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Cold Tolerance and Overwintering Physiology of the Salvinia Weevil (Cyrtobagous salviniae): Improving the Biological Control of Giant Salvinia in Temperate Louisiana

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COLD TOLERANCE AND OVERWINTERING PHYSIOLOGY OF THE SALVINIA WEEVIL (*CYRTOBAGOUS SALVINIAE*): IMPROVING THE BIOLOGICAL CONTROL OF GIANT SALVINIA IN TEMPERATE LOUISIANA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
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in

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by
Alana D. Russell
B.S., University of Connecticut, 2013
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To my fiancé Steve, for without his love and support (and countless hours of help counting weevils), I would not have survived this endeavor.

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ABSTRACT

Cyrtobagous salviniae is widely used for biological control of *Salvinia molesta*. Despite success in tropical and subtropical regions, the effectiveness of *C. salviniae* on *S. molesta* is inconsistent in temperate regions, indicating the need for a better understanding of the thermal biology of this agent. The objectives of this study were to compare cold tolerance of *C. salviniae* populations from the temperate native range and Louisiana, and characterize the overwintering physiology and population dynamics of *C. salviniae* in Louisiana.

Surveys of the Lower Paraná-Uruguay Delta resulted in the first record of *C. salviniae* in Uruguay, and revealed the most southern distribution of this species in Argentina and Uruguay. Survival at 0°C was 1.5-times greater, chill coma recovery time was 1.8-times faster, and SCP was 1.2-times lower in the Argentine population compared to the Louisiana population. These findings show that the Argentine provenance should be considered for managing *S. molesta* in temperate regions. Besides host range tests, cross breeding between the Louisiana and Argentine populations should be investigated to determine the life histories of any possible hybrid.

Laboratory assays demonstrated phenotypic plasticity in the cold tolerance of populations from central and southern Louisiana when acclimated to winter conditions. Survival at 0°C was 1.8- and 1.7-times greater, critical thermal minimum was 1.2- and 1.3-times lower, and chill coma recovery time was 2.7- and 1.5-times faster in the winter treatments compared to summer treatments, for both populations (central and south, respectively). Seasonal changes in reproductive status, fat body, and water content were evident from the field study, and are speculated to contribute to the overwintering success of adults at both sites. Adult and larval

densities showed that populations were most vulnerable in the late winter/early spring, presumably when water temperatures and host plant quality have not yet recovered. Seasonal monitoring of the physiological status and population dynamics should be conducted to improve the timing of releases and predicting the success of *S. molesta* control. In conclusion, the management of *S. molesta* in northern Louisiana should incorporate releases of cold tolerant populations, and seasonal monitoring of physiology and population dynamics of *C. salviniae*.

CHAPTER 1. INTRODUCTION

1.1 Giant Salvinia: One of the World's Worst Aquatic Weeds

Giant salvinia, *Salvinia molesta* Mitchell (Salviniales: Salviniaceae), is a free floating aquatic fern that has invaded the waterways of tropical and subtropical regions throughout the world (Julien et al. 2009, McFarland et al. 2004). Owing to its aggressive nature and ability to cause profound ecological and economic impacts, *S. molesta* is considered one of the world's worst aquatic weeds (Koutika and Rainey 2014).

Salvinia molesta is one of seven species of free floating aquatic ferns belonging to the family Salviniaceae (USDA-NRCS 2017). Together with *S. molesta, S. auriculata* Aubl.*, S. herzogii* de la Sota*,* and *S. biloba* Raddi constitute a taxonomic group referred to as the *S. auriculata* complex (Forno 1983). *Salvinia molesta* colonies are comprised of an intricate network of free-floating plants (Room 1983, McFarland et al. 2004). The most basal unit is termed a ramet, and is characterized by two floating fronds and a "root-like" submersed frond that are interconnected by branching rhizomes (Forno 1983). The ovate floating fronds contain rows of trichomes (hairs) that branch off and conjoin back together at the tip in an "eggbeater" shape (McFarland et al. 2004). The eggbeater-shaped trichomes are characteristic of all species in the *S. auriculata* complex, and distinguish these species from *S. minima* Baker, which has hairs that do not conjoin after branching (Forno 1983, Knutson and Mukherjee 2012). In natural settings, *S. molesta* has three growth stages, based on the degree of crowding or leaf shape: primary, secondary, and tertiary (Mitchell and Tur 1975). Primary stage plants have small invading fronds that lay flat in open water, secondary stage plants appear in clumps with boat-shaped fronds,

and tertiary stage plants have vertically folded fronds that form dense mats (Mitchel and Tur 1975).

Native to southeastern Brazil (Forno and Harley 1979, Forno 1983), *S. molesta* currently plagues waterways in over 20 countries in North America, the Caribbean, Africa, Australia and Oceania (McFarland et al. 2004, Julien et al. 2009). It was first introduced to the United States in 1995 in a small pond in South Carolina through the aquatic plant trade (Johnson 1995), but was quickly eradicated using chemical treatments (Jacono 1999). However in 1998, *S. molesta* was rediscovered invading the Toledo Bend Reservoir along the border of Texas and Louisiana (Jacono 1999), subsequently spreading uncontrollably to lakes, ponds, and reservoirs throughout the southeastern USA (Tipping and Center 2003). *Salvinia molesta* has been reported in 13 states in the USA, with the largest infestations observed in Texas and Louisiana (EDDMapS 2016). In Louisiana, the current distribution ranges from 29°N to 33°N, invading nearly every parish in the state (Alex Perret, LDWF, personal communication). *Salvinia minima* is the only other member of the genus that has invaded the USA (Madeira et al. 2006), and it was first reported in Florida in 1928. Subsequently, *S. minima* has been reported in Alabama, Georgia, Louisiana, and Texas, where infestation levels range from minimal to aggressive (Small 1931, Landry 1981, Jacono et al. 2001, Madeira et al. 2006).

Salvinia molesta is found in slow moving waters including lakes, ponds, ditches, streams, rivers, and swamps (Horner 2002) where, under favorable conditions, it can form mats up to one meter thick, and double in size in as little as 2-3 days (McFarland et al. 2004, Thomas and Room 1986). Vegetative reproduction by fragmentation, rapid growth rates, and human dispersion are major factors in the formation of dense mats of this noxious weed (Jacono 1999,

McFarland et al. 2004, Thomas and Room 1986). Thick mats of *S. molesta* can pose both ecological and economic threats, including restriction of commercial and recreational boating access, reduction of habitat for game birds, displacement of native vegetation, and reduction of water quality (McFarland et al. 2004).

Salvinia molesta is managed through mechanical, chemical, and biological control methods. Mechanical control methods include physical removal, barriers, and water-level drawdowns (McFarland et al. 2004). Physical removal involves the use of hand pulling or harvester machines to directly remove plants (Thomas and Room 1986). While sufficient in controlling small patches of *S. molesta*, this form of management is quite costly and ineffective due to rapid growth rates and large biomass production of infestations (Thomas and Room 1986). Moreover, large-scale removal may be impractical in inaccessible backwater areas blocked by vegetation (McFarland et al. 2004). Mechanical barriers such as booms and nets can be used to confine *S. molesta* infestations and restrict downstream migration; but the use of these tools requires constant inspection to assess for breakage and maintenance needs (Oliver 1993). Water-level drawdowns aim to reduce salvinia biomass by lowering water levels and thereby exposing target plants to desiccation or lethal temperatures (Cooke et al. 1986). This method of aquatic weed control has been effectively implemented in Louisiana in the past (Lantz et al 1964); however, the level of biomass reduction depends on the size and structure of the infestation (McFarland et al. 2004).

To date, the most common herbicides used to control *S. molesta* in the USA are glyphosate and diquat, which are applied to the fronds in combination with surfactants (Mudge et al. 2016). Studies have found that these herbicides can effectively suppress *S. molesta*, but

regrowth poses a risk to the effectiveness of control (Nelson et al. 2001). In Louisiana, chemical control of *S. molesta* is costly, averaging $60-70 /acre for one application (Jillian Day, personal communication). Some field studies have also found that chemical control programs are ineffective over the long-term time scale, and at risk to resistance due to over-reliance on few herbicides (Thomas and Room 1986, Tipping et al. 2008, Mudge et al. 2016). Overall, the effectiveness of mechanical and chemical control methods in Louisiana is dependent on size and thickness of salvinia infestations, and repeated applications and maintenance require a costly budget for the Louisiana Aquatic Plant Control Program of $8 million (Jillian Day, LDWF, personal communication).

Alternatively, biological control utilizes host-specific populations of natural enemies to suppress pest levels (van Driesche et al. 2008). Biological control can be a sustainable, cost-effective, and environmentally safe alternative to other control methods, and is often the most feasible measure in the control of widespread, inaccessible weed infestations (Culliney 2005, van Driesche et al. 2008).

1.2 Biological Control of Giant Salvinia Using the Salvinia Weevil

Biological control of *S. molesta* using the salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae) has been successfully used in several countries (Cilliers 1991, Joy 1986, Room et al. 1981, Thomas and Room 1986). One of two closely related species that use *Salvinia* spp. as a host, *C. salviniae* was originally misidentified as *C. singularis* Hustache (Calder and Sands 1985). However, researchers later determined that they were separate species, based on differences in feeding strategies, larval behavior, and morphology, and failure to hybridize (Sands 1983, Calder and Sands 1985, Sands and Schotz 1984). Adult *C. salviniae* are

morphologically differentiated from *C. singularis* by (1) subcircular yellowish scales on the dorsal surface of the rostrum that are not sunken into punctures and (2) rounded sclerite of the male genitalia (Calder and Sands 1985).

Cyrtobagous salviniae is a holometabolous insect. Eggs are 0.5 × 0.24 mm, milky white, and jelly bean shaped (Forno et al. 1983). Larvae are milky white with a brown, sclerotized head, and range from 1 to 2.6 mm in length, depending on the instar (Forno et al. 1983). Pupae are 2 × 2.6 mm and enclosed in a brown cocoon interwoven with the root-like submerged fronds of *S. molesta* (Forno et al. 1983, May and Sands 1986). Adult *C. salviniae* are 2 to 3.5 mm long and have a shiny brown coloration when newly emerged (teneral) that changes to black when mature (Calder and Sands 1985). Generally, females tend to be larger than males, however it is difficult to distinguish between male and female based on external morphology (Forno et al. 1983).

