfactant, was evaluated for salvinia control by Nelson *et al.*, (2001). Komeen® showed excellent activity against *Salvinia molesta* during the first week after a single application, but plant mortality declined over time. Percent control after 42 days was 81.3 %. A safer but more expensive copper-based herbicide, Clearigate® (Applied Biochemists, Germantown, WI), achieved an 88% level of mortality after 7 days at a concentration of 20%, but mortality was only 73% after 42 days (Glomski *et al.*, 2003). Applications of 0.5 %, 1 %, and 2% were ineffective. At rates of 5-10%, significant reduction of biomass occurred, but not enough to prevent reinfestation from undamaged tissue. Application rates of 15% and 20% initially produced an 85% and 92% reduction of biomass, respectively, but

again, percent mortality declined over time. Since copper is a metal it is not biodegradable, and tends to be persistent in the environment. It is also moderately toxic to fish and aquatic invertebrates (Murphy and Barrett, 1990). Copper based herbicides are relatively expensive. Chilton *et al.* (1999) estimated the cost of treating a salvinia infestation at Toledo Bend Reservoir with copper-based herbicides to be \$316-714/ha (\$128.00-\$300.00/ acre). Mixtures of diquat and copper chelates gave results similar to diquat alone (Nelson *et al.*, 2001). Herbicide resistance may develop in plants exposed to persistent herbicides (like copper) over long periods of time (Koschnick *et al.*, 2006), rendering these chemicals less effective.

Fluridone was found to be an effective control agent against salvinia in tank studies in New Zealand when applied at rates of 100 mgs/L (Wells *et al.*, 1986). At lower concentrations plant death (determined by bleaching of plant tissue) did not consistently take place. A further complication is that fluridone is very slow acting and must be in contact with the plants for 30 to 90 days to be effective (Ross and Lembi, 1999e). Plants begin to show symptoms 7-10 days after treatment, as nutritional reserves are exhausted and not replaced due to the inhibition of chlorophyll formation (Murphy and Barrett, 1990b). This characteristic requires an entire enclosed water body to be treated to prevent re-infestation, which, due to the expense and logistics, means that fluridone is not usually suitable for large water bodies (Masser *et al.*, 2001; Ross and Lembi, 1999e). Fluridone, while regarded as one of the safer options, is also the most expensive. Chilton *et al.* (1999) estimated comprehensive treatment costs at Toledo Bend to be \$2292.13/ha (\$927.99/acre).

Glyphosate can be an effective herbicide for salvinia, and its acute toxicity to aquatic species is relatively low (Henry *et al.*, 1994; Beyers, 1995). However, glyphosate is also relatively

slow-acting. Salvinia treated with glyphosate (at 8.97 kg/ha) averaged 78.3% mortality after 28 days, as opposed to 100% mortality in diquat treatments applied at a rate of 1.12 kg/ha (Nelson *et al.*, 2001). The length of time required for effective treatment, and the costs associated with applying 8.97 kg/ha make glyphosate a fairly expensive option. Chilton *et al.* (1999) estimated that treatment of the Toledo Bend infestation with glyphosate, including labor and equipment, would cost \$506.35/ha (\$205.00/acre), or \$225/ river km (\$375.00 per river mile). A 'cocktail' of glyphosate and diquat is currently being used by spray crews in Texas. Louisiana crews are spraying diquat (Pers. Comm., D. Sanders). Some commonly used surfactants for aquatic herbicides can be detrimental to aquatic organisms (Wojtaszek *et al.*, 2004).

Other adjuvants may be introduced unintentionally, and these can have profound effects. In mesocosm studies, Relyea (2005a) found that glyphosate-based herbicides (single dosage of 3.8 mg/l) containing a polyethoxytallowamine surfactant (Roundup®), reduced species richness by 22% and nearly exterminated 3 species of frogs. Roundup® is not certified for aquatic use, but overspray is not uncommon, and many amphibians spend a considerable portion of their life cycle in terrestrial environments (Relyea, 2005b). Glyphosate is primarily broken down by bacteria, which are less active at lower temperatures. The half-life of Roundup® is 7-70 days (Relyea, 2005a).

Nelson *et al.* (2001) conducted a comprehensive study involving 32 herbicide treatments on salvinia. The treatments included two forms of endothall, as well as diquat, glyphosate, imazapyr, and copper products. These chemicals, and mixtures of these chemicals, were applied with different application rates, techniques, and surfactants, to salvinia grown in tanks at the Corps of Engineers (LAERF) research site in Lewisville, TX. Each chemical was applied as a

single application. Diquat, formulated as diquat dibromide, was by far the most effective herbicide, whether it was used alone or in combination with other agents. Applied at a rate of 1.12 kg/ha, all of the diquat treatments gave complete control (100%) within 42 days.

Glyphosate, and mixtures containing glyphosate, provided nearly complete control (99.3%) in that same time period, but an application rate of 8.97 kg/ha was required to be effective.

Campbell *et al.* (1999) reported that diquat is relatively safe to workers, fish and other wildlife if applied at label rates. However, Paul *et al.* (1994) found that 96-hr LC50s of 0.74-4.9 mg/l was toxic to very early life stages of walleyed pike, and both large- and smallmouth bass. Both diquat and glyphosate are adsorbed by soil particles, so their effectiveness is reduced in muddy water (Masser *et al.*, 2001; Funderburk and Lawrence, 1963).

Due to its overall properties, diquat has become the most frequently used treatment for *S. molesta* infestations. Even so, the cost of chemical control can be considerable for a large infestation. Chilton *et al.* (1999) estimated the cost of diquat treatment at \$185.25/ha (\$75.00/acre) (chemical alone) or \$427.31/ha (\$173.00/acre) including equipment and labor. At the 1999 price, enough diquat for a complete, one time treatment of a 485.82 ha (1200 acre) mat of salvinia (like the one found on Toledo Bend Reservoir), would therefore cost \$90,000; with labor and equipment included; \$207,600. The other herbicides, excluding 2,4-D, would cost more.

Even if the cost were not prohibitive, herbicides alone are unlikely to provide effective control of major infestations of giant salvinia. Creagh (1991) calculated that a single ramet, under optimal conditions, could become a mat covering 16.2 ha (40 square miles) in three months. Considering the difficulty of spraying every ramet in every slough and inlet, and given

salvinia's prodigious growth rate, a small relict population with sufficient nutrients could return to problem size within a short time. Not surprisingly, Wendell Lorio, the resident biologist at our Gheens, LA, field site, noted that a salvinia-infested canal reverted to its prior state 2 weeks after being sprayed (Pers. Comm., Dr. Wendell Lorio, Biologist, Golden Ranch Plantation, Gheens, LA).

Mitchell and Tur (1975) estimated that salvinia (live) biomass could range from 45-109.5 tons/ha/year. So, if applicators did manage to kill all the salvinia in a 1200 acre mat (like the one at Toledo Bend, LA, in 1998), the huge load of organic matter decomposing in the water would tend to drive the dissolved oxygen in that area to anoxic levels (Ross and Lembi, 1999e). This could be harmful to fish and other aquatic life (Tasker, 2001; Oliver, 1993), and possibly cause substantial mortality to fish populations (Kannan, 1979; Murphy and Barrett, 1990c).

Other than small or isolated infestations, herbicides are only a temporary solution for salvinia control (Charudattan, 2001). The chemical alternatives are either to spray herbicides on a regular basis, indefinitely, in order to reduce the severity of the infestation, or to risk causing anoxic conditions that could result in extensive damage to aquatic ecosystems. Maintenance control is a program defined as "a method for the control of non-indigenous plants in which control technologies are utilized in a coordinated manner on a continuous basis in order to maintain the plant population at the lowest possible level as determined by the Florida Department of Environmental Protection" [Florida Statutes 369.22 (2) (d), 2009]. Maintenance control has been used to good effect to control water hyacinth with 2,4-D in Florida (Simberlof, 1996). The goal is to maintain surface coverage of water hyacinth at 5%, which has been shown to halve herbicide use and improve sedimentation and dissolved oxygen values, compared to

water bodies whose surface is covered (Schardt, 1997). Presumably, control technologies would include biocontrol, mechanical procedures, herbicides, and water level management, all of which are used to mitigate infestations of aquatic weeds in the U.S. (Chilton *et al.*, 1999). However, the most common practice is the frequent application of herbicides (Center *et al.*, 1999). The cost is considerable, however, and infestations can rebound quickly if herbicide use is discontinued (Center *et al.*, 1999). Indiscriminate herbicide application may cause widespread mortality of natural enemies of aquatic weeds, but host plant quality (especially nitrogen content) may benefit from careful herbicide use because of reduced competition among a less crowded host plant population (Center *et al.*, 1999). Host plant quality is one of the important drivers of population growth of *C. salviniae* (Julien *et al.*, 1987). Therefore, control efforts may benefit from the judicious application of IPM principles (Hill and Olckers, 2001). Most herbicides target non-specific processes (such as photosynthesis), which can alter community structure when applied continuously, and confound efforts to restore native habitat (Murphy and Barrett, 1990c).

Biocontrol Efforts with *Cyrtobagous salviniae*

Surveys conducted in the years 1978-81 identified three potential phytophagous biological control agents: *Cyrtobagous singularis* Hustache, *Paulinia acuminata* (De Geer), and *Samea multiplicalis* (Gueneé) (Forno and Bourne, 1984). None of these ultimately turned out to be an effective biocontrol agent when deployed as a control measure for *S. molesta* (Sands, 1983). A fourth candidate, *Cyrtobagous salviniae*, was initially misidentified as *Cyrtobagous singularis* and deployed along with *Cyrtobagous singularis* as part of a successful control program against *S. molesta* in Australia. It was not until a population of weevils composed

predominantly of *C. singularis* was released unsuccessfully against *S. molesta* in Papua New Guinea that further research led to the correct identification of *C. salviniae* (Calder and Sands, 1985). The successful release of *C. salviniae*, and the subsequent reduction of the floating mat to 1% of its original size, established that *C. salviniae* was a potentially useful biocontrol agent against *S. molesta* (Thomas and Room, 1986), and the insect responsible for a similar feat on Lake Moondarra, in northwest Queensland, Australia (Julien *et al.*, 2002).

Cyrtobagous salviniae is a specialized herbivore whose larvae burrow in the rhizomes of the plant. Adults consume buds and leaves. Floating mats of salvinia may sink from the cumulative damage caused by the two feeding stages (Thomas and Room, 1986). *Cyrtobagous salviniae* has been deployed successfully against *Salvinia molesta* throughout its introduced range (Calder and Sands, 1985; Tasker, 2001). *Cyrtobagous singularis* does feed on *S. molesta*, but was found to be ineffective due to its relatively low reproductive rate and feeding characteristics. *Cyrtobagous singularis* larvae feed on the outside of roots and rhizomes, which does not result in destruction of those structures. Along with the adults, larval feeding damages leaves and buds, but the plant is able to compensate, because damage to meristematic tissue is minimal (Thomas and Room, 1986). *Cyrtobagous singularis* was collected from, and is adapted to, *Salvinia auriculata* Aublet, which has higher nitrogen content, so reduced nitrogen levels have a greater impact on the reproductive rate of *Cyrtobagous singularis* (Sands *et al.*, 1986).