Development from egg to adult is completed in approx. 45 d at 26°C (Forno et al. 1983). Eggs are deposited singly by adult females in cavities in the bud or suspended in the root mass, and hatch in approx. 10 d (Forno et al. 1983). Earlier instar larvae are found externally on the fronds, and later instars are found tunneling inside the rhizome (Sands et al. 1983). After approx. 23 d, larvae exit the plant to enter the pupal stage attached to the fronds underwater (Forno et al. 1983). Adults emerge from the pupal stage after approx. 12 d (Forno et al. 1983). Adults are found on or between fronds, feeding preferentially on nitrogen rich buds (Forno et al. 1983). Adults are also found among the roots of *S. molesta,* respiring underwater from a film of air contained ventrally between the legs and abdomen (Forno et al. 1983, Julien et al. 2009). Development is greatly affected by temperature and nitrogen (N) content of plant tissues

(Sands et al. 1983, Julien et al. 2009). For example, larval development and oviposition is promoted by higher levels of N in plant tissues, with a concentration of 2-3% N considered optimal (Forno et al. 1983, Sands et al. 1983, Sands et al. 1986). Furthermore, larvae fail to develop below 17°C, and eggs are not laid and cannot hatch below 19°C (Sands et al. 1983, Hennecke and Postle 2006, Julien et al. 2009).

Cyrtobagous salviniae adults and larvae feed aggressively on *S. molesta*. Adults feed preferentially on apical buds, producing "shotgun" hole feeding scars, whereas larvae mainly feed inside the rhizome and damage is distinguished by browning fronds (Forno et al. 1983, Sands et al. 1986, Julien et al. 2009). While adult feeding suppresses plant growth, the tunneling behavior exhibited by larvae disconnects the flow of nutrients from submersed fronds to the emergent fronds and buds, causing the plant to disintegrate and sink (Forno et al. 1983, Sands et al. 1983, Julien et al. 1987). Adults feed between 13 and 33°C, with the number of feeding scars and area of frond consumed increasing linearly with temperature (Forno et al. 1983).

The reported native range of *C. salviniae* includes Paraguay, southern Brazil, and northern Argentina, between 21 and 33°S (Calder and Sands 1985, Forno et al. 1983). Field observations in the native range have reported *C. salviniae* exclusively using *S. biloba, S. auriculata, S. minima, S. herzogii* and *S. molesta* (Madeira et al. 2006). Furthermore, extensive host range experiments demonstrated that adults and larvae are host specific to the genus *Salvinia* (Forno et al. 1983, Flores and Wendel 2001), which qualified *C. salviniae* as a good candidate in biological control. Consequently, *C. salviniae* is now widely used for biological control of *S. molesta* in approx. 20 countries worldwide, including Australia, South Africa and

the United States (Julien et al. 2009). Successful control of *S. molesta* following releases of *C. salviniae* has been achieved in as little as three months to four years, depending on the size of infestation and environmental conditions (Forno 1987, Thomas and Room 1986, Room and Fernando 1992, Tipping et al. 2008).

In 1980, *C. salviniae* originating from Joinville, Brazil (26° S), were first released for the biological control of *S. molesta* in Australia in Lake Moondarra (21° S), where effective biological control of the weed was achieved (Forno and Bourne 1984, Forno 1987, Room et al. 1981). Following outbreaks of *S. molesta* in the USA, biological control practitioners first released a Florida population of *C. salviniae* that was discovered on *S. minima* in the 1960's (Kissinger 1966). Releases of this population were discontinued after widespread control of *S. molesta* was not obtained (Tipping and Center 2003), and later molecular analysis called this population a 'Florida' biotype of *C. salviniae* that was suspected to be better adapted to *S. minima* as a host (Madeira et al. 2006). Following unsuccessful control with the Florida biotype, *C. salviniae* of the 'Brazilian' biotype (originating from Joinville, Brazil) were collected from Wappa Dam in Queensland, AUS (26°S, Flores and Wendel 2001) and released in the USA at Toledo Bend Reservoir (31°N, Louisiana /Texas) and Lake Texana (29°N, Texas) in 2001 (Tipping and Center 2003). Since the original releases of the Brazilian biotype, *C. salviniae* has been shown to effectively control *S. molesta* in southern Louisiana and Texas (Tipping and Center 2003, Tipping et al. 2008).

Despite success at tropical and subtropical latitudes, biological control of *S. molesta* has been inconsistent in temperate regions of the adventive range (Julien et al. 2009, Mukherjee et al. 2014, Sullivan and Postle 2010). In northern Louisiana and Texas, *C. salviniae* populations

suffered high winter mortality in 2010-2011 and ultimately failed to establish north of 32°N (Mukherjee et al. 2014). Likewise, biological control has failed south of 34°S in Australia (Julien et al. 2009), and has been inconsistent between 31 and 34°S in South Africa (Cilliers 1991). One limiting factor in the biological control of *S. molesta* in temperate regions is hypothesized to be mortality due to freezing temperatures in winter (Mukherjee et al. 2014). One approach to overcome this deficiency is to search for cold tolerant populations of *C. salviniae* in temperate climates and therefore could be able to survive freezing winters. Moreover, the native range distribution of several species in the *S. auriculata* complex extends beyond 34°S (Forno 1983) making it possible that there are natural enemies of the plant at higher latitudes.

1.3 Project Rationale

Understanding the cold tolerance of a biological control agent has important implications towards effective management of that species, especially when establishment is limited by freezing temperatures (Chown and Nicholson 2004, Sinclair et al. 2015). Previous studies have examined the lower lethal temperature and critical thermal minimum of a *C. salviniae* population from South Africa (Allen et al. 2014), and compared the cold tolerance of populations from Florida, Louisiana, Texas, and Australia (Mukherjee et al. 2014). However, surveys for cold tolerant populations from the temperate native range have not been conducted, despite the reported native distribution of *C. salviniae* extending to 33°S (Calder and Sands 1985, Madeira et al. 2006). In addition, studies aimed to characterize the physiological mechanisms underlying cold tolerance in *C. salviniae* have not been conducted.

Therefore, the objectives of this study were to:

1. Compare cold tolerance of *C. salviniae* populations from the temperate native range and Louisiana.
2. Characterize the overwintering physiology and population dynamics of *C. salviniae* in Louisiana

This study reports cold tolerant *C. salviniae* populations in the temperate native range, and determines the physiological mechanisms conferring cold tolerance. These results are used to propose strategies to improve the management of *S. molesta* in temperate regions and discuss avenues of further research.

1.4 References

Allen, J.L., Clusella-Trullas, S., Chown, S.L., 2014. Thermal tolerance of *Cyrtobagous salviniae*: a biocontrol agent in a changing world. BioControl 59, 357-366.

Calder, A.A., Sands, D.P.A., 1985. A new Brazilian *Cyrtobagous* Hustache (Coleoptera: Curculionidae) introduced into Australia to control salvinia. J. Aust. Entomol. Soc. 24, 57-64.

Chown, S.L., Nicolson, S., 2004. Insect physiology and ecology: mechanisms and patterns. Oxford University Press, Oxford.

Cilliers, C.J., 1991. Biological control of water fern, *Salvinia molesta* (Salviniaceae), in South Africa. Agr. Ecosyst. Environ. 37, 219-224.

Cooke, G.D., Welch, E.B., Peterson, S.A., Newroth, P.R., 1986. Lake and reservoir management. Butterworth Publishers, Stoneham, MA, USA.

Culliney, T.W., 2005. Benefits of classical biological control for managing invasive plants. Cr. Rev. Plant Sci. 24, 131-150.

EDDMapS, 2016. Early Detection & Distribution Mapping System. The University of Georgia - Center for Invasive Species and Ecosystem Health. http://www.eddmaps.org/. (Accessed 10.05.15).

Flores, D., Wendel, L.E., 2001. Proposed field release of the salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Curculionidae: Coleoptera) a host-specific biological control agent of giant salvinia, *Salvinia molesta* D.S. Mitchell (Salviniaceae: Polypodiophyta) a federal noxious weed indigenous to southeast Brazil. http://www.salvinia.org/LCR/proposedrelease.pdf. (Accessed 11.06.16).

Forno, I.W., 1983. Native distribution of the *Salvinia auriculata* complex and keys to species identification. Aquat. Bot. 17, 71-83.

Forno, I.W., 1987. Biological control of the floating fern *Salvinia molesta* in north-eastern Australia: plant-herbivore interactions. B. Entomol. Res. 77, 9-17.

Forno, I.W., Harley, K.L.S., 1979. The occurrence of *Salvinia molesta* in Brazil. Aquat. Bot. 6, 185-187.

Forno, I.W., Bourne, A.S., 1984. Studies in South America of arthropods on the *Salvinia auriculata* complex of floating ferns and their effects on *S. molesta*. B. Entomol. Res. 74, 609-621.

Hennecke, B., Postle, L., 2006. The key to success: an investigation into oviposition of the salvinia weevil in cool climate regions, in: C. Preston, et al., 15th Australian Weeds Conference, Papers and Proceedings, September 2006, Adelaide, South Australia.

Horner, T., 2002. Field release of the salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Curculionidae: Coleoptera) for control of giant salvinia, *Salvinia molesta* Mitchell (Hydropteridales: Salviniaceae). http://www.salvinia.org/LCR/salviniaEA2.pdf. (Accessed 14.05.2016).

Jacono, C.C., 1999. *Salvinia molesta* (Salviniaceae), new to Texas and Louisiana. Sida 18, 927-928.

Jacono, C.C., Davern, T.R., Center, T.D., 2001. The adventive status of *Salvinia minima* and *S. molesta* in the southern U.S. and the related distribution of the weevil *Cyrtobagous salviniae*. Castanea 66, 214-226.

Johnson, D., 1995. Giant salvinia found in South Carolina. Aquatics 17, 22.

Joy, P.J., 1986. Salvinia control in India. Biocontrol News and Information 7, 142.

Julien, M.H., Bourne, A.S., Chan, R.R., 1987. Effects of adult and larval *Cyrtobagous salviniae* on the floating weed *Salvinia molesta*. J. Appl. Ecol. 24, 935-944.

Julien M.H., Hill, M.P., Tipping, P.W., 2009. *Salvinia molesta* D.S. Mitchell (Salviniaceae), in: Muniappan, R., Reddy, G.V.P., Raman, A. Biological control of tropical weeds using arthropods. Cambridge University Press, Cambridge, pp. 378–407.

Kissinger, D.G., 1966. *Cyrtobagous* Hustache, a genus of weevils new to the United States fauna (Coleoptera: Curculionidae: Bagoini). Coleopterist's Bull. 20, 125-127.

Knutson, A., Mukherjee, A., 2012. Giant salvinia, salvinia weevil, and biological control of giant salvinia, pp. 1-8. In A. Knutson and J. Nachtrieb (eds.), A guide to mass rearing the salvinia weevil for biological control of giant salvinia. Texas A&M AgriLife Extension Service, Special Publication ESP-475. http://cise.tamu.edu/media/355740/gs_manual_10-15-12.pdf.

Koutika, L-S, Rainey, H.J., 2015. A review of the invasive, biological and beneficial characteristics of aquatic species *Eichhornia crassipes* and *Salvinia molesta*. Appl. Ecol. Env. Res. 13, 263-275.

Landry, G.P., 1981. *Salvinia minima* new to Louisiana. Amer. Fern. J. 68, 95.

Lantz, K. E., Davis, J.T., Hughes, J.S., Shafer, H.E., 1964. Water level fluctuation: its effects on vegetation control and fish population management. Proc. Southeast Assoc. Game Fish Comm. 18, 482-494.

Madeira, P.T., Tipping, P.W., Gandolfo, D.E., Center, T.D., Van, T.K., Obrien, C.W., 2006. Molecular and morphological examination of *Cyrtobagous* sp. Collected from Argentina, Paraguay, Brazil, Australia, and Florida. BioControl 51, 679-701.

May, B.M., Sands, D.P.A., 1986. Descriptions of larvae and biology of *Cyrtobagous* (Coleoptera: Curculionidae): agents for biological control of salvinia. P. Entomol. Soc. Wash. 88, 303-312.

McFarland, D., Nelson, L., Grodowitz, M., Smart, R., Owens, C., 2004. *Salvinia molesta* DS Mitchell (Giant Salvinia) in the United States: a review of species ecology and approaches to management. http://acwc.sdp.sirsi.net/client/search/asset/1002855. (Accessed 15.05.2016).

Mitchell, D.S., Tur, N.N., 1975. The rate of growth of *Salvinia molesta* (*S. auriculata* Aubl.) in laboratory and natural conditions. J. Appl. Ecol. 12, 213-225.

Mudge, C.R., Perret, A.J., Winslow, J.R., 2016. Evaluation of foliar herbicide and surfactant combinations for control of giant salvinia at three application timings. J. Aquat. Plant Manage. 54, 32-36.

Mukherjee, A., Knutson, A., Hahn, D.A., Heinz, K.M., 2014. Biological control of giant salvinia (*Salvinia molesta*) in a temperate region: cold tolerance and low temperature oviposition of *Cyrtobagous salviniae*. BioControl 59, 781-790.

Nelson, L.S., Skogerboe, J.G., Getsinger, K.D., 2001. Herbicide evaluation against giant salvinia. J. Aquat. Plant Manage. 39, 48-53.

Oliver, J.D., 1993. A review of the biology of giant salvinia (*Salvinia molesta* Mitchell). J. Aquat. Plant Manage. 31, 227-231.

Room, P.M., 1983. 'Falling apart' as a lifestyle: the rhizome architecture and population growth of *Salvinia molesta*. J. Ecol. 71, 349-365.

Room, P.M., Harley, K.L.S., Forno, L.W., Sands, D.P.A., 1981. Successful biological control of the floating weed salvinia. Nature 294, 78-80.

Sands, D.P.A., 1983. Identity of *Cyrtobagous* sp. (Coleoptera: Curculionidae) introduced into Australia for biological control of salvinia. J. Aust. Ent. Soc. 22, 200.

Sands, D.P.A., Schotz, M., Bourne, A.S., 1983. The feeding characteristics and development of larvae of a salvinia weevil *Cyrtobagous* sp. Entomol. Exp. Appl. 34, 291-296.

Sands, D.P.A., Schotz, M., 1984. Control or no control: a comparison of the feeding strategies of two salvinia weevils, in: E.S. Delfosse (ed.), Proc. VI Int. Symp. Biol. Contr. Weeds, August 1984, Vancouver, Canada.

Sands, D.P.A., Schotz, M., Bourne, A.S., 1986. A comparative study on the intrinsic rates of increase of *Cyrtobagous singularis* and *C. salviniae* on the water weed *Salvinia molesta*. Entomol. Exp. Appl. 42, 231-237.

Sinclair, B.J., Coello Alvarado, L.E., Ferguson, L.V., 2015. An invitation to measure insect cold tolerance: Methods, approaches, and workflow. J. of Therm. Biol. 53, 180-197.

Small, J.K., 1931. Ferns of Florida: being descriptions of and notes on the fern-plants growing naturally in Florida (illustrated). The Science Press, New York.

Sullivan, P.R., Postle, L.A., 2010. Low temperature reproduction of *Cyrtobagous salviniae*: good news for biological control of salvinia in temperate climate. J. Aquat. Plant. Manage. 48, 92-96.

Thomas, P.A., Room, P.M., 1986. Taxonomy and control of *Salvinia molesta*. Nature 320, 581–584.

Tipping, P.W., Center, T.D., 2003. *Cyrtobagous salviniae* (Coleoptera: Curculionidae) successfully overwinters in Texas and Louisiana. Fla. Entomol. 86, 92-93.

Tipping, P.W., Martin, M.R., Center, T.D., Davern, T.M., 2008. Suppression of *Salvinia molesta* Mitchell in Texas and Louisiana by *Cyrtobagous salviniae* Calder and Sands. Aquat. Bot. 88, 196-202.

van Driesche, R., Hoddle, M., Center, T., 2008. Control of pests and weeds by natural enemies: an introduction to biological control. Blackwell Publishing, Malden, MA.

USDA-NRCS, 2016. The PLANTS Database. https://plants.usda.gov/core/profile?symbol=SAMO5. (Accessed 15.05.2016)

CHAPTER 2. SURVEYS IN ARGENTINA AND URUGUAY REVEAL *CYRTOBAGOUS SALVINIAE* (COLEOPTERA: CURCULIONIDAE) POPULATIONS ADAPTED TO SURVIVE TEMPERATE CLIMATES IN SOUTHEASTERN USA[1]

2.1 Introduction

Historically, biological control of floating aquatic weeds has been highly successful (McFadyen 1998) although several programs have failed through lack of establishment of agents in some geographical regions (May and Coetzee 2013, Mukherjee et al. 2014, Zhao et al. 2015). The distribution of a biological control agent is often more restricted than that of its respective target in the adventive range due to lack of adaptation to the ecological conditions (Morin et al. 2009, van Driesche et al. 2008, Zhao et al. 2015). Temperature has been reported as a limiting climatic factor in biological control of aquatic weeds including *Alternanthera philoxeroides* (Mart.) Griseb (Caryophyllales: Amaranthaceae), *Eichhornia crassipes* (Mart.) Solms (Commelinales: Pontederiaceae), and *Pistia stratiotes* L. (Alismatales: Araceae) (May and Coetzee 2013, Zhao et al. 2015). When biological control agents are limited by climatic factors, a possible solution is to search for new biotypes from different geographic locations with closer climatic matches (van Driesche et al. 2008). For example, *Trioxys pallidus* Haliday (Hymenoptera: Aphidiidae) from Iran was introduced in northern California to improve the biological control of *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae), where establishment of the original French biotype was limited by climate (van den Bosch et al. 1970). When successful in finding new biotypes in the native range, biological control practitioners are challenged by a lack of protocols for ensuring their safe release. Because of the absence of a

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clear science-driven regulatory protocol for the handling of new biotypes, potential challenges could arise, including misidentification of cryptic species, hybridization, and non-target attacks (Hoffmann et al. 2002, Morin et al. 2009, Paterson et al. 2016). To improve the success in aquatic weed biological control, practitioners need to develop a pre- and post-release framework to understand how to handle biotypes of an agent from different geographic regions.

The salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), is widely used for the biological control of *S. molesta* in several countries (Cilliers 1991, Joy 1986, Room et al. 1981, Thomas and Room 1986). The reported native range of the *C. salviniae* includes southern Brazil and northern Argentina (Calder and Sands 1985, Forno et al. 1983). In 1980, *C. salviniae* originating from Joinville, Brazil (26° S), were first released for the biological control of *S. molesta* in Lake Moondarra, Queensland, Australia (21° S) where effective biological control of the weed was achieved (Forno and Bourne 1984, Forno 1987, Room et al. 1981). Following its success in Australia (Room et al. 1981, Room et al. 1984), *C. salviniae* collected from Wappa Dam in Queensland (26°S, Flores and Wendel 2001) were released in the United States at Toledo Bend Reservoir (31°N, Louisiana /Texas) and Lake Texana (29°N, Texas) in 2001 (Tipping and Center 2003). Since the original releases, *C. salviniae* has been shown to effectively control *S. molesta* in southern Louisiana and Texas (Tipping and Center 2003, Tipping et al. 2008).

However, biological control of *S. molesta* has been inconsistent in temperate regions (Julien et al. 2009, Mukherjee et al. 2014, Sullivan and Postle 2010). For example, *C. salviniae* populations in northern Texas and Louisiana suffered high winter mortality in 2010-2011, failing

to establish north of 32°N (Mukherjee et al. 2014). One approach to overcome mortality due to freezing temperatures is to search for cold tolerance populations of *C. salviniae* in temperate climates. Because the native distribution of several species in the *S. auriculata* complex extends beyond 34°S (Forno 1983), we hypothesize that there are populations of *C. salviniae* at higher latitudes that are able to survive freezing winters.

To compare the cold tolerance among insect populations, investigators have measured survival at low temperatures, chill coma recovery time, and supercooling point (SCP). Cold tolerance variables are chosen based on how they broadly represent thermal tolerances and practicality to the organism in question (Sinclair et al. 2015). Survival at low temperatures can be described using the lethal times (LT_x), defined as the time it takes for a particular percentage of a population to die when exposed to a set temperature (Sinclair et al. 2015). The chill coma recovery time of an insect is the time observed for an insect to recover after being exposed to a temperature-time combination that induces a chill coma (Sinclair et al. 2015). The SCP of an insect is defined as the lowest temperature experienced before the formation of ice in the tissues of the body (Denlinger and Lee 2010).