A population of *C. salviniae* in Florida, believed to be introduced accidentally, was also incorrectly identified as *C. singularis* (Kissinger, 1966). Weevils from the Florida population are smaller than weevils from the Brazilian strain, and though they differ genetically, the significance of this finding is not entirely clear at present (Goolsby *et al.*, 2000). Madeira *et al.*

(2006) reported that there are many more similarities than differences when the genomes of the two weevils were compared. The genetic distance was five times greater between *C. singularis* and *C. salviniae* than that between the Florida and Brazilian populations of *C. salviniae*. Florida weevils are currently considered an ecotype (Madeira *et al.*, 2006).

It has been suggested that the Florida population of *C. salviniae* is responsible for the less invasive nature of *S. minima* infestations in Florida, as opposed to those in Louisiana and Texas (Jacono *et al.*, 2001), and that they could help to control infestations of both species in other states (Madeira *et al.*, 2006). Since the 'Florida weevil' is apparently naturalized, this would also be a less risky option, ecologically speaking (Jacono *et al.*, 2001). Tipping and Center (2005a) showed that both the Brazilian and Florida strains preferred large (tertiary) plants in the laboratory, but neither exhibited a preference for either *S. minima* or *S. molesta*. Their data, however, is based on small sample sizes, and was not significant at $\text{ALPHA}=.05$. In a 2005 study, Tipping and Center (2005b) examined the population dynamics of *C. salviniae* on *S. minima* in South Florida, but did not relate it to biomass values obtained from sampling treated vs. untreated plots. Consequently, the effectiveness of the Florida strain of *C. salviniae* as a control measure for *S. molesta* is difficult to evaluate.

In 1999, weevils from the Florida population were released at a major infestation at Toledo Bend Reservoir, in Sabine Parish, LA, and other infestations in Texas and Louisiana (Tipping and Center, 2003). Unfortunately, the introductions were not successful, so weevils from the Brazilian strain were substituted (Tipping and Center, 2003). These did become established at a number of locations, and *S. molesta* biomass was dramatically reduced at several of the infestations. At most of the release sites, however, stochastic events (floods,

hurricanes, vandalism) complicated efforts to introduce *C. salviniae* or collect data (Tipping *et al.*, 2008). Brazilian weevils were also introduced unsuccessfully at four *S. minima* infested field sites in Louisiana. The locations were at Joyce Wildlife Management Area, Maurepas Wildlife Management Area, and on private land in Vacherie, La., all in 2003, and at Cypress Lake (at Moss Bluff, LA), in 2005 (Pers. Comm., S. Johnson). Florida weevils were released at an infestation of *Salvinia minima* at Jean Lafitte National Historical Park and Preserve in Jefferson Parish, LA, in 2002–2005. This colony did appear to become established, at least initially. However, little activity was seen in the spring of 2004, and the project was abandoned after Hurricane Katrina. Sampling by Dr. Seth Johnson, of the LSU Agcenter, indicated that there was a significant number of Florida weevils present at the Jean Lafitte site in 2008. Further sampling in September of 2009 indicated that weevils were present, and the population appeared to be established (Pers. Comm., S. Johnson). Substantial numbers of weevils were also found in 2010 samples (Pers. Obs.)

Life History of *C. salviniae*

Oviposition begins 6-14 days after emergence at 25.5°C. In laboratory studies at constant temperatures, oviposition did not occur below 21°C (Forno *et al.*, 1983). Mated females typically deposit one egg every 2-5 days in shallow holes excavated in plant tissue (Fig. 4a). Forno *et al.* (1983) reported that females can continue to oviposit for at least 60 days, but Sands *et al.* (1986) found that weevils maintained on nitrogen-rich salvinia oviposited 374 times in 14.5 weeks, and Jayanth (1989) recorded 290 ovipositions over a nine month period. Eggs hatch in 10 days, and the larvae (Fig. 4a & 4b), which are about 1 mm in length, feed on roots and young buds. However, eggs do not hatch below constant temperatures of 20 °C or above

36° C (Forno *et al.*, 1983). The neonate larvae rapidly begin to burrow within the rhizomes, where they undergo three developmental instars in 23 days. Both the developmental rate and the amount of tunneling that takes place are dependent on host quality and temperature; more tunneling takes place if plants are low in nitrogen (Forno *et al.*, 1983). This can increase tissue damage, but the larvae are smaller and take longer to develop. Females are also less fecund. The population rate of increase is lower as a result (Forno and Semple, 1987). Larvae cannot survive at constant temperatures below 16.3° C. When pupation ensues, the cocoons can be found underwater, attached to roots or rhizomes. Pupation lasts 12.6 days at 25.5°C (Sands *et al.*, 1983). Adult *C. salviniae* weevils (Fig. 4d) are light ivory in color when they emerge. They turn light brown within 1-2 days, and then darken to black after 5-6 days. Females are approximately 2.2 mm (L) x 1.2 mm (W), and males are approximately 1.8 mm (L) x 0.9 mm (W), measured from the margin of the pronotum to the apex of the elytra (Calder and Sands, 1985). Adults are found on or among leaves, or underwater among roots. When they are underwater, they breathe by means of a plastron—an air bubble anchored by stiff setae on the ventral surface of the weevil (Forno *et al.*, 1983). Both male and female adults engage in multiple matings for 5-26 days after emergence. Adults feed at temperatures between 13 and 33° C (Forno *et al.*, 1983). *Cyrtobagous salviniae* is gregarious; populations may reach a density of 300 adults and 900 larvae per sq. meter, which was considered necessary by Room and Thomas (1985), and Room (1988) to achieve a desirable level of control. Large scale releases, however, may not be necessary for *C. salviniae* to become established. Tipping *et al.* (2008) reported a significant reduction in salvinia biomass and surface coverage, as well as increased bud damage at three *C. salviniae* release sites at the Toledo Bend infestation, despite the

introduction of low numbers of weevils (5,069) and cold weather. Weevils were released over a four year period. Biomass at the release sites was $2.6 \text{ k/m}^2 (\pm 0.4 \text{ S. E.})$, compared to $8.8 \text{ k/m}^2 (\pm 0.6 \text{ S. E.})$ at the controls. The percentage of damaged buds at the release sites was $41.0 (\pm 4.7 \text{ S. E.})$, while damaged buds at the control sites were $26.3 (\pm 3.3 \text{ S. E.})$. The percentage of the

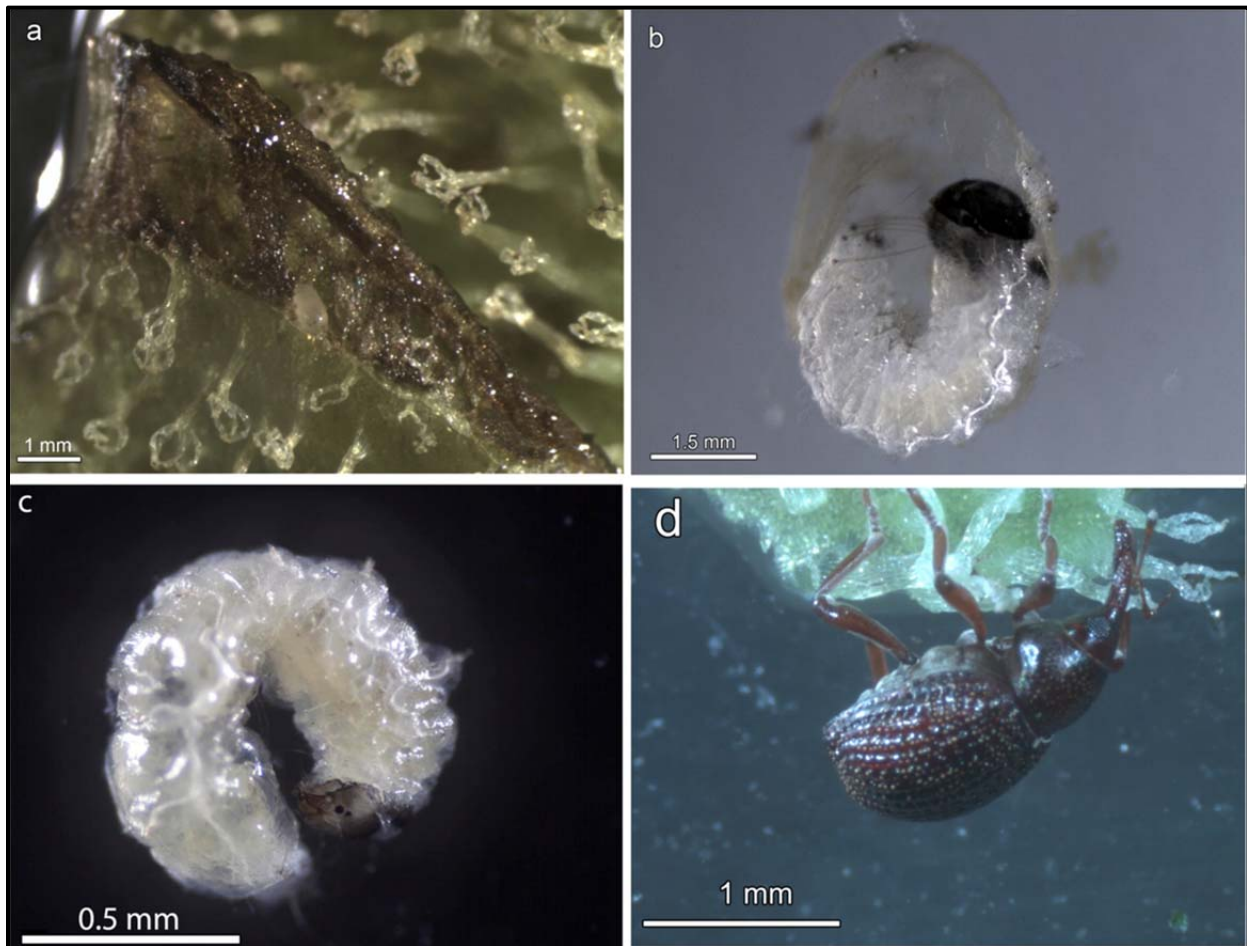


Figure 4. Developmental Stages of *C. salviniae*. (a) An egg in the keel of the leaf-a common oviposition site. (b) An egg containing a larva nearly ready to hatch. (c) The mature larvae. (d) A newly emerged (teneral) weevil.

surface covered with *S. molesta* at the control sites was $41.1 (\pm 5.5 \text{ S. E.})$, as opposed to $91.9 (\pm 2.7 \text{ S. E.})$ at the controls. *C. salviniae* population density (weevils/kilo) was not assessed.