The objectives of this study were to (1) determine the regions of South America that are climatically similar to *S. molesta* habitats in temperate Louisiana, (2) conduct surveys in these regions to search for *C. salviniae*, and (3) compare the survival at 0°C, chill coma recovery time, and supercooling point of populations of *C. salviniae* from Louisiana, USA and the Lower Paraná-Uruguay Delta, South America. We report the presence of *C. salviniae* in temperate regions in the native range and propose steps to utilize these new biotypes to improve the management of *S. molesta* in temperate regions.

2.2 Materials and Methods

2.2.1 Species distribution modeling

The Maximum Entropy Species Distribution Model (Phillips et al. 2006), hereafter referred as MaxEnt, was used to map regions in South America with climatic similarity to *S. molesta* sites in Louisiana. MaxEnt is an ecological niche model that predicts a species' geographic distribution based on point occurrences (latitude and longitude) and environmental data layers (Phillips et al. 2006). This modelling approach characterizes environmental conditions, such as temperature, that are associated with confirmed occurrences of a species (Mukherjee et al. 2010). By predicting areas with climatic similarity between South America and Louisiana, we prioritized areas for foreign exploration of cold tolerant populations of *C. salviniae*. Although *S. molesta* had not been reported from Argentina and Uruguay, this region was surveyed because there are other host species in the *S. auriculata* complex known to occur there (Forno 1983).

Forty spatially unique point occurrences of *S. molesta* occurring in Louisiana north of 32°N were used, based on reports from 2010 to 2011 where *C. salviniae* failed to establish (Mukherjee et al. 2014). Point occurrences were acquired using distribution data from the Early Detection and Distribution Mapping System (EDDMapS 2016). One bioclimatic environmental data layer, minimum temperature of coldest month (BIO6), was downloaded from the WORLDCLIM (Hijmans et al. 2005) database and incorporated into the model. Bioclim layers are derived from monthly values of temperature and precipitation, and represent annual trends (Mukherjee et al. 2010). The minimum air temperature of the coldest month was chosen based on the importance of cold temperature in *S. molesta* management in Louisiana. Air

temperature was used due to lack of available water temperature data, despite the floating nature of *S. molesta* plants. However the use of air temperature data should not affect predictions, in light of a study that determined that there was no significant difference between temperature data from local climate stations, on-site air recordings, and microclimate recordings within *E. crassipes* patches (Coetzee 2012). Moreover, Devaney et al. (2009) found that non-water specific environmental data successfully predicted the distribution of Eurasian cyprinid fishes.

2.2.2 Surveys in Argentina and Uruguay

Exploratory surveys were conducted along the Lower Paraná-Uruguay Delta in Argentina and Uruguay. Surveys were performed in eastern Uruguay in November 2015 (spring), and western Uruguay in March 2016 (late summer) and June 2016 (winter). Surveys in northeastern Argentina occurred continually from July 2015 (winter) to February 2016 (summer). During surveys, *Salvinia* plants were collected using fishing nets, placed in plastic buckets, and brought back to the laboratory for processing for presence of *C. salviniae*. *Salvinia* species were identified in the laboratory using field keys (Forno 1983). Plants were placed in Berlese funnels for 48h to extract live arthropods associated with each sample of S*alvinia*. Geographical coordinates of each collection locality were recorded with a handheld GPS.

Cyrtobagous salviniae adults were sorted from samples with a dissection microscope and identified based on size, color, and punctures and scales on the elytra (Calder and Sands 1985). Ten specimens from each site were sent to Dr. Charles O'Brien at the University of Arizona, Entomology Department in Tucson, AZ for morphological identification. Sequence analysis of the 28S rRNA D2 expansion domain was conducted at the Invasive Plant Research

Laboratory, USDA-ARS in Fort Lauderdale, FL. Both morphological and molecular identification methods were used to distinguish *C. salviniae* from *C. singularis* Hustache, a closely related species with similar morphology (Calder and Sands 1985, Madeira et al. 2006). *Cyrtobagous salviniae* is morphologically differentiated from *C. singularis* by (1) subcircular yellowish scales on the dorsal surface of the rostrum that are not sunken into punctures and (2) rounded sclerite of the male genitalia (Calder and Sands 1985). Voucher specimens were deposited at the Louisiana State Arthropod Museum in Baton Rouge, LA.

2.2.3 Climatic description in adventive and native range

To understand the climatic conditions between Louisiana and selected regions of the Lower Paraná-Uruguay Delta, we compared temperature ranges and accumulated degree days. Historical climate data from 2011-2015 were acquired from weather stations from the Instituto Nacional de Tecnología Agropecuaria (INTA, Campana, Argentina), Instituto Nacional de Investigacion Agropecuaria (INIA, La Estanzuela, Uruguay), and the National Oceanic and Atmospheric Administration (NOAA, Caddo Lake, Toledo Bend Reservoir, Texas, and Thibodaux, Louisiana, USA). These stations were chosen based on proximity to confirmed presence of *C. salviniae* in the native range and target climatic areas of *S. molesta* control in the adventive range (Louisiana, USA). Mean annual temperature, minimum temperature of coldest month, maximum temperature of warmest month, and annual precipitation were recorded for each year and then averaged over the five years for comparison of climatic conditions.

Daily minimum and maximum air temperatures were averaged for the five years, providing 365 values for each weather station for degree-day calculation. Accumulated degree days for *C. salviniae* were calculated using the degree day calculator developed by the

University of California-Davis (http://ipm.ucanr.edu/WEATHER/index.html). This application calculates the accumulated degree-days by incorporating lower and upper developmental thresholds for the target organism with daily average minimum and maximum temperatures and using the single sine method. Lower and upper developmental thresholds for *C. salviniae* were 19 and 31°C, respectively (Forno et al. 1983, Sands et al. 1983). To compare the accumulated degree days between sites, we calculated accumulated degree days for two additional sites in each region (south, central, and north LA; and Lower Paraná-Uruguay Delta) and analyzed the data using ANOVA and Tukey's HSD (JMP®, Version 13). In addition, we used the United States Plant Hardiness Zone Map (USDA PHZM 2012) to describe the winter conditions at the sites in Louisiana. The United States Plant Hardiness Zone Map divides the United States in 10-degree Fahrenheit zones based on average annual extreme minimum winter temperature during a 30-year period (USDA PHZM 2012).

2.2.4 Cold tolerance

Adult *C. salviniae* were collected from the field in Henderson Swamp, Louisiana, USA (30.3064°, -91.7568°) and the Lower Paraná-Uruguay Delta, South America (approx. 33.4° S), hereby referred to as the Louisiana and Lower Paraná-Uruguay Delta populations, respectively. The Louisiana population was collected from *S. molesta* plants in February 2016. The Lower Paraná-Uruguay Delta population was collected from *S. biloba* Raddi plants in March 2016. Adult *C. salviniae* were extracted from host plants in Berlese funnels for 48 h. All insects were acclimated on *S. molesta* plants in a growth chamber for 72 h at 13°C before cold tolerance experiments (Hennecke and Postle 2006). All acclimations and experiments were conducted in the dark to maintain consistent temperatures.

To measure survival at 0°C, adult *C. salviniae* were placed in groups of twenty on *S. molesta* plants in plastic containers containing 300 mL of reverse osmosis water. A group of twenty weevils comprised one replication, and each treatment was replicated two times, for a total of 40 adults per treatment. Weevils were exposed to 0°C inside a growth chamber for 1, 3, 5, 7, 9, 11, and 14 d. Preliminary experiments were performed to determine optimal frequency of exposure times and duration of the experiment. Immediately after the exposure time, experimental groups were removed from the chambers and placed in a new chamber programmed to 25°C to allow for recovery. Mortality was assessed after 24 h of recovery by removing weevils from experimental plants and then placing them on damp filter paper in a Petri dish at room temperature (approx. 21°C). An individual was determined to be dead if it could not right itself after tactile stimulation (Mukherjee et al. 2014). Mortality data were analyzed using the Probit function in SAS to yield LT_{50} and LT_{90} values (Zhao et al. 2015), and differences between populations were noted by non-overlapping 95% confidence intervals (Payton et al. 2003).

Chill coma recovery time was observed for a total of 50 adult *C. salviniae* from each population, with each insect representing a replicate. Groups of 25 adults were placed inside 15 mL graduated tubes. Graduated tubes and weevils were secured within a horizontal grip rack and submerged in an ARCTIC SC100-A28 Temperature Controlled Refrigerated Bath (Fischer Scientific, Inc., Waltham, MA) containing ethylene glycol diluted with water. Starting from 13°C (acclimation temperature), weevils were cooled at a rate of -1°C/min until 0°C, then held at this temperature for 4 h (Mukherjee et al. 2014). Immediately after 4 h, insects were removed from the refrigerated bath and placed ventral side up on a damp paper towel in a Petri dish at room

temperature (21°C). The time in minutes and seconds required for each insect to stand up and regain coordination was recorded with a digital stopwatch. Recovery times were tested for normality using a Shapiro-Wilk test and analyzed using a Kruskal-Wallis test (JMP®, Version 13).

The supercooling point (SCP) was determined for a total of 56 adult *C. salviniae* from each population, with each insect representing a replicate. Individual adult *C. salviniae* were attached to Type-J thermocouples using Dow Corning high vacuum grease and inserted into 2 mL cryotubes. Cryotubes containing insects and thermocouples were placed into Nalgene Cryo 1°C freezing containers. The use of the freezing containers ensured a constant cooling rate of -1°C/min. The freezing containers were subsequently placed into a freezer programmed to -25°C, and body temperatures of individuals were recorded by a DI-1000TC-8 channel data recorder (DATAQ Instruments, Akron, OH) that transferred the data to a computer using Windaq Data Acquisition software. The body temperature of individuals was recorded until the heat of crystallization was observed, as indicated as a steep rebound in temperature on the thermal curve. The SCPs of the two populations were tested for normality using a Shapiro-Wilk test and analyzed using a Kruskal-Wallis test (JMP®, Version 13).

2.3 Results

2.3.1 Species distribution modeling

Moderate to high (50-96%) suitability of *S. molesta* occurrence was recorded for Argentina south of the Lower Paraná-Uruguay Delta border (AUC test value = 0.977; Figure 2.1). Moderately to low (0-50%) suitability was recorded for the majority of Uruguay.

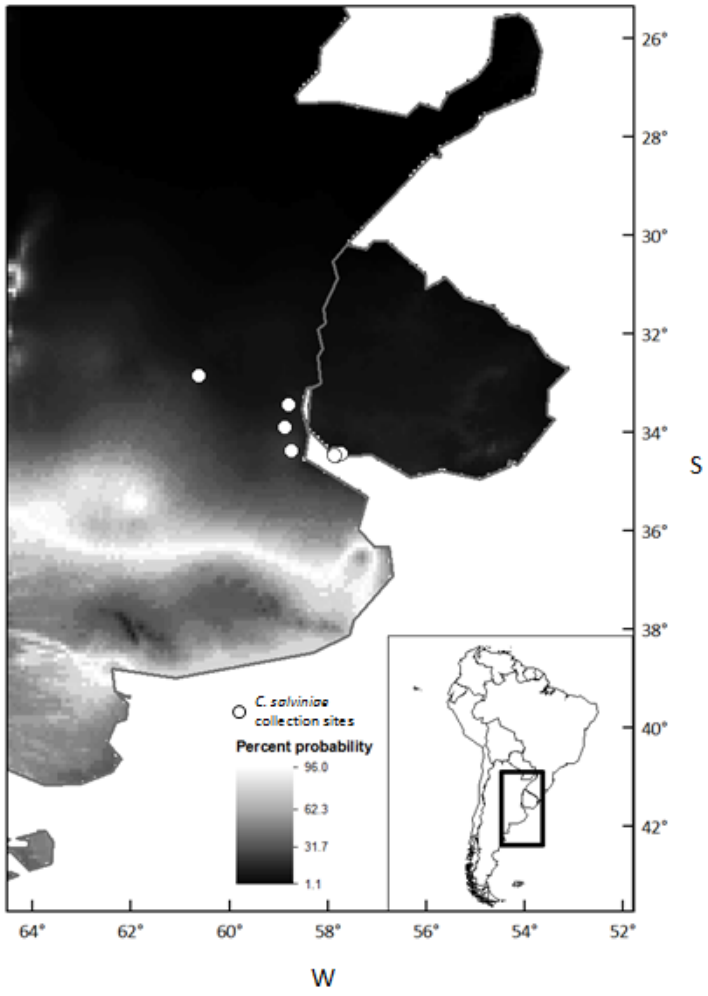

Figure 2.1. Projected distribution of *Salvinia molesta* in Argentina and Uruguay based on *S. molesta* distribution in Louisiana. Circles indicate collection sites of *C. salviniae*. Lighter colors indicate higher probabilities of occurrence.

2.3.2 Surveys in Argentina and Uruguay

Areas of the Lower Paraná-Uruguay Delta explored included the Buenos Aires, Entre Ríos and Santa Fe provinces in Argentina, and Colonia, Treinta y Tres, and Rocha departments in Uruguay. Hydrologic components of this delta region included main rivers, tributaries, small streams, ponds, wetlands, marshes, rice canals and ditches. Plant communities in these aquatic systems comprised of submersed vegetation and floating aquatic vegetation (FAV). FAV including Lemnoideae (duck weeds), *Pistia* sp., *Ludwigia* sp., *E. crassipes*, and *A. philoxeroides*

were recorded existing both in the presence and absence of *Salvinia* spp. at sites. A total of 20 *Salvinia* sites were discovered in the Lower Paraná-Uruguay Delta region (Table A.1). *Salvinia biloba*, synonymous to *S. herzogii* (de la Sota 1995), and *S. minima* Baker were found throughout this region. *Salvinia molesta* was not found at any of the sites studied. *Salvinia* plants were found together with other FAV species, as well as in monotypic patches. *Cyrtobagous salviniae* was found at nine of the 20 *Salvinia* sites, but it was not found at all on *S. minima* (Table A.1). In Argentina, *C. salviniae* was found at five sites containing *S. biloba*: two in the Entre Ríos province, one in the Santa Fe province, and one in the Buenos Aires province in the northeast. In Uruguay, *C. salviniae* was found at four sites containing *S. biloba* within the Colonia department in the southwest. Three of the four *S. biloba* sites which had *C. salviniae* in Colonia were located in ephemeral pools and drainages along the Río de la Plata. *Cyrtobagous salviniae* was not found in the Treinta y Tres and Rocha departments in the northeast.

2.3.3 Climatic description in adventive and native range

Accumulated degree-days (dd) between the three Louisiana sites (Thibodaux, Toledo Bend Reservoir, and Caddo Lake) were not significantly different ($F_{2,6}$= 2.75, P = 0.14), ranging from 1282 dd in the north (Caddo Lake, Mooringsport) to 1360 dd in the south (Thibodaux) (Table 2.1). However, accumulated degree-days in northeastern Argentina and southwestern Uruguay were significantly lower than the regions in Louisiana by 1.9- and 2.3-times, respectively ($F_{3,7}$ = 39.18, P < 0.001). Temperatures were above the developmental threshold of 19°C for *C. salviniae* throughout the spring and early summer in Louisiana, whereas temperatures remained closer to the lower developmental threshold in Argentina and Uruguay. A clear differentiation of plant hardiness zones exists between south, central, and north

Louisiana (Table 2.1). North Louisiana (Caddo Lake, zone 8a) experiences extreme minimum temperatures ranging from -12.2 to -9.4°C, whereas central Louisiana (Toledo Bend Reservoir, zone 8b) experiences -9.4 to -6.7°C, and south Louisiana (Thibodaux, zone 9a), experiences -6.7 to -3.9°C.

Table 2.1. Climatic conditions from 2011-2015 at *Salvinia* sites in the United States and South America.

City, Country	Coordinates	Mean annual air temp. (°C)	Min. temp. of coldest month (°C)	Max. temp. of warmest month (°C)	Accumulated Degree Days	US Plant Hardiness Zone [d]
Campana, AR [a]	-34.17380°, -58.86613°	17.1	-3.9	37.6	729	n/a
Semillero, UY [b]	-34.33833°, -57.69053°	16.7	-0.8	37.4	594	n/a
Caddo Lake, LA, USA [c]	32.41720°, -93.63887°	18.6	-9.1	40.9	1282	8a (-12.2 to -9.4°C)
Toledo Bend Reservoir, LA, USA [c]	31.2°, -93.5697°	19.5	-4.9	37.8	1301	8b (-9.4 to -6.7°C)
Thibodaux, LA, USA [c, d]	29.7547°, -90.7748°	20.2	-4.7	35.9	1360	9a (-6.7 to -3.9°C)

[a] Data source: Instituto Nacional de Tecnología Agropecuaria, Paraná Delta Experimental Station. Host plant species: *Salvinia biloba*.
[b] Data source: Instituto Nacional de Investigación Agropecuaria, La Estanzuela- Colonia Climate Station. Host plant species: *S. biloba*.
[c] Data source: NOAA Climate Data Online (https://www.ncdc.noaa.gov/cdo-web/). Host plant species: *S. molesta*.
[d] Data source: USDA Plant Hardiness Zone Map (http://planthardiness.ars.usda.gov)

2.3.4 Cold tolerance

The Lower Paraná-Uruguay Delta population was more cold tolerant than the Louisiana population. Survival at 0°C was approximately 1.5-times greater in the Lower Paraná-Uruguay Delta population than the Louisiana population (Table 2.2). The LT_{50}s (95% fiducial limit) for the Lower Paraná-Uruguay Delta and Louisiana populations were 7.3 (6.6 – 8.0) d and 4.6 (4.1 – 5.2)

d, and the LT_{90}s were 11.7 (10.7 – 13.2) d and 7.7 (6.9 – 8.9) d, respectively. Non-overlapping 95% fiducial limits infer significant differences between the populations.

Table 2.2. Survival of *Cyrtobagous salviniae* populations from Lower Paraná-Uruguay Delta and Louisiana at 0°C. LT_{50} and LT_{90} values represent the time in days required to kill 50 and 90% of the populations during exposure to 0°C. Non overlapping 95% CI (confidence interval) indicate differences in populations. χ^2 is the Wald test for the hypothesis that the slope parameter is zero.

Population	n	Slope ± SE	LT_{50} (95% CI) d	LT_{90} (95% CI) d	χ^2
Lower Paraná-Uruguay Delta	240	0.50 ±0.05	7.3 (6.6 – 8.)	11.7 (10.7 – 13.2)	98.07
Louisiana	240	0.71 ±0.08	4.6 (4.1 – 5.2)	7.7 (6.9 – 8.9)	73.41

The chill coma recovery time was 1.8-times faster for the Lower Paraná-Uruguay Delta population (5.0 ± 0.19 min, mean ± SE) in comparison to the Louisiana population (8.8 ± 0.96 min) (χ^2 = 6.11, P = 0.0134) (Figure 2.2). The SCP of the Lower Paraná-Uruguay Delta population (-16.3 ± 0.29°C) was approximately 1.2-times greater than the Louisiana population (-13.5 ± 0.22°C) (χ^2 = 49.99, P < 0.0001) (Figure 2.2).