The Classes of Reproductive Development

The most common and reliable techniques for obtaining physiological age estimates are based on the changes that occur in the female reproductive system over time (Tyndale-Biscoe, 1984; Hayes and Wall, 1999). Assessment is based on characteristics of the fat body, development of the various aspects of the ovaries, the presence, location, and development of follicles, and the presence or absence of follicular relics (Tyndale-Biscoe, 1984). Follicular relics are the remnants of the epithelial tissue that surrounds the follicle during its development in the vitellarium. Changes in the characters of the follicular relics are particularly relevant to differentiating the parous stages (Hayes and Wall, 1999). Parous classes describe the reproductive system once oviposition has been initiated. Nonparous stages describe those stages that occur prior to oviposition. The following system has been described by Grodowitz *et al.* (1997), working with *Neochetina eichorniae*:

N1 : Ovaries small, highly tracheated. No distinct follicles present within ovaries. Follicular relics absent. Fat body fills body cavity, creamy white in color, lacks discrete cell clusters. Cuticle is soft, untanned.

N2: Ovaries intermediate in size, between N1 and parous stages. Tracheae present in large numbers. Clearly differentiated follicles are present, more mature follicles evident at proximal end of vitellarium. Proximal follicles lack yolk (transparent). Fat body reduced in volume, still smooth, and does not completely obscure ovaries. Cuticle partially hardened.

N3: Ovaries larger than N2, with less tracheation. Proximal follicle has fully developed yolk. No clear areas, and nucleus is indistinct. Fat body does not fill body cavity and ovaries visible. Discrete fat cells visible at low magnification. No follicular relics present. Cuticle is hardened.

P1: Ovaries larger than any nonporous stage, with a limited number of tracheae. Distinct follicles are present in the vitellarium and may be present in the lateral and common oviduct. Limited number of ovipositions have taken place. Fat body does not fill body cavity, and contains adult cell

clusters. Follicular relics are present, but do not completely encircle the base of the ovariole.

P2: Similar to P1 in most regards, but the vitellaria contain a higher number of follicles in a progressive state of development. Follicular relics surround ovariole base, but no distinct darkened particles present. Significantly higher number of ovipositions have taken place. Fat body of “lean” type.

P3: Characters similar to P2, but darkened particles are apparent within the follicular relics, which may or may not fill the ovariole base.”

Note: These characters may change as the ovaries degenerate due to age or environmental conditions. This can make it difficult to distinguish between individuals that have degenerate ovaries because of environmental challenge, and those that undergo physiological age-related senescence. Physiological age-grading can be used to estimate numbers of ovipositions, and differentiate between parous and nonparous individuals, or between individuals that have oviposited a few vs. a large number of eggs. Natural variation prohibits activities other than assignment to physiological age classes and other approximations.

Development of the Follicle

In the germarium, each oogonium divides and produces two daughter cells, one of which is a functional stem cell, and one that is an oocyte (Chapman, 1998). The oocyte enlarges as it migrates toward the margin of the germarium, where it is embedded in prefollicular tissue as it enters the vitellarium. Initially, there are three or four layers of prefollicular tissue; these divide rapidly as the follicle grows (Chapman, 1998). Eventually, the follicular epithelia form a single layer of cuboid or columnar cells, which become stretched into a squamous layer as the oocyte undergoes rapid growth during yolk accumulation (Chapman, 1998). Nuclear division

continues in the follicular epithelial cells after mitosis has ceased, producing multiple nuclei that synthesize minor yolk proteins, enzymes, and ecdysone. These eventually fuse into a germinal vesicle. As the oocyte develops, follicular epithelial tissue produces the vitelline envelope, and eventually, the chorion (Chapman, 1998).

An oocyte with its attendant epithelia is termed a follicle. The follicle grows rapidly in size, accumulating yolk as it transits the vitellarium. This process is greatly accelerated as the follicle assumes the terminal position, adjacent to the lateral oviducts (Chapman, 1998). The majority of yolk proteins and lipids are produced by the fat body (Chapman, 1998), and transported to the oocyte. As the mature follicle exits the vitellarium, the epithelial tissue that encapsulates the oocyte is stripped off by the pedicel, and remains in the base of the vitellarium (Chapman, 1998). These are termed follicular relics, and they accumulate over time (with each oviposition), which allows a rough estimate of the number of ovipositions that have taken place. A developmental continuum forms within the vitellarium over the reproductive life of the female (Chapman, 1998). *Cyrtobagous salviniae* has a telotrophic reproductive system, in which nurse (sister) cells remain in the germarium and form a trophic core that provides nutrients to the developing follicles through a nutritive cord (Chapman, 1998).

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The Reproductive Morphology and Development, Oviposition, and Age-Grading of
Cyrtobagous salviniae Calder and Sands

INTRODUCTION

The invasive floating fern, *Salvinia molesta*, has caused profound ecological, environmental and infrastructural problems in more than twenty tropical and subtropical countries (Storrs and Julien, 1996). *Salvinia* was first reported in North America in 1995 (Johnson, 1995). Its North American range is primarily in the Deep South, but it has also been found in California and in Virginia (Jacono *et al.*, 2001). In Louisiana, *S. molesta's* range has grown to an estimated 70,000 acres in 2010 (Johnson *et al.*, 2010). In 2009, it was estimated that there were 17,292 acres of *salvinia* in state managed water bodies, but repeated herbicide applications, along with a hard freeze in the winter of 2009-2010 reduced that figure to 2,706 acres (Pers. Com., A. Perret, Louisiana Department of Wildlife and Fisheries). However, the surviving *salvinia* has been proliferating rapidly (Pers. Obs.), and control efforts continue at Lake Bistineau, Caddo Lake, Spanish Lake, the Atchafalaya Basin, Toledo Bend Reservoir (Fig. 5), and other locations. Current control efforts often utilize a number of different techniques, applied simultaneously to address infestations in different habitats, such as cypress swamp, mud flats, open water, and flooded grasslands. Control methods for *salvinia* include chemical herbicides, biological control, mechanical harvesting, and cultural methods such as drawdowns (Tasker, A. V., 2001). Chemical control is the primary means of control in the U.S., and is widely employed. However, chemical control is an expensive and often difficult option that must be conducted on a continual basis to achieve a significant reduction in *salvinia* biomass (Charudattan, 2001; Center *et al.*, 1999; Simberlof, 1996). This can be especially difficult in complex environments like cypress swamps, where deploying spray equipment in dense underbrush can be challenging (Schardt, 1997). Cultural methods can be very helpful in the short term; drawdowns strand and

dehydrate large quantities of salvinia on dry ground, which reduces the scale of an infestation. This has proven to be very useful at Lake Bistineau in northern Louisiana, an effect that was enhanced by two consecutive, unusually cold, winters. Repeated herbicide applications also played a role. (Pers. Com., A. Perret). It is not usually possible to draw down an entire water body; hence re-infestation may be rapid when the impounded salvinia encounters nutrient-rich water and open spaces after re-flooding. Drawdowns also kill native aquatic organisms, and preclude recreational use. Mechanical harvesting is time-consuming, subject to breakdowns, and ineffective (Mitchell, 1979; Chilton *et al.*, 1999). Biological control is a promising option, using an herbivorous weevil, *C. salviniae*, the only biocontrol agent that has been shown to have a significant impact throughout most of the introduced range of *S. molesta* (Julien *et al.*, 2002;



Figure 5. A salvinia infestation at Saunders Creek, Toledo Bend Reservoir, LA, in 2008. Photo courtesy of Howard Elder, Texas Parks and Wildlife Department.

Thomas and Room, 1986). However, the weevil has failed to provide a consistent level of control in temperate regions (Hennecke and Postle, 2006), or under climatic extremes such as drought (Tipping *et al.*, 2008). In other cases, the reasons are unknown (Skeat, 1990). Research that would enhance our understanding of the physiological age structure of *C. salviniae* field populations could be of great utility in the successful deployment of this important biocontrol agent, as would information on the relationship between host plant nutrition and oviposition.

Physiological age-grading provides a means for investigating these parameters, as well as contributing factors that may drive *C. salviniae*'s reproductive rate. In order to develop a physiological age-grading system, it is necessary to describe the general morphology of *C. salviniae*, identify characters (if present) that allow reproductive development to be grouped into classes, and link those classes to the number of eggs oviposited by individuals whose physiological age is known. The first experiment addresses the first two requirements, and the second experiment addresses the third.

OBJECTIVES

- Describe in detail the female reproductive morphology.
- Observe the changes that occur over time in the reproductive system of *C. salviniae* in order to identify characters that can be used to group individuals into classes that reflect the continuum of reproductive development.
- Quantify oviposition, and relate it to the physiological age classes.

MATERIALS AND METHODS

The Reproductive Morphology and Development of *Cyrtobagous salviniae*

A colony of *C. salviniae* weevils (referred to hereafter as weevils), was established at The Range Campus Greenhouses, located at 436 S. Campus Dr., on the Louisiana State University campus in Baton Rouge, LA (30° 24' 40.870"N, 91° 10' 20.658"W). The founding population was obtained from a field site (a six acre pond) at Golden Ranch Plantation, in Ghens, LA, that was used to mass-rear weevils to release at giant salvinia infestations around the state. The weevils were held under greenhouse conditions, on salvinia grown in 567.8 l (150 gallons) tanks (cattle troughs, Rubbermaid Commercial Products, Rubbermaid Corporation, Winchester, VA) (Fig. 6) containing pure water (pH 7) produced by a reverse osmosis filter (model LCRO, 200 gpd, Culligan International Co., Rosemont, IL). The greenhouses were covered with 70% shade cloth (Aluminet, Polysack U.S.A., Inc., San Diego, CA), and equipped with fans and evaporative coolers, as well as heaters for the winter months. Temperatures in the greenhouse ranged from 27° C to 35° C in the daytime during the warmer months (when the population is growing rapidly), and dropped to 16° C to 20° C at night. Heat was set to 26° C in the winter, but daytime temperatures sometimes reached 35° C. The tanks were fertilized twice weekly in order to maintain a concentration of 2 ppm of nitrogen, using Miracle-Gro® Liquid Lawn Food (Scotts Miracle-Gro Company, Marysville, OH). This product has a NPK rating of 36-6-6 (mass %), and contains 0.325 chelated iron. Thuricide® (Bonide Products, Oriskany, NY) was sprayed twice weekly to control *Samea multiplicalis* Guenee', whose larvae feed on salvinia. To ameliorate extremes of temperature, the sides and rim of each tank was insulated with bubblewrap/ foil insulation (Reflectix Inc., Markleville, IN). Also, fresh salvinia



Figure 6. Salvinia in the greenhouse in the summer of 2011

was added to culture tanks as needed to replace vegetation consumed by herbivores. High intensity discharge (HID) lamps (models ED28 and ED18, LunaPro Lighting, Miami, FL) were used to extend photoperiod to 14:10 (L:D) in the winter and early spring months to keep females reproductively active after the autumnal equinox. To obtain weevils for dissection, weevil-infested salvinia was collected from a field site at Golden Ranch Plantation, in Ghens, LA, and desiccated in Berlese funnels (Fig. 7) using reptile lamps (Fluker Farms Inc., Port Allen, LA) equipped with 125 watt brooder bulbs (Model #125BR401RP, Osram Sylvania Inc., Danvers, MA). The Berlese funnels were fabricated by LSU Facilities Services personnel. Weevils were then collected from a ramet of salvinia floating in two inches of water in a 10.2 cm x 21.6 cm (4" x 8.5") Whirlpak bag (Nasco Whirl-Pak, Ft. Atkinson, WI) secured to the stem of the funnel.