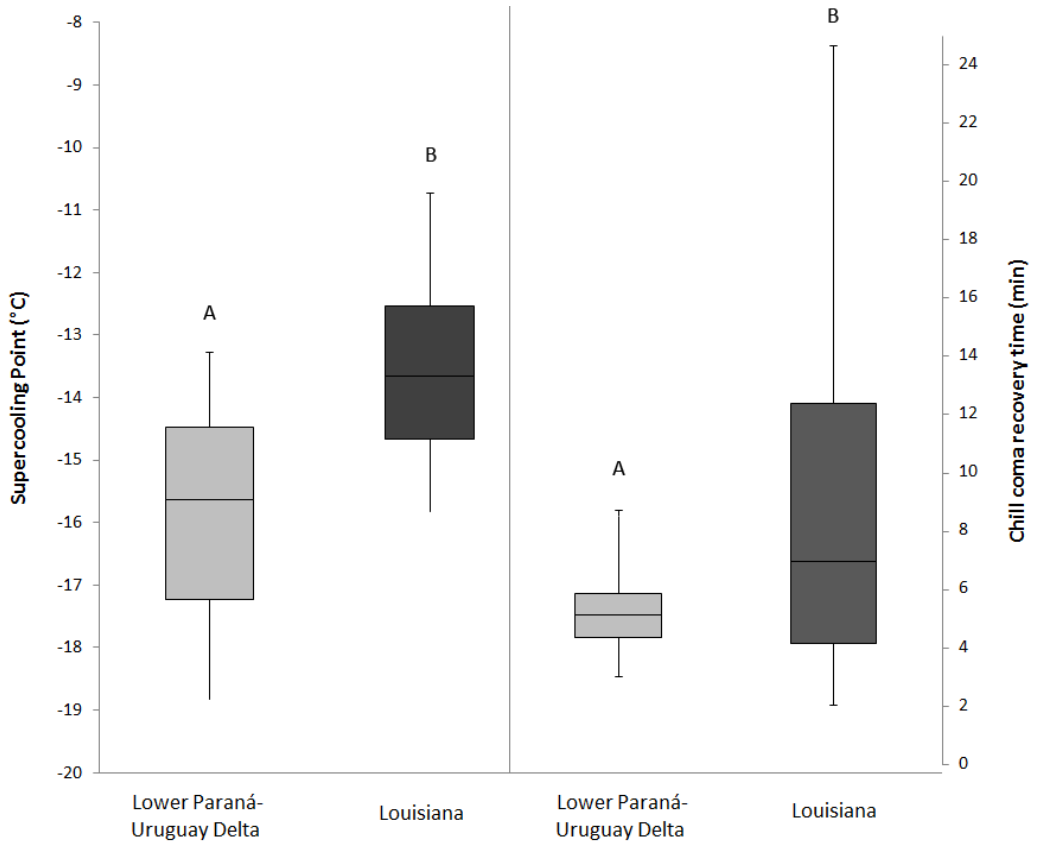

Figure 2.2. Supercooling points (°C) and chill coma recovery times (minutes) for *Cyrtobagous salviniae* populations from the Lower Paraná-Uruguay Delta and Louisiana. Bars with different letters are statistically significant ($\chi^2 > 6.11$, $P < 0.05$).

2.4 Discussion

Climate matching can be applied as a useful tool to search for natural enemies or new biotypes used in the biological control of invasive species (Mukherjee et al. 2010). We combined results from MaxEnt with historical collection data to find regions with *C. salviniae* in the temperate native range. However, suitability varied throughout the Lower Paraná-Uruguay Delta region. In an analysis of temperature between the sites in Argentina and Uruguay averaged from 2011-2015, we found that the Argentine site had a colder minimum temperature of coldest month by more than 3°C (Table 2.01). Additionally, the Argentine site experienced almost 4.8-times more freezes per year than the Uruguayan site (AR= 18, UY=

3.8/year). From these data, we suspect that the lower suitability rating in Uruguay is due to warmer winter temperatures as a result of coastal location of the country. The distribution of *C. salviniae* sites discovered extended as far south as 34.5° S in Colonia, Uruguay, and probably represent the most southern distribution of *C. salviniae* in South America known to date. However, reports of *S. auriculata* Aubl. existing around 37-38° S in Argentina (Gabriela Giudicce, personal communication) justify further exploration for *C. salviniae* in this region. This study presents the first record of *C. salviniae* in Uruguay (Forno et al. 1983). We suspect that most of the *C. salviniae* found in drainages and pools along the Rio de la Plata in Colonia, Uruguay had washed there from the headwaters of the Paraná River during rainstorms earlier in the month (June 2016), due to the ephemeral state of these habitats. However, *S. biloba* and *C. salviniae* were found consistently growing in one pond in Colonia, demonstrating the ability of *C. salviniae* and its host to establish in this region.

The climate differed between the Lower Paraná-Uruguay Delta sites and Louisiana sites. Based on accumulated degree days, *C. salviniae* populations in the Lower Paraná-Uruguay Delta experience shorter growing seasons compared to populations in Louisiana. At the Lower Paraná-Uruguay Delta sites, temperatures remain closer to the lower developmental threshold for longer throughout the spring, therefore slowing the rate of degree-day accumulation and suggesting that there are fewer generations per year. Populations exposed for longer durations to cold temperatures should therefore be more adapted to these conditions, because they must be able to survive, feed, and reproduce (Denlinger and Lee 2010). In Louisiana, temperatures increase well above the lower developmental threshold for larvae earlier in the spring, and remain at these temperatures for longer into the fall compared to the Lower

Paraná-Uruguay Delta sites. Seasonal temperature fluctuations varied between north and south Louisiana. In north Louisiana, populations of *C. salviniae* experience warmer summers and colder winters (zone 8a, -12.2 to -9.4°C) while populations in south Louisiana experience milder winters (zone 9a, -6.7 to -3.9°C). Obeysekara et al. (2015) described the climate in north Louisiana as characterized by periodic cold fronts that decrease water temperatures below the critical thermal minimum during several hours or days. We speculate that the north Louisiana thermal regime leads to population growth in the summer followed by population crashes from winter mortality, whereas the south Louisiana thermal regime leads to more sustainable populations that recover faster from mild winters (Micinski et al. 2016). These vast latitudinal differences in thermal regimes in Louisiana suggest that the successful use *C. salviniae* for management of *S. molesta* will require the implementation of region-specific approaches.

The Lower Paraná-Uruguay Delta population was more cold tolerant than the Louisiana population based on its survival at 0°C, chill coma recovery time and SCP. Similarly, Mukherjee et al. (2014) also compared the chill coma recovery time and survival at 0°C of *C. salviniae* populations from the United States (Texas, Louisiana, and Florida) and Australia (Camden), and found that the Australian population was more cold tolerant. The authors suggested that the two founding events that the United States population underwent (Brazil to Australia in 1980, Australia to USA in 2001) could have caused a genetic bottleneck, resulting in the loss of cold tolerant genotypes from the gene pool. We speculate that the Lower Paraná-Uruguay Delta population was more cold tolerant than the Louisiana population because of greater genetic diversity and longer time exposed to winter conditions selecting for cold tolerant genes (Chen and Walker 1993, Grewal et al. 1996). The three cold tolerance variables compared in this study

(survival at 0°C, chill coma recovery time and SCP) were chosen because of the high informational output they provided with relatively low costs (Sinclair et al. 2015). However, there are several other variables that can be used to measure insect cold tolerance. For example, Allen et al. (2014) conducted a study on the critical thermal minimum (CT_{min}), the threshold at which an insect loses the ability to move due to cold exposure (Sinclair et al. 2015), of a *C. salviniae* population from South Africa. We suggest that the CT_{min} of the populations in this study should also be determined, to compare to the data from South Africa. We also suggest that the lower developmental threshold, or the temperature at which development ceases (Denlinger and Lee 2010), is another variable with much ecological relevance. Experiments following the longevity, feeding activity, and fecundity of insects after cold exposure should also be considered because of their ecological relevance (Obeysekara et al. 2015).

Because of the large degree of biotype variation found in *C. salviniae* (Madeira et al. 2006, Russell et al. 2017), we suggest that an abbreviated host range testing should be conducted on the Lower Paraná-Uruguay Delta biotype of *C. salviniae* before consideration for release. Flores and Wendel (2001) performed a host range test on *C. salviniae* collected from Joinville, Brazil, and concluded that biotype was host specific to *Salvinia* spp. and not a threat to any non-target plants tested. Because previous host range tests have found *C. salviniae* to be host specific to the genus *Salvinia* (Forno et al. 1983), we suggest that a host range test of the Lower Paraná-Uruguay Delta populations should be simplified to only the families of true ferns, Salviniaceae and Marsileaceae, excluding taxonomically unrelated plants tested by Flores and Wendel (2001), including *Sagittaria sanfordii* E. Greene (Alismatales: Alismataceae) and *Zizania*

aquatic L. (Poales: Poaceae). Volchansky et al. (1999) found that two biotypes of the biological control agent *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) were host-specific to different species of *Opuntia* (Caryophyllales: Cactaceae), resulting in inconsistent control of *Opuntia* species in South Africa. Furthermore, Hoffmann et al. (2002) later determined that the biotypes of *D. opuntiae* were able to hybridize, producing non-*Opuntia* specific F_1 progeny, but some host-specific nymphs were produced from the F_2 crosses. The authors concluded that the hybridization of these biotypes was not effective for the biological control program, and cautioned practitioners about the consequences of not studying the effects of hybridizing biotypes of biological control agents. We suggest that host preference studies should be conducted to determine if *S. molesta* is a suitable host for the Lower Paraná-Uruguay Delta biotype. Moreover, cross breeding studies between the Lower Paraná-Uruguay Delta and Louisiana biotypes should be conducted to determine the effectiveness of the hybrids as biological control agents of *S. molesta*.

In conclusion, surveys in the temperate native range of *C. salviniae* revealed a population from the Lower Paraná-Uruguay Delta that was more cold tolerant than the Louisiana population. Phylogenetic analysis determined that this population is a different biotype than the Brazilian biotype found in Louisiana. *Cyrtobagous salviniae* from the Lower Paraná-Uruguay Delta should be considered as a new tool to improve the efficacy of the biological control of *S. molesta* in temperate Louisiana, with precaution and further testing undertaken to ensure its fidelity to the genus *Salvinia*.

2.5 References

Allen, J.L., Clusella-Trullas, S., Chown, S.L., 2014. Thermal tolerance of *Cyrtobagous salviniae*: a biocontrol agent in a changing world. BioControl 59, 357-366.

Calder, A.A., Sands, D.P.A., 1985. A new Brazilian *Cyrtobagous* Hustache (Coleoptera: Curculionidae) introduced into Australia to control salvinia. J. Aust. Entomol. Soc. 24, 57-64.

Chen, C.P., Walker, V.K., 1993. Increase in cold-shock tolerance by selection of cold resistant lines in *Drosophila melanogaster*. Ecol. Entomol. 18, 184-190.

Cilliers, C.J., 1991. Biological control of water fern, *Salvinia molesta* (Salviniaceae), in South Africa. Agr. Ecosyst. Environ. 37, 219-224.