Initial efforts to obtain specimens for dissection were problematic. In order to provide healthy specimens for dissection, a cohort of 120 weevils was then initiated in a lab incubator (Intellus Environmental Controller, model # 136VL Percival Scientific, Inc., Perry, IA) to describe female reproductive morphology and identify the classes that characterize reproductive development. A protocol derived from a pilot study (unpublished data) was used to maintain



Figure 7. Large Berlese Funnels with brooder lamps that desiccate salvinia samples and allow the retrieval of live *C. salviniae* weevils for dissection or other research purposes.

the cohort in 266 ml jars (Qorpak, Inc., Bridgeville, PA), each of which contained a mated female. These were held in the environmental chamber at $29.5^{\circ}\text{C} (\pm .5 \text{ S. E.})$, with a 12:12 photoperiod (L:D). The pilot study indicated that females did not need a male present after mating in order to oviposit. If a resident weevil died, the jar then received a newly emerged

(teneral) weevil, so that specimens of different physiological classes would be available for dissection, and their morphological characters could be identified. Weevils intended for the cohort were harvested by collecting samples of giant salvinia from weevil-infested tanks in the greenhouse. The samples were dessicated in Berlese funnels, using the method described above. The Berlese sample was searched for weevils in the lab under a Leica MZ75 dissecting microscope (Leica, Inc., Basil, Switzerland), using an angled microdissection forceps (FST 11063-07, Fine Science Tools, Inc. (USA), Foster City, CA). The weevils were separated into black (mature) and brown (teneral) groups. The brown weevils were held overnight in 125 x 25 mm Petri dishes (Nun-Tek, Fisher Scientific, Pittsburgh, PA), in groups of twenty, to allow the weevils to mate. The black weevils were returned to the greenhouse colony. The following day, the weevils were placed in jars with their lower half masked with duct tape (Ace Hardware Corporation, Oak Brook, IL), and Tulle mesh (Mandel Fabrics LLC, Johnstown, NY) secured over the top with rubber bands to prevent escape.

The jars were provisioned with salvinia from the greenhouse, floating in 150 ml of reverse osmosis water. Every week each of the ramets were placed in a Petri dish. The jar was rinsed, and scrubbed vigorously with a test tube brush (Fisherbrand, Fisher Scientific, Pittsburgh, PA). On rare occasions, it was necessary to wash the jar(s) with Palmolive Pure and Clear® dish detergent (Colgate-Palmolive, NY, NY) to remove algal growth. The jar was then filled to the top of the duct tape (about half-way) with reverse osmosis water, and a fresh ramet with at least one bud was placed in the jar. The weevil was then located and transferred to the new ramet with a microdissection forceps. The Tulle mesh was re-fastened over the top of the jar, which was labeled with the date on a piece of colored lab tape (VWR International

LLC, Radnor, PA), and returned to the environmental chamber. After 20 weeks, all of the weevils in this cohort were dissected by the following method: A ramet, along with its resident weevil, was removed from a jar. The ramet was placed in a Petri dish, and the weevil was transferred to a 10.2 x 10.2 cm (4" x 4") Kimwipe (Kimberly-Clark, Roswell, GA) on another Petri dish to dry. A five mm drop of Instant Krazy Glue (distributed by Elmer's Products, Columbus, OH) was applied to a microscope slide, stirred, and allowed to partially dry. At this point the weevil was placed, ventral side up, in the glue, and pressed lightly to secure it to the slide. The glue was then allowed to dry thoroughly before beginning the dissection. Sometimes it was necessary to hold the weevil down until the glue began to set. The number of the jar was written on the slide. Sets of 4-6 slides were prepared before starting, and one or more weevils were glued between dissections to lessen prep time. When it was ready, the slide containing the weevil was placed on the stage of a dissecting microscope (Leica MZ125, Leica, Inc., Basel, Switzerland) and covered with Phosphate Buffered Saline (pH 7.4) (Sigma-Aldrich, St. Louis, MO). A microdissection forceps was used to steady the weevil while the edges of the elytra were carefully cut away from the abdominal tergites with a disposable scalpel (Sharpoint, Surgical Specialties Corporation, Reading, PA). The terminalia was grasped with a second microdissection forceps, and pulled horizontally out of the abdomen until the entire reproductive system was visible. The terminal ligaments were then severed with a Superfine Vannas Scissors (World Precision Instruments, Sarasota, FL). The ovaries were kept in the drop of Phosphate Buffered Saline, and pulled gently toward the edge of the drop, where they could lay flat. Phosphate Buffered Saline was removed with the corner of a rolled Kimwipe®

(Kimberly-Clark Global Sales, Roswell, GA) and the ovaries manipulated so that they could be further investigated or photographed under a cover slip. The gross morphology of each individual's ovaries was observed. The appearance and number of follicles in the vitellaria was noted. Somatic characters were also assessed. Fat body was graded as fat (filling the haemocoel), intermediate (1/3-2/3 full) or lean ($< 1/3$). The nonparous classes could be quickly recognized by a soft brown cuticle. A stage constructed of microscope slides, and secured with Krazy Glue® was used to elevate the specimen, which allowed light to be transmitted from below. This helped to obtain micrographs of transparent structures that were difficult to photograph.

Oviposition and Physiological Age-Grading

A cohort of 100 female *Cyrtobagous salviniae* weevils was maintained in jars, using the methods detailed above. Newly emerged (teneral) weevils were obtained from the same greenhouse colony, using the same foundation stock, Berlese funnels and techniques. The weevils were maintained in a separate incubator, but the protocol was very similar to that used for the cohort used to investigate morphology, which is detailed above. However, oviposition cohort jars were labeled with a number as well as a date, in order to keep track of the number of eggs oviposited by each individual. In addition, the ramets removed from the jars each week were dissected and searched for eggs, using the following method: After the roots and leaves were removed, the rhizome was cut into 3-6 mm pieces with a cuticle scissors (La Crosse Division, District Delaware Laboratories, Inc., Uniondale, NY), and lined up on microscope slides. The leaves were pulled apart at the keel and arrayed on a Petri dish lid. Both leaves and rhizomes were moistened to prevent dessication, and covered with the inverted Petri dish until

needed. They were then examined under a MZ75 dissecting microscope (Leica, Inc., Basel, Switzerland). It was necessary to remove most of the tissue from the rhizomes with a microdissection forceps to find all the eggs. This was accomplished with a series of grasping motions while the rhizomes were stabilized with a Rubis # 2 forceps (Rubis Company, Switzerland).

At the end of the study, all of the females from the oviposition study were dissected, using the same techniques detailed above. The number and location of eggs were recorded for each weevil. The mean and standard error were calculated for each class, and ANOVA and Tukey-Kramer procedures were performed (SAS Institute, Cary, N. Carolina). Weevils that produced no eggs for three weeks were replaced with teneral weevils from the greenhouse colony, so that specimens of different classes would be available for dissection, and their characters identified. Some, but not all, of those removed were dissected and found to be misidentified males. Similarly, weevils that died without producing eggs were replaced with brown weevils from the greenhouse.

No reliable method of determining the sex of a *C. salviniae* weevil had been tested at that time. Females are, on average, larger than males, but there is a great deal of overlap in the distribution of the size of the two sexes. Larger weevils were selected for the lab colony, but the only way to determine if a weevil would produce eggs was to hold them for three weeks (after mating) and see if oviposition commenced. To complicate things further, some intra-colony mortality was inevitable, but how much was impossible to predict. If weevils oviposited before they died, they were dissected and classified (by reproductive characters) at that time, provided they had not decomposed too badly to identify characters. If they had not oviposited, another

weevil was placed in the jar, and the process began all over again. All living individuals were held for the duration of the study. Information on the earlier parous classes (P1 and P2) was obtained from weevils that died prematurely, or were added to the colony to replace nonproductive individuals.

RESULTS AND DISCUSSION

The Reproductive Morphology of *Cyrtobagous salviniae*

The reproductive system of a female salvinia weevil is similar to that of the rice weevil, *Sitophilus oryzae* (L) (Perez-Mendoza, 2004), the granary weevil, *Sitophilus granarius* (L) (Richards, 1947), and the boll weevil, *Anthonomus grandis* Boheman (Grodowitz and Brewer, 1987). All four are meroistic and telotrophic. The ovaries of telotrophic insects have trophocytes (specialized nurse cells) that remain in the germarium and supply nutrients to follicles through a nutritive cord (Chapman, 1998). Although no clear photos of the nutritive cord were obtained, only a few primitive orders have panoistic ovaries, and the distinctive clusters of nurse cells found in polytrophic ovaries (Chapman, 1998) were not present. The reproductive system of a female salvinia weevil (Fig. 8a) consists of a pair of ovaries, each of which is composed of two tubular ovarioles. The ovarioles are joined at the proximal end, where they are connected to the lateral oviduct. The lateral oviducts unite to form the common oviduct through which eggs must pass for oviposition to take place. An ovariole is composed of two layers, an outer ovariole sheath (tunica externa) and an inner tunica propria, which forms the walls of the germarium vitellarium (Chapman, 1998). An ovariole has two regions, a distal germarium and a proximal vitellarium (Fig. 8a). Oogonia in the germarium produce the follicles, which are surrounded by epithelial cells prior to entering the vitellarium (Chapman, 1998).

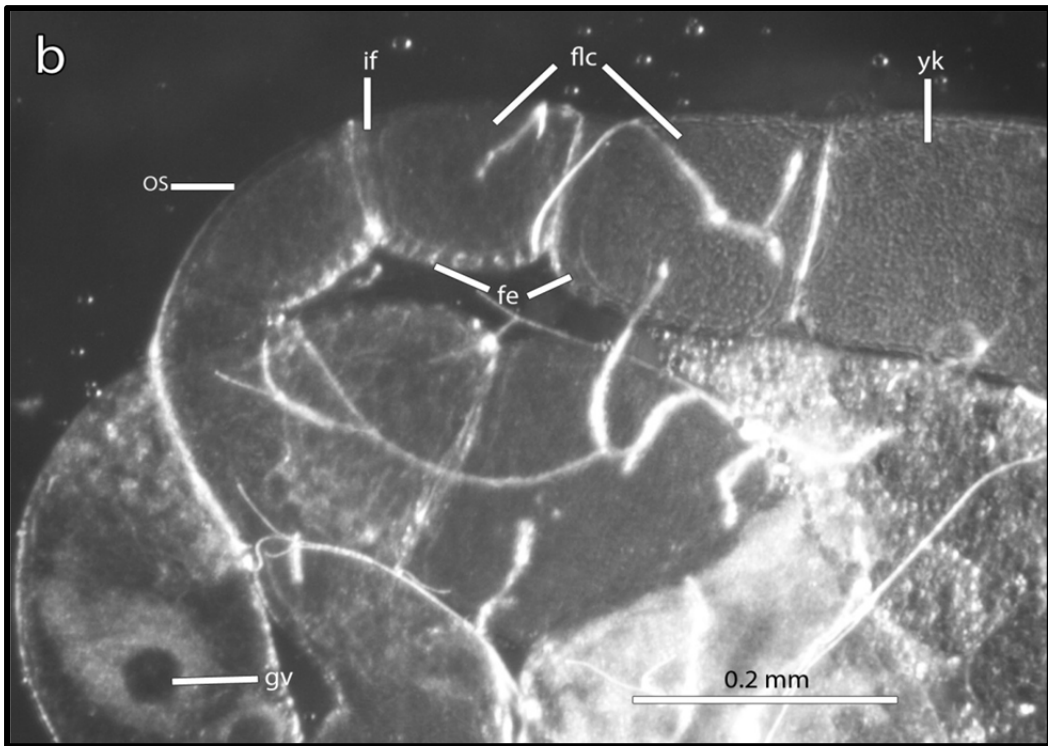
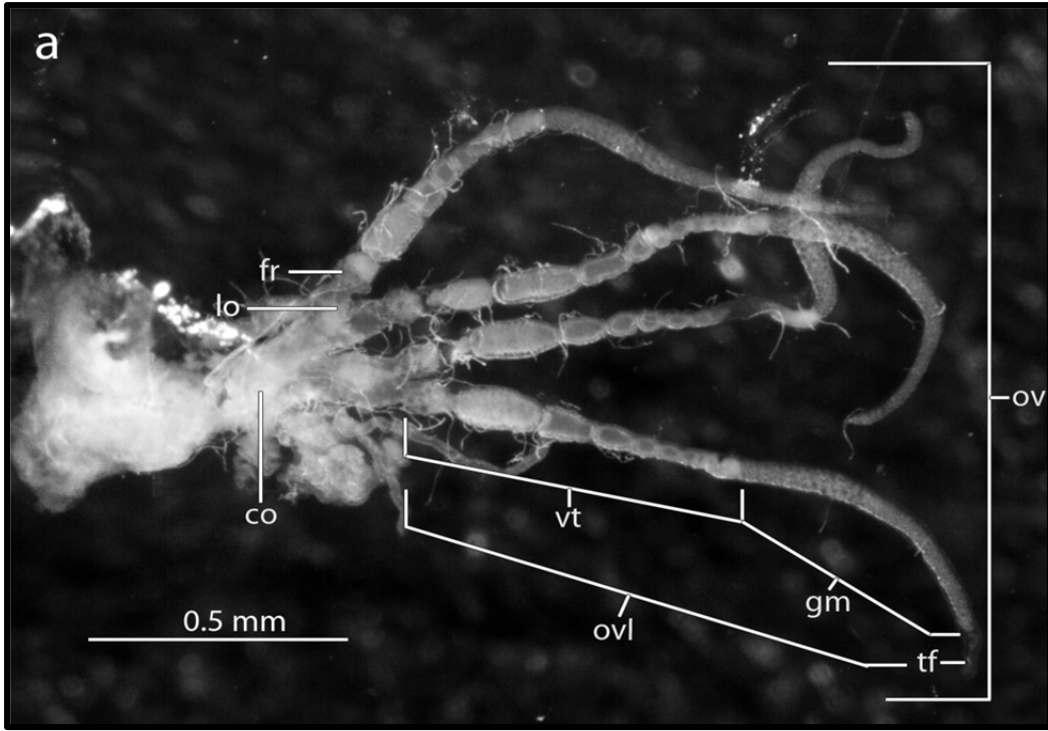


Figure 8a. The Reproductive Morphology of *C. salviniae*. ov, ovaries; tf, terminal filament; fr, follicular relics; lo, lateral oviducts; co, common oviducts; vt, vitellarium; gm, germarium; ovl, ovariole. Fig. 8b. Detail of Follicle. os, ovariole sheath; if, interfollicular tissue; flc, follicle; yk, yolk; gy, germinal vesicle; fe, follicular epithelia

The vitellarium is an elongated chamber that houses the developing follicles. As follicles mature, they move toward the lateral oviducts, so a progression of developing follicles develops over time, with newly evolved follicles adjacent to the germarium, and the most mature follicles proximal to the lateral oviduct (Fig. 8a). The follicle is initially clear, and then becomes translucent as it fills with yolk while developing in the vitellarium (Fig. 8b). The ovariole walls taper at the proximal end of the vitellarium. According to Tyndale-Biscoe (1984) the outer epithelial layer is stripped off the follicle as the oocyte exits the vitellarium and enters the lateral oviducts. The retained material is known as a follicular relic (Fig. 9a). Follicular relics collect at the proximal end (base) of the ovariole, where they eventually form a slim yellow ring that can be seen under low (12.8 X) magnification. The yellow color is due to the presence of β -Carotene (Tyndale-Biscoe, 1984). This is a common occurrence that has been observed in many different orders, and is often used to identify mature reproductives (Tyndale Biscoe, 1984). The ring becomes broader over time. Initially the follicular relics are pale yellow; eventually they turn a deeper yellow, and then darken to an amber color as more ovipositions take place (Fig. 11 a, b). When a greater number of ovipositions have taken place, dark specks begin to appear in the follicular relics, due to compression from oocytes squeezing through the accumulated mass of follicular relics at the base of the ovariole (Tyndale-Biscoe, 1984). Since changes in the appearance of the follicular relics are associated with oviposition, such changes may provide a means of differentiating the parous classes from each other, and from the nonparous classes as well. A rough estimate of the number of ovipositions may also be obtained. However, females may re-absorb yolk and proteins from their follicles in response to environmental stress (Chapman, 1998). In that case, yolk and proteins are removed from the

oocyte and the follicular epithelium collapses into a resorption body, which persists in the proximal vitellarium, (Chapman, 1998), and is indistinguishable from ordinary follicular relics (Grodowitz *et al.*, 1997). They may also be forced into the lateral oviducts during ovulation (Spurgeon *et al.*, 2003). Because of this variation, the follicular relics may only be used to group individuals into classes on the basis of shared characteristics, such as follicular relics that encircle the base of the vitellarium, or dark specks in the follicular relics. Below the junction with the vitellarium, the lateral oviducts of each ovary extend and join to form the common oviduct, a hollow tube (Figure 8a) through which eggs must pass to be oviposited. The spermathecal duct (Fig. 9b) enters the common oviduct about halfway along its length. This is where fertilization typically takes place (Chapman, 1998). The duct is connected to a translucent brown, sclerotized spermatheca (Fig. 9b) that stores sperm. For most of its length, the spermatheca is roughly sickle-shaped. It terminates in a bilobed, pouchlike structure. The

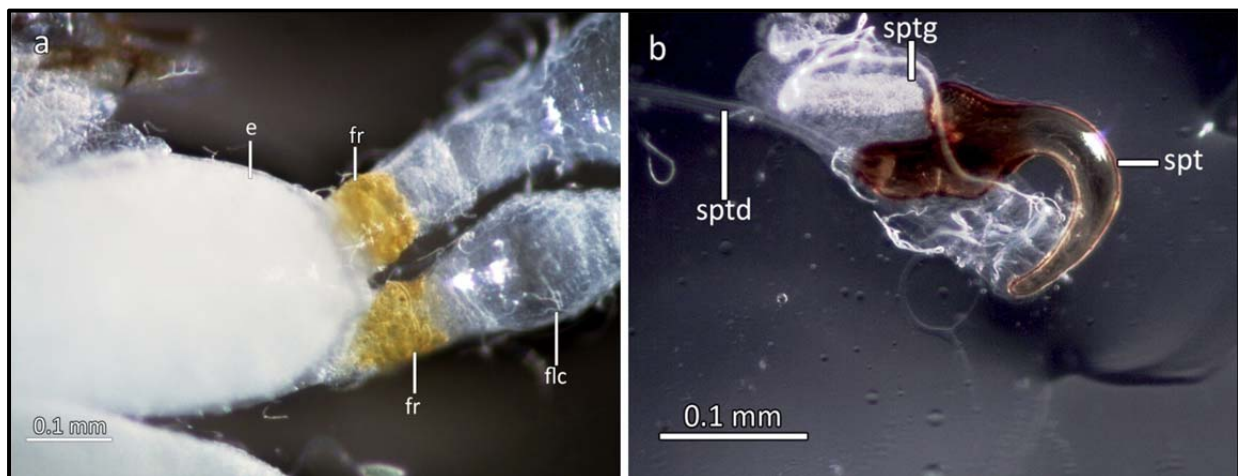


Figure 9. The Follicular Relic and Spermatheca of *C. salviniae*. (a) follicular relics. e, egg; fr, follicular relics; flc, follicle. (b) The spermatheca of *Cyrtobagous salviniae*. sptd, spermathecal duct; sptg, spermathecal gland; spt, spermatheca

presence of masses of motile sperm may be confirmed through the spermathecal walls under magnification of 100-160 X, though sperm may be visible at a lower magnification (12.8 X).

Masses of sperm are visible within the spermatheca in Fig. 10a and b. Sperm is absent in Fig. 9b. Muscle bands are attached to the spermatheca (Fig. 10a), and a spermathecal gland is present (Fig. 10b). In most insects, the spermathecal gland is known to secrete a fluid in which the sperm are conveyed to eggs in the common oviduct, aided by the contraction of the muscle bands (Fig. 10a) (Chapman, 1998). There are no apparent accessory glands. The fat body typically fills the haemocoel and obscures the organs in the nonparous classes of reproductive

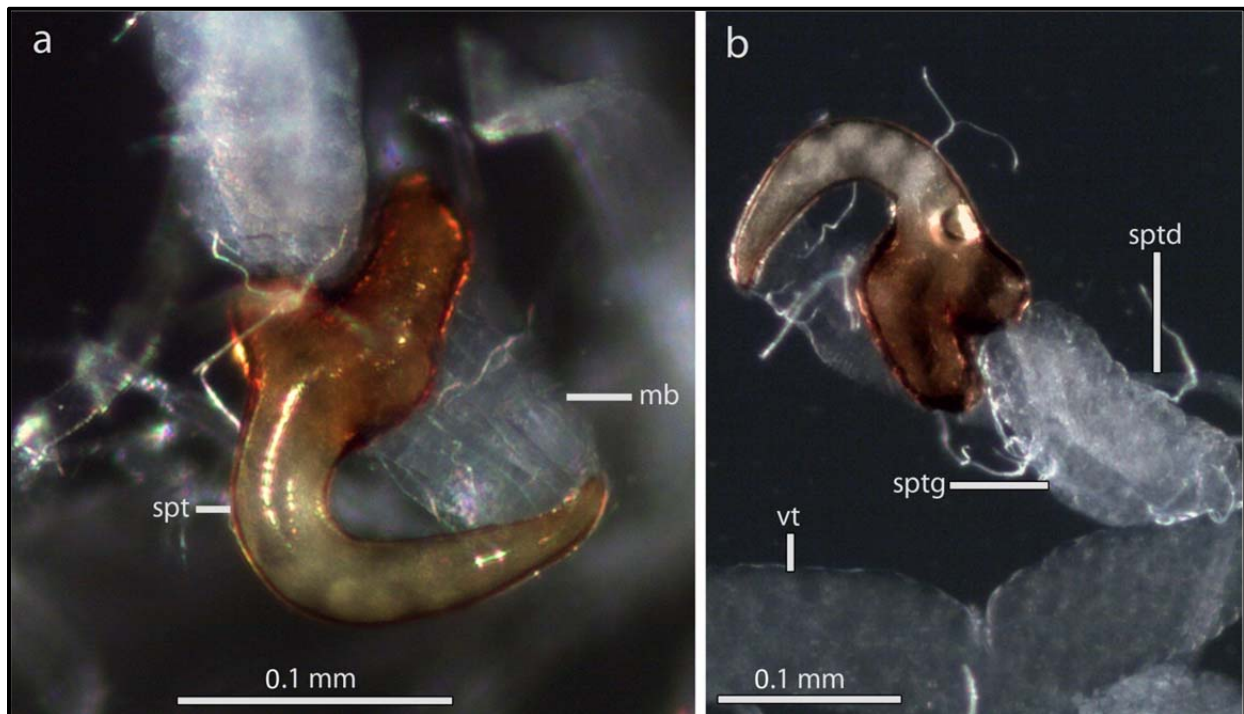


Figure 10. The Spermatheca of *C. salviniae*. (a) spt, spermatheca; mb, muscle bands; (b) vt, vitellarium; sptg, spermathecal gland; sptd, spermathecal duct.