Coetzee, J.A., 2012. Meteorological weather station data can be used in climate matching studies of biological control agents. Biocontrol Sci. Tech. 22, 419-427.

Denlinger, D. L., Lee, R.E., 2010. Low Temperature Biology of Insects. Cambridge University Press, Cambridge.

DeVaney, S.C., McNyset, K.M., Williams, J.B., Peterson, A.T., Wiley, E.O., 2009. A tale of four "carp": Invasion potential and ecological niche modeling. PLoS ONE 4, e5451. http://dx.doi.org/10.1371/journal.pone.0005451.

EDDMapS, 2016. Early Detection & Distribution Mapping System. The University of Georgia - Center for Invasive Species and Ecosystem Health. http://www.eddmaps.org/. (accessed 10.05.15).

Flores, D., Wendel, L.E., 2001. Proposed field release of the salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Curculionidae: Coleoptera) a host-specific biological control agent of giant salvinia, *Salvinia molesta* D.S. Mitchell (Salviniaceae: Polypodiophyta) a federal noxious weed indigenous to southeast Brazil. http://www.salvinia.org/LCR/proposedrelease.pdf. (accessed 11.06.16).

Forno, I.W., 1983. Native distribution of the *Salvinia auriculata* complex and keys to species identification. Aquat. Bot. 17, 71-83.

Forno, I.W., 1987. Biological control of the floating fern *Salvinia molesta* in north-eastern Australia: plant-herbivore interactions. B. Entomol. Res. 77, 9-17.

Forno, I.W., Sands, D.P.A., Sexton, W., 1983. Distribution, biology and host specificity of *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae) for the biological control of *Salvinia molesta*. B. Entomol. Res. 73, 85-95.

Forno, I.W., Bourne, A.S., 1984. Studies in South America of arthropods on the *Salvinia auriculata* complex of floating ferns and their effects on *S. molesta*. B. Entomol. Res. 74, 609-621.

Grewal, P.S., Gaugler, R., Wang, Y., 1996. Enhanced cold tolerance of the entomopathogenic nematode *Steinernema feltiae* through genetic selection. Ann. appl. Biol. 129, 335-341.

Hennecke, B., Postle, L,. 2006. The key to success: an investigation into oviposition of the salvinia weevil in cool climate regions, in: C. Preston, et al., 15th Australian Weeds Conference, Papers and Proceedings, September 2006, Adelaide, South Australia.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G., Jarvis, A., 2005. Very high resolution interpolated climate surfaces for global land areas. Int. J. Climatol. 25, 1965-1978.

Hoffmann, J.H., Impson, F.A.C., Volchansky, C.R., 2002. Biological control of cactus weeds: implications of hybridization between control agent biotypes. J. Appl. Ecol. 39, 900-908.

JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2007.

Joy, P.J., 1986. Salvinia control in India. Biocontrol News and Information 7, 142.

Julien M.H., Hill, M.P., Tipping, P.W., 2009. *Salvinia molesta* D.S. Mitchell (Salviniaceae), in: Muniappan, R., Reddy, G.V.P., Raman, A. Biological control of tropical weeds using arthropods. Cambridge University Press, Cambridge, pp. 378–407.

Madeira, P.T., Tipping, P.W., Gandolfo, D.E., Center, T.D., Van, T.K., Obrien, C.W, 2006. Molecular and morphological examination of *Cyrtobagous* sp. Collected from Argentina, Paraguay, Brazil, Australia, and Florida. BioControl 51, 679-701.

May, B., Coetzee, J., 2013. Comparisons of the thermal physiology of water hyacinth biological control agents: predicting establishment and distribution pre- and post-release. Entomol. Exp. Appl. 147, 241-250.

McFadyen, R.E.C., 1998. Biological control of weeds. Annu. Rev. Entomol. 43, 369-393.

Micinski, S., Fitzpatrick, B.J., Ferro, M.L., Johnson, S.J., Johnson, B., Williams, S., 2016. Flight activity of *Cyrtobagous salviniae* Calder and Sands in Louisiana. Southwest Entomol. 41, 313-320.

Morin, L., Reid, A.M., Sims-Chilton, N.M., Buckley, Y.M., Dhileepan, K., Hastwell, G.T., Nordblom, T.L., Raghu, S., 2009. Review of approaches to evaluate the effectiveness of weed biological control agents. Biol. Control 51, 1-15.

Mukherjee, A., Christmas, M.C., Overholt, W.A., Cuda, J.P., 2010. Prioritizing areas in the native range of hygrophila for surveys to collect biological control agents. Biol. Control 56, 254-262.

Mukherjee, A., Knutson, A., Hahn, D.A., Heinz, K.M., 2014. Biological control of giant salvinia (*Salvinia molesta*) in a temperate region: cold tolerance and low temperature oviposition of *Cyrtobagous salviniae*. BioControl 59, 781-790.

Obeysekara, P.T., Knutson, A., Mukherjee, A., Heinz, K.M., 2015. Repeated cold exposure effects on mortality and feeding activity of the salvinia weevil, *Cyrtobagous salviniae* (Coleoptera: Curculionidae). Environ. Entomol. 44, 1590-1598.

Paterson, I.D., Mangan, R., Downie, D.A., Coetzee, J.A., Hill, M.P., Burke, A.M., Downey, P.O., Henry, T.J., Compton, S.G., 2016. Two in one: cryptic species discovered in biological control agent using molecular data and crossbreeding experiments. Ecol. Evol. http://dx.doi.org/10.1002/ ece3.2297.

Payton, M.E., Greenstone, M.H., Schenker, N., 2003. Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? J. Insect Sci. 3, 34 http://dx.doi.org/10.1093/jis/3.1.34.

Phillips, S.J., Anderson, R.P., Schapire, R.E., 2006. Maximum entropy modeling of species geographic distributions. Ecol. Model. 190, 231-259.

Room, P.M., Harley, K.L.S., Forno, L.W., Sands, D.P.A., 1981. Successful biological control of the floating weed salvinia. Nature 294, 78-80.

Room, P.M., Forno, I.W., Taylor, M.F.J., 1984. Establishment in Australia of two insects for biological control of the floating weed *Salvinia molesta*. B. Entomol. Res. 74, 505-516.

Russell, A., Johnson, J., Cibils, X., McKay, F., Moshman, L., Madeira, P., Blair, Z., and Diaz, R., 2017. Surveys in Argentina and Uruguay reveal *Cyrtobagous salviniae* (Coleoptera: Curculionidae) populations adapted to survive temperate climates in southeastern USA. Biol. Control 107, 41-49.

Sands, D.P.A., Schotz, M., Bourne, A.S., 1983. The feeding characteristics and development of larvae of a salvinia weevil *Cyrtobagous* sp. Entomol. Exp. Appl. 34, 291-296.

Sinclair, B.J., Coello Alvarado, L.E., Ferguson, L.V., 2015. An invitation to measure insect cold tolerance: Methods, approaches, and workflow. J. of Therm. Biol. 53, 180-197.

de la Sota, E.R., 1995. Nuevos sinónimos en Salvinia ség. (Salviniaceae, Pteridophyta). Darwiniana 33, 309-313.

Sullivan, P.R., Postle, L.A., 2010. Low temperature reproduction of *Cyrtobagous salviniae*: good news for biological control of salvinia in temperate climate. J. Aquat. Plant. Manage. 48, 92-96.

Thomas, P.A., Room, P.M., 1986. Taxonomy and control of *Salvinia molesta*. Nature 320, 581–584.

Tipping, P.W., Center, T.D., 2003. *Cyrtobagous salviniae* (Coleoptera: Curculionidae) successfully overwinters in Texas and Louisiana. Fla. Entomol. 86, 92-93.

Tipping, P.W., Martin, M.R., Center, T.D., Davern, T.M., 2008. Suppression of *Salvinia molesta* Mitchell in Texas and Louisiana by *Cyrtobagous salviniae* Calder and Sands. Aquat. Bot. 88, 196-202.

USDA PHZM, 2012. Agricultural Research Service, U.S. Department of Agriculture. http://planthardiness.ars.usda.gov. (accessed 05.05.16).

van den Bosch, R., Frazer, R.D., Davis, C.S., Messenger, P.S., Hom, R., 1970. *Trioxys pallidus*: an effective new walnut aphid parasite from Iran. Calif. Agr. 24, 8-10.

van Driesche, R., Hoddle, M., Center, T., 2008. Control of pests and weeds by natural enemies: an introduction to biological control. Blackwell Publishing, Malden, MA.

Volchansky, C.R., Hoffmann, J.H., Zimmermann, H.G., 1999. Host-plant affinities of two biotypes of *Dactylopius opuntiae* (Homoptera: Dactylopiidae): enhanced prospects for biological control of *Opuntia stricta* (Cactaceae) in South Africa. J. Appl. Ecol. 36, 85-91.

Zhao, L., Jia, D., Yuan, X., Guo, Y., Zhou, W., Ma, R., 2015. Cold hardiness of the biological control agent, *Agasicles hygrophila*, and implications for its potential distribution. Biol. Control 82, 1-5.

CHAPTER 3. OVERWINTERING PHYSIOLOGY, COLD TOLERANCE, AND POPULATION DYNAMICS OF *CYRTOBAGOUS SALVINAE* POPULATIONS FROM SOUTH AND CENTRAL LOUISIANA

3.1 Introduction

When faced with short- or long-term exposure to cold, insects must either undergo physiological changes to maintain homeostasis, or succumb to mortality (Lee 1989). An organism's capacity to survive exposure to cold is referred to as cold tolerance (Lee 1989, Denlinger and Lee 2010). Understanding insect cold tolerance has implications towards controlling and managing species of concern, such as predicting the potential spread of a pest or the successful establishment of a biological control agent (Chown and Nicholson 2004, Sinclair et al. 2015). Low temperatures have limited the establishment of weed biological control agents including *Eccritotarsus catarinensis* (Carvalho) (Hemiptera: Miridae), *Agasicles hygrophila* Selman and Vogt (Coleoptera: Chrysomelidae), and *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), where cold tolerance and climate matching studies were not conducted before their release (Coetzee et al. 2007, Mukherjee et al. 2014, Zhao et al. 2015). Post-release studies have investigated the cold tolerance of agents to explain lack of establishment in the adventive range (Coetzee et al. 2007, May and Coetzee 2013). Moreover, researchers have compared the cold tolerance of geographically distinct populations of an agent, proposing to release the cold-adapted populations in regions where winter survival is problematic (Mukherjee et al. 2014). Despite the importance of winter management, few studies on the overwintering physiology and cold tolerance of weed biological control agents have been conducted.

To prepare for overwintering, insects use cues including decreasing temperature and/or photoperiod (Teets et al. 2013). Most insects can be classified into two groups based on overwintering strategy: freeze-tolerant or freeze-intolerant (Denlinger and Lee 2010). Freeze-tolerant insects can survive extracellular formation of ice within the tissues, whereas freeze-intolerant insects cannot (Lee 1989). For the purpose of this study, the mechanisms conferring cold tolerance in freeze-intolerant insects will be discussed. Freeze-intolerant insects must employ an array of physiological and behavioral adaptations to avoid mortality due to freezing (Denlinger and Lee 2010). For example, freeze-intolerant insects may remove ice nucleating agents, adjust their biochemistry, and/or synthesize proteins associated with freeze prevention and recovery (Denlinger and Lee 2010). Many of these adjustments act to maintain a supercooled state, where body fluids remain unfrozen below the melting point (Salt 1961, Zachariassen 1985). The supercooling point (SCP) of an insect is defined as the temperature at which spontaneous freezing occurs (Zachariassen 1985).

Ice nucleating agents (INAs) are externally or internally derived molecules that promote ice nucleation in body fluids (Block et al. 1990, Denlinger and Lee 2010). The removal or masking of INAs can increase the supercooling capacity by preventing ice activity (Zachariassen 1985, Denlinger and Lee 2010). INAs can be sourced from mineral particles, microorganisms, food particles, and extracellular organic macromolecules (Block et al. 1990), and the cessation of feeding and evacuation of gut contents is suggested as a mechanism to remove many INAs (Salt 1961).

Insects may also undergo biochemical changes such as accumulation of cryoprotectants and cell membrane adjustments (Teets et al. 2013). Cryoprotectants are low molecular weight

sugars and polyols, including glycerol, sorbitol, and trehalose that function to prevent ice formation and stabilize membranes and proteins (Block et al. 1990, Denlinger and Lee 2010, Teets et al. 2013). By reducing body water content, the concentration of cryoprotectants is increased, further contributing to the supercooling capacity (Zachariassen 1985, Denlinger and Lee 2010). Insects are also hypothesized to undergo homeoviscous adaptation, where they biochemically adjust their membrane composition to maintain consistent membrane fluidity at low temperatures (Sinensky 1974, Teets et al. 2013). Homeoviscous adaptation may be maintained by increasing proportions of unsaturated fatty acids in the membrane or increasing the ratio of short-chain to long-chain fatty acids (Teets et al. 2013).

On the molecular level, genes and proteins have been identified in association with freeze prevention and recovery (Teets et al. 2013). Among these, antifreeze proteins (AFPs) inhibit freezing by depressing the difference between melting and freezing points, a phenomenon known as thermal hysteresis (Block et al. 1990, Denlinger and Lee 2010). Antifreeze proteins function according to the adsorption-inhibition mechanism (Raymond and DeVries 1977), where they adsorb onto the surface of an ice crystal and inhibit growth (Block et al. 1990, Denlinger and Lee 2010). Alternatively, heat-shock proteins (HSPs) are proteins that are expressed in response to environmental stresses, including cold shock (Feder and Hofmann 1999), and serve as molecular chaperones that bind to denatured proteins and mitigate the effects of misfolding (Rinehart et al. 2007). With improvements in transcriptomics and proteomics, researchers are still studying the molecular underpinnings of cold tolerance (Teets et al. 2013).

Some insects undergo behavioral changes to prepare for overwintering and the consequences associated with low temperatures, such as lack of food and poor nutrition (Block et al. 1990). To avoid injury during adverse conditions, non-diapausing insects may enter a state of quiescence, which is defined as a reversible state of suppressed activity (Tauber et al. 1986). Differing from diapause, an insect may recover from a quiescent state immediately upon the return of favorable conditions (Nation 2008). Prior to overwintering, feeding and development may cease, and energy reserves such as lipids and glycogen in fat body are accumulated to account for reduced metabolism and declining quality food (Tauber et al. 1986, Block et al. 1990).

Analysis of the population dynamics of overwintering populations can contribute to the effective management of a species at low temperatures. Characterizing metrics of population dynamics, such as reproductive status, physiological age class, and adult/larval densities can help researchers evaluate the health of a population (Block et al. 1990, Hayes and Wall 1999). Furthermore, evaluating reproductive status and physiological age can provide an estimate of population fecundity (Hayes and Wall 1999). For example, Grodowitz et al. (1997) used a physiological age-grading system to evaluate the reproductive health of the biological control agent *Neochetina eichhorniae* (Warner) (Coleoptera: Curculionidae). In addition, a comprehensive analysis of population dynamics can assist researchers in formulating management strategies based on phenology.

The salvinia weevil, *Cyrtobagous salviniae* Calder and Sands, is used in several countries worldwide for the biological control of the invasive free floating fern giant salvinia, *Salvinia molesta* Mitchell (Salviniales: Salviniaceae) (Cilliers 1991, Julien et al. 2009, Tipping and Center

2003). Despite successes at tropical and subtropical latitudes (Cilliers 1991, Julien et al. 2009, Room et al. 1981), *C. salviniae* has failed to control *S. molesta* in temperate regions, such as in northern Louisiana, USA, where populations have reportedly failed to overwinter (Mukherjee et al. 2014, Sullivan and Postle 2010). To overcome this deficiency, researchers have sought to characterize the cold tolerance of *C. salviniae* populations (Allen et al. 2014, Mukherjee et al. 2014, Russell et al. 2017). Moreover, researchers have suggested to release cold tolerant populations of *C. salviniae* in northern Louisiana, in light of recent studies that reported populations from Camden, Australia were more cold tolerant than populations from Louisiana (Mukherjee et al. 2014). Despite major improvements in the knowledge of *C. salviniae* cold tolerance, there is a lack of research focusing on (1) the physiological mechanisms that confer cold tolerance in *C. salviniae* (Mukherjee et al. 2014), and (2) the population dynamics of overwintering *C. salviniae* populations (Mukherjee et al. 2017).

The goal of this study was to characterize the overwintering physiology and population dynamics of *C. salviniae* populations from Louisiana to improve the understanding and management of *C. salviniae* during low temperatures. Specifically, this study sought to (1) evaluate the effects of summer and winter conditions on the physiology and cold tolerance of *C. salviniae* in the laboratory, and (2) characterize the physiological changes and population dynamics of *C. salviniae* in the field over time. Populations from southern and central Louisiana were studied to discern latitudinal differences in physiology and cold tolerance. This study reports trends that may confer cold tolerance in *C. salviniae* from Louisiana, and proposes strategies to improve the management of *C. salviniae* in northern Louisiana.

3.2 Materials and Methods

3.2.1 Effects of summer and winter conditions on physiology and cold tolerance

Insect collection, acclimation, and exposure

Cyrtobagous salviniae adults were collected from field sites in Lena, LA (31.5184°, -92.7310°) and Grand Chenier, LA (29.7121°, -92.7654°) over five sampling occasions per population between September and October 2016. *Cyrtobagous salviniae* and *S. molesta* plants were harvested using rakes and immediately transported to the laboratory for extraction and processing. At the laboratory, host plants were placed within Berlese funnels for 24 h to extract adult *C. salviniae* (Boland and Room 1983). A total of 1000 adult *C. salviniae* from each site were collected for the purpose of this study.

Immediately following extraction, *C. salviniae* adults from each population were placed in groups of 100 on host plants inside plastic containers containing 7 L of reverse osmosis (RO) water fertilized to 5 ppm N (Miracle-Gro® Water Soluble Lawn Food, 36N-0P-6K). Insects were acclimated for 72 h at a temperature of 24°C and photoperiod of 12:12 h [L:D] to ensure consistency in baseline conditions. Following acclimation, the insects were divided into two groups: baseline and experimental. Sixty *C. salviniae* from each population were collected for the baseline group to evaluate baseline conditions. Baseline conditions were characterized by female fat body, reproductive status, fresh weight, water content, and lipid content, using methods stated below. The remaining experimental *C. salviniae* (n= 940/site) were transferred into plant growth chambers for exposure to either summer or winter treatments. Summer conditions were simulated in a chamber programmed to 28°C with a photoperiod of 14:10h (L:D), and winter conditions were simulated in a chamber programmed to 20°C with a

photoperiod of 10:14h (L:D). Summer and winter temperatures were determined by calculating the mean daily temperature of each season in central Louisiana (NOAA 2016). However, the winter treatment was increased from 16 to 20°C due to high mortality at 16°C in a pilot study, presumably due to starvation. Experimental groups were placed in plastic containers containing 20 salvinia plants and 7 L of RO water and exposed to the aforementioned treatments for 20 d. Three fresh *S. molesta* plants were added every 7 d. HOBO temperature data loggers were placed in all chambers to ensure that temperatures remained ± 1°C of the treatment. At the completion of the exposure, *C. salviniae* were removed from host plants, and the physiological changes and associated cold tolerance were assessed using the methods below.

Characterization of physiological indices

Physiological indices were characterized for baseline and experimental groups of *C. salviniae* to determine the physiological changes between summer and winter. The indices used were: female fat body, female reproductive status, and water content.

To assess female fat body and reproductive status, 20 female *C. salviniae* from each treatment were dissected using a dissecting microscope. Specimens were dissected live inside a Petri dish containing <1 mL tap water using methods adapted from Grodowitz et al. (1997) and Eisenberg (2011). Live dissection was necessary to avoid damage to reproductive tissues caused by fixatives or preservatives (Grodowitz et al. 1997). To expose the internal anatomy, the head and thoracic segments were first removed with a pair of microdissection forceps. Next the elytra and hindwings were separated from the abdominal sternites, exposing the internal organs. The quantity of fat body in each individual was characterized into three categories: (1) lean, fat body occupied < 1/3 of the haemocoel, (2) intermediate, fat body occupied 1/3 to 2/3

of the haemocoel, and (3) fat, fat body occupied > 2/3 of the haemocoel (Grodowitz et al. 1997, Eisenberg 2011). Reproductive status was assessed