development. Teneral fat body is a creamy white or ivory color, and is present in a smooth semisolid form (Fig. 11). As the individual ages and produces follicles, the fat body diminishes, occupies a smaller portion of the haemocoel, and becomes distinctly granular or “clumped”. It may also take on a greenish cast. A similar sequence of events was observed by Grodowitz (1997) in parous *N. eichorniae*, except that the percentage of individuals that were fat

increased in P2 and P3 individuals. Spurgeon *et al.*, (2003), noted that fat body associated with diapause was intermediate to fat, but cautioned that the development of the reproductive organs and contents of the hindgut may influence perception of fat body status. Lenz *et al.* (2007) reported that that the fat body of *Hydrellia pakistanae* Deonier was initially diffuse and green, and then became clustered and green within one to two hours of emergence. Fat body eventually became white and difficult to observe as it became lean.



Figure 11. Teneral fat body is white, diffuse, and fills the haemocoel in contrast to adult fat body is clumped, yellowish-green, and does not obscure the internal organs.

With advanced age, individuals may exhibit signs of a degenerate or nonfunctional reproductive system. These include a grossly enlarged germarium, a narrowed vitellarium that contains fewer follicles, dense, speckled follicular relics, follicles that may not be maturing in

sequence, and follicles with clear areas (Fig. 13d). These clear areas are due to resorption of yolk, and the resorption bodies thus created are difficult to distinguish from follicular relics due to ovulation (Spurgeon *et al.*, 2003) in *A. grandis*. Grodowitz *et al.* (1997) also noted that, while the relative accumulation of follicular relics typically indicated a higher number of ovipositions, resorption bodies that accumulated at the base of the vitellarium could not be distinguished from ordinary follicular relics. Perez-Mendoza (2004) reported that follicular relics were present at the base of the ovariole in both mated and unmated female *Sitophilus oryzae*.

The physiological classes and their diagnostic characters are listed in Table 1. Ovarioles in the first nonparous, or N1 class (Fig. 12a) can be identified by the absence of visible follicles within the vitellarium, which is a slim, clear tube at this stage of development, with no clear differentiation between germarium and vitellarium. N1 fat body covers the internal organs and is creamy white in color. A secondary characteristic that can be used to confirm a nonparous individual is a soft brown cuticle, indicating recent (i.e., less than one week) emergence. Grodowitz *et al.* (1997) noted that 80% of nonparous *A. grandis* females had soft cuticles, compared to only 10% of parous individuals. Sclerotization of the cuticle takes 5-6 days in *C. salviniae* so some individuals had both black and brown regions of a partially sclerotized cuticle. Follicles first differentiate in the vitellarium during the N2 class (Fig. 12b) and enlarge as they accumulate yolk. A continuum develops over time, with less developed follicles located toward the distal ends of the vitellarium, and more developed follicles closer to the proximal end, near the lateral oviducts. The vitellarium may contain 8 or more follicles. Fat body remains smooth and creamy (i.e., juvenile or larval fat), and still partially obscures the organs. There are no follicular relics because no oviposition has taken place (Fig. 12b). There is a clear differentiation

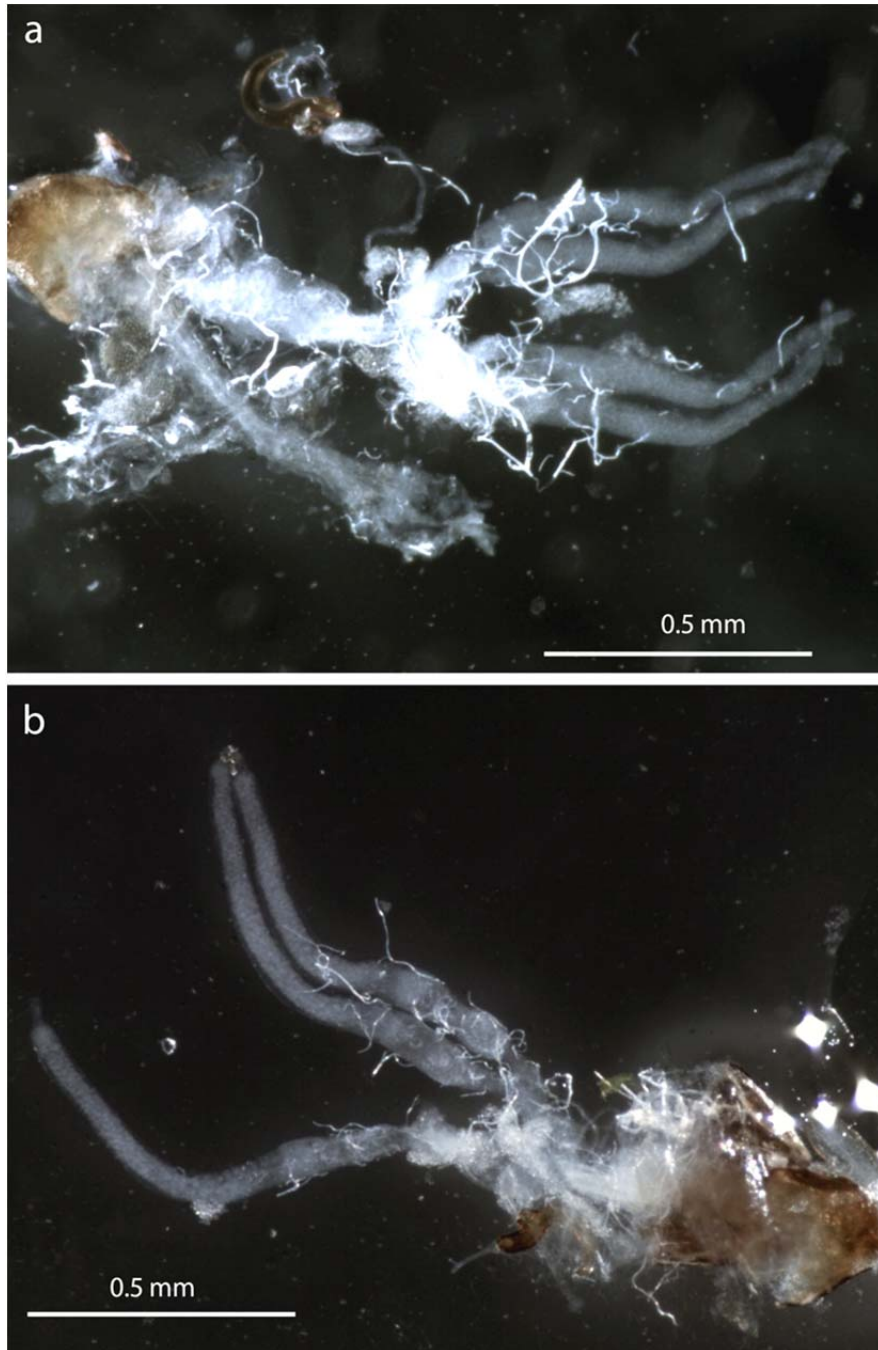


Fig. 12. The Nonparous Classes of Reproductive Development. (a) N1. Note the absence of follicles in the vitellarium. (b) N2. Follicles are present in the vitellaria, but no follicular relics are present.

between germarium and vitellarium. In the P1 class (Fig. 13a), follicular relics are pale yellow and do not completely encircle the vitellarium. Initially, follicular relics loosely occupy a region

at the base of the vitellarium. The ovarioles contain a progression of developing follicles. The terminal follicle is usually larger. A mature P1 female may have up to 8 follicles in the vitellarium (see table 1). Eggs may be present in the lateral or common oviducts. The fat body has a granular or clumped appearance and is intermediate in quantity (fills half the haemocoel). The cuticle is fully sclerotized (hardened and black). An ovary in the P2 class (Fig. 13b) has follicular relics that fully encircle the bases of the vitellaria. They are typically deeper yellow,

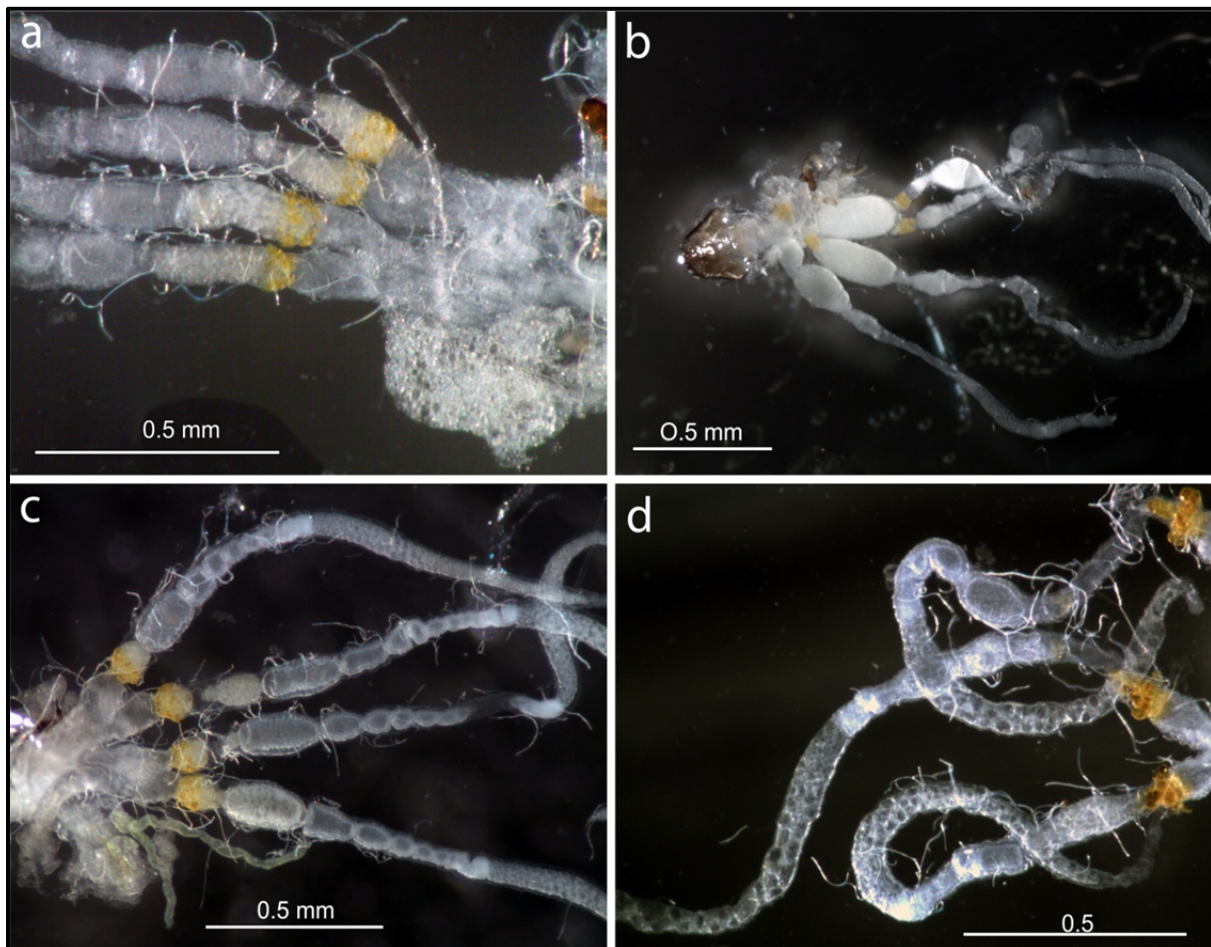


Fig. 13. The parous classes of reproductive development. (a) P1; follicular relics do not completely encircle the vitellaria (contrast was enhanced to show the incomplete ring of follicular relics). (b) P2; follicular relics do encircle the base of the vitellaria. (c) P3; dark specks appear in the follicular relics due to compression from a high number of ovipositions. (d) Degenerate ovaries; very dark follicular relics and very few follicles are characteristic of the ovaries of this aged individual, but are may be lighter if fewer oviposition have taken place.

and broader in width than P1 follicular relics (see fig. 9a, 13b, and 14a and b). No dark inclusions (particles) were present. The continuum of developing follicles and the overall appearance of the ovarioles is similar to that of a P1 individual. The vitellaria contain 8-10 follicles. Fat body is intermediate to lean, and the cuticle tends to be black and harder than P1. Ovaries that are in the P3 class (Fig. 13c) resemble P2 ovaries, except that inclusions (dark specks) are present, indicating compression of the follicular relics from a high number of follicles that have shed their epithelial layer as they passed out of the vitellarium. There may be follicles in the lateral oviducts and the common oviduct. Fat body is lean (little or no visible fat

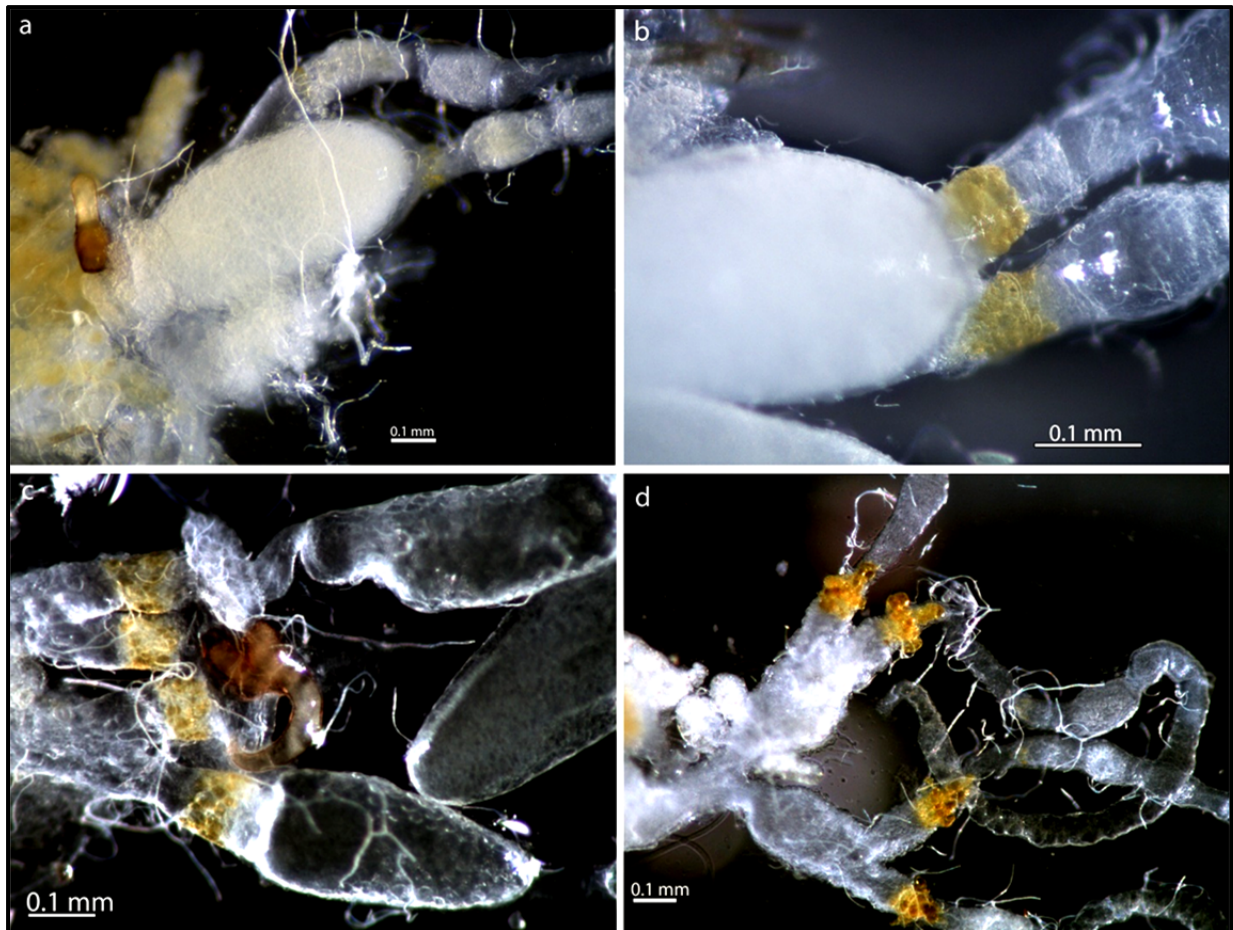


Figure 14. Close-up of Follicular Relics. (a) P1; very light in color, and do not encircle base of vitellaria. (b) P2; do encircle base. (c) P3; dark specks are apparent. (d) degenerate; follicular relics are very dark and compressed in this aged individual.

body), and the cuticle is very hard and black. There may be as many as 8-11 follicles within the vitellaria. However, as weevils approached 20 weeks of age, the number of follicles declined sharply as individuals began to exhibit signs of senescence. The degenerate ovaries of aged individuals (in the late stages of the P3 class) contain few, if any, healthy follicles (Fig. 13d). Other signs of deterioration are often present, such as long slender germaria, and reduced vitellaria that contain follicles that were not sequential in their development. Clear follicles that were not filled with yolk appeared between larger, more developed follicles. Follicular relics were often highly compressed, contained numerous inclusions and were more deeply colored than earlier P3 ovaries. Some of these individuals oviposited at a lower rate, or oviposited with one ovary while the other degenerated. Detail of follicular relics may be seen in Fig. 14. The number of follicles per vitellarium increased from the N2 class to the P2 class, then declined, an indication there were a significant number of P3 individuals whose ovaries were degenerate. Eggs were occasionally located in the oviducts of parous individuals. Follicular relics were by far the most reliable indicator of parity (100%), and their absence as reliable an indicator of nonparity. The characters of the follicular relics that were used to distinguish the classes were also reliable, with the exception of four individuals in the P1 and P2 class that had follicular relics with some characters that resembled P3's (95.6 accurate, $n=92$). This is presumably due to resorption of follicles, which is often caused by insufficient nitrogen or other nutrients in the diet, but also occurs for reasons unknown (Chapman, 1998). The amount of sperm in the spermatheca did not appear to be a good indicator of physiological age, because weevils were held individually in jars for five months after a 24 hour mating period. Individuals in the P1 class had full spermathecae, P2 had a moderate amount, P3 had little or no sperm. However, field

collected females are known to engage in multiple matings for 5-26 days after emergence (Forno *et al.*, 1983), and parous individuals with empty spermathecae were rarely seen, so this was an artifact of experimental design. A review of micrographs of field-collected weevils from Gheens, LA, showed that 127 of 129 parous weevils (98.4%) had sperm in the spermatheca. Somatic changes were also assessed (Table 2). Fat body characteristics were a fairly reliable means of confirming physiological age; 89.7% of P3 individuals (52 of 58) had lean fat body. Cuticle color was found to be reliable 97.8% of the time for distinguishing the nonparous and parous classes. Cuticle hardness was less so. While 74% of parous cuticles (68 of 92) were hard, the cuticles of weevils held in the incubator never did get as hard as field collected weevils. It should be noted that these somatic characters may only be used to confirm observations on physiological age. Fat body may not be an accurate means of age-grading in insects that feed as adults, and cuticle hardness (and color) can only be used to identify newly emerged individuals (Tyndale-Biscoe, 1984). Physiological age, based on characters related to reproductive development, is far more accurate means of age-grading (Hayes and Wall, 1999).

Describing the reproductive morphology and reproductive development of a species is the first step in developing a physiological age-grading system, an investigative tool that can be used to evaluate the reproductive health of weevil populations in the field. For example, if monitoring revealed that a majority of parous females were reabsorbing their follicles, this would be a good time to evaluate host plant quality, especially nitrogen content. A population whose age structure is skewed toward P2 and P3 may indicate an unhealthy population whose reproductive rate is insufficient to produce population densities (weevils/kilo) high enough to be an effective biocontrol agent.

Table 1. Physiological Characters of the Reproductive System of *Cyrtobagous salviniae*

	N1	N2	P1	P2	P3
Number of Follicles, Vitellarium, $\bar{x} \pm S.E.$	0	3.5 (± 2.1)	7.6 (± 1.3)	7.0 (± 1.4)	6.2 (± 2.7)
Eggs, oviduct	No	No	Possibly	Possibly	Possibly
Follicular Relics	No	no	Yes	Yes	Yes
Encircle Base	No	no	No	Yes	Yes
Inclusions	No	No	no	No	Yes
Sperm in spermatheca	No	Possibly	Yes	Yes	Yes

Table 2. Somatic Characters used for Age-Grading *Cyrtobagous salviniae*

	N1	N2	P1	P2	P3
Fat Body	Fat	Fat	Intermediate	Lean	Lean
Cuticle Hardness	Soft	Soft	Hard	Hard	Hard
Cuticle Color	Brown	Brown	Black	Black	Black

Too many water bodies throughout the south are afflicted with salvinia to expect that eradication is possible—at least in the short term. Biological control can be a useful component of a comprehensive program to mitigate the impact of invasive salvinia. Expanding the knowledge base with regard to *C. salviniae* and its interactions with *S. molesta* can augment the chances of a successful outcome. A physiological age-grading system is a valuable tool that can be used to investigate factors that shape a population, such as the effects of nutritional stress (Room and Thomas, 1985), diapause, temperature and developmental cues (Spurgeon *et al.*, 2003), behavior, pesticide application and population age structure (Hayes and wall, 1999), as well as other crucial drivers that may limit the successful deployment of this important biocontrol agent (Tyndale-Biscoe, 1984).

Oviposition and Physiological Age-Grading

This study investigated the oviposition of *C. salviniae* under stable conditions at moderate nutrient levels. Mean oviposition values for the three parous classes were widely separated—22.5, 84.3, and 208.3. ANOVA indicated that there was a significant difference in oviposition among the classes ($F=176.51$, $p<.0001$, $DF=2, 44.9$). Means separation with Tukey-Kramer revealed that all three sample means were significantly different from each other (Table 3). Ordinarily, physiological age and chronological age are not directly related, but when temperature and nutrition are controlled, physiological age and chronological age are approximately equal (Perez-Mendoza, 2004). The mean (chronological) age of the P3 class was 16 weeks. The mean number of eggs oviposited by P2 females was 1.10 per day, and the mean number of eggs oviposited by the P1 class was 0.41 per day. The values obtained in this oviposition study were used to link oviposition with the morphological and physiological age-grading data obtained in the first experiment to develop an age-grading system. The data is presented in Table 3. The distribution of oviposition values is reported in Fig. 15. Only females from the initial group of 100 were used to calculate oviposition. Eight individuals did not oviposit, despite a series of replacements, so the total number of weevils was 92. The majority

Table 3. Oviposition Ranges, Means, and Chronological Age by Physiological Age Class. Values followed by different letter are significantly different (Tukey's).

Physiological Age Class	Ovipositions (range)	Ovipositions (Sample mean \pm S.E.)	Mean Age (in Weeks)
P1	1-59	22.5 \pm 4.2 <i>a</i>	7.9
P2	34-123	84.3 \pm 6.5 <i>b</i>	10.9
P3	90-396	208.3 \pm 9.1 <i>c</i>	16.0

of living weevils (58) were in the P3 class at the end of the study. Because the weevils were dissected, and did not live out their natural lives, it was not possible to obtain a value for fecundity. However, 97.7 % of the total ovipositions had taken place by the 18th week, and many of those left alive had degenerate ovaries when they were dissected. At two months (8

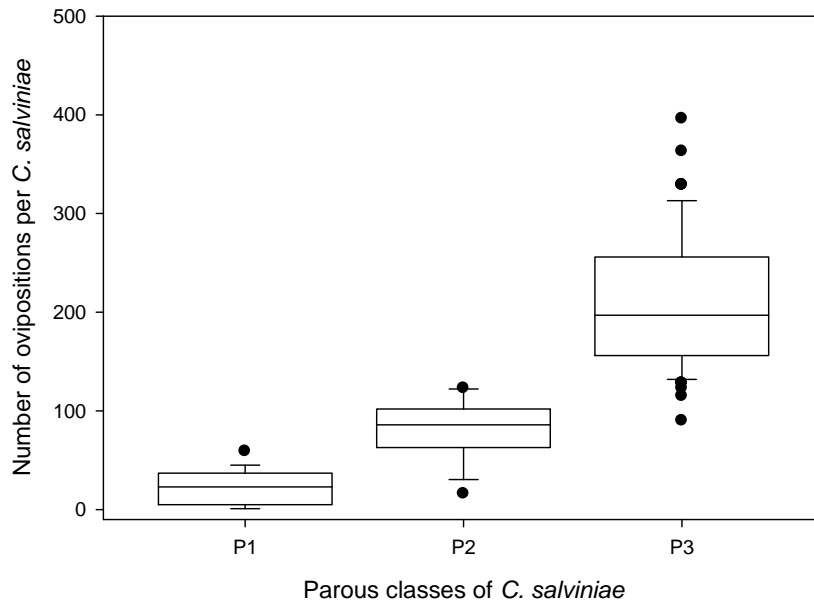


Fig. 15. The Distribution of Oviposition per Weevil by Physiological Age Class. Horizontal bars represent the 95% confidence interval. Black circles represent outliers.

weeks) most of the weevils were entering the P3 class, by far the most productive in terms of oviposition. The number of ovipositions per weevil per week varied widely. Some weevils did not oviposit during a weekly period, in contrast to the findings of Sands *et al.*, (1986), who reported that oviposition was continuous in all individuals. The highest weekly number of ovipositions by a female was 63. This was unexpected, as there are relatively few studies that address oviposition, and there are no reports of a weevil ovipositing 9 eggs a day, except those maintained on salvinia that was “very rich in nitrogen” for short periods (Sands *et al.*, 1986,). Weevils in this study oviposited up to 15 times as many eggs as reported by Forno *et al.* (1983)

in their 60 day study. Female weevils oviposited 32.1 % of their eggs in the keel of the leaf.

There is only a passing mention of this location in the literature, except for Jayanth (1989), who reported finding 59.3% of eggs in the keel of the leaf in his fecundity study. The most common site for oviposition was in the rhizomes of mature ramets. Very few eggs were located in buds or young rhizomes, which are often consumed by larvae and adults. We found no eggs on the outside of leaves or on roots, although a similar egg, probably hemipteran, was sometimes encountered.

Many weevils in the P3 class were showing signs of becoming degenerate (physiological age-related senescence) at twenty weeks. Many of these individuals had laid hundreds of eggs, and had ovaries that in many ways resembled those of nonparous individuals, but with some important differences. For example, aged individuals often had enlarged germaria and reduced vitellaria, often containing a few follicles that were not progressive in their development. These characters were difficult to quantify. Interestingly, some aged weevils showed signs of deterioration in one ovary, but were still producing eggs with the other (although fertility was probably affected). Some signs of deterioration were also evident. Follicular relics in this class were deep amber, and contained numerous, and larger inclusions (dark specks), than earlier class P3 weevils.

A wide range of oviposition values has been reported for *C. salviniae* in the literature (Table 4). Weevils that are raised in the lab under ideal conditions may provide values for oviposition that seem overly optimistic. On the other hand, the low reproductive rate often assumed to be possible (Forno *et al.*, 1983) does not explain the rapid population growth that has been observed in successful releases of *C. salviniae*). The values obtained in this study are

quite possible if field conditions are favorable, or if the timely intervention of man can enhance host quality. Although this was, of necessity, a lab study, there were several factors that make it relevant to field populations. First, moderate regimes for nitrogen content (1.48 ppm in tissue), photoperiod (12:12), and temperature (29.5°C) were selected. In lab studies, many of those left alive had degenerate ovaries when they were dissected. At two months (8 weeks) most of the weevils were entering the P3 class, by far the most productive in terms of parameters such as these are stable and so do not reflect the perturbations so often associated

Table 4. Comparison of Oviposition Studies of *C. salviniae*

Author & Date	Mean Ovipositions	Length of Parous Stages	Nitrogen content (H2O/host)	Temperature	N=	Maximum survival in weeks
Forno et al. 1983	20-25(60 days)	Unknown	Not Tested	25.5 °C	86	15
Sands et al. 1986	374	14.5	3 ppm 2.1 ppm	27 °C	10	38
Jayanth 1989	290	37.6	Not Tested	Greenhouse Temperatures	10	40.6
Current Study 2011	208.4	16	2 ppm 1.5 ppm	29.5 °C	92	20+

with natural systems. However, in this case, host plant quality was not consistent throughout the five month period (due to equipment failure), and there was no natural population of salvinia that could be used to replenish the greenhouse supply during the winter months. Host plant quality, as influenced by temperature, nutrition, and sunlight is a key factor that drives oviposition, in concert with it *C. salviniae's* inherent reproductive potential. Research by Forno *et al.* (1983), Sands *et al.* (1986), and Jayanth (1989) either did not control nitrogen content, or had unrealistic concentrations (Table 4). There were also problems with algal infestation in the

jars, which may have caused increased mortality among the resident weevils. Both Cary and Weerts (1983) and Fairchild *et al.* (2002) reported problems with algal infestation on salvinia in the greenhouse. Some algal strains produce toxins powerful enough to kill livestock, so weevil mortality may not be a remote possibility (Repavich *et al.*, 1990). Host plant quality may have been diminished as well. Some of the mortality may have been due to poor handling. It was impossible to obtain an exact figure for mortality related to poor handling, but mortality typically increased with an influx of new student workers, and declined over time as they became more skilled. Mortality was very high in the population of weevils introduced later in the study to replace those identified as males, or lost to attrition. Some of this coincided with the period of declining host plant quality mentioned above, which may indicate a period of vulnerability in early adulthood, as weevils engage in energy-expensive processes like reproductive development and egg production. Weevils can apparently reabsorb their follicles in response to environmental challenge (Chapman, 1998), but this, too, would require energy, at a time when resources may be scarce. There are some indications that female weevils can become reproductively active if fed high quality diet (Pers. Com., M. Grodowitz)

Physiological age-grading techniques can be used to obtain data on population age structure (Tyndale-Biscoe, 1984), or gain insight into the reproductive health, of a population. Some of this information, for instance, assessments of reproductive health, may be difficult, or impossible to obtain by other methods (Hayes and Wall, 1999). For example, Grodowitz *et al.*, (1997) described the reproductive morphology of *Neochetina eichorniae* and devised an age-grading system that allowed the assessment of the age structure of a field population of *N. eichorniae*. With this information, it was possible to discern that parous females were at their

highest levels in March, and decreased to low levels in June before rising again in September. There was no apparent resorption of follicles, an indication that host plant nutrition was adequate. Tyndale-Biscoe *et al.*, (1981), working with *Onthophagus granulatus* Boheman, identified a number of important factors related to population dynamics, including threshold temperatures for development, optimal brood temperature, and drought related stress, by quantifying the distribution of individuals in various reproductive classes. Guillot *et al.* (1988) reported that parous females of a population of horn flies, *Haematobia irritans* (L.), were less likely to disperse than newly mated flies, and that immigrant populations of flies had different age structures. Spurgeon *et al.* (2003) related female reproductive development, fat body characters, and environmental conditions to the onset of diapause in the boll weevil, *Anthonomus grandis*.

A baseline study that links the physiological development and oviposition of *C. salviniae* provides a powerful tool for research. In the case of *C. salviniae*, whose efficacy with regard to *S. molesta* is a function of its population growth, information on oviposition, lifespan, and reproductive development can provide valuable insight about this important biocontrol agent. Data derived from sampling programs could be used to recognize benchmark events that allow a more refined assessment of population age structure. The timing and spatial characteristics of releases could be investigated using physiological age-grading techniques, along with possible density dependent mechanisms like follicle reabsorption and changes in sex ratio. Information derived from studies employing physiological age-grading techniques could be used to anticipate problems in the release program, which needs to be expanded if it is to be effective against this rapidly growing threat to aquatic ecosystems.

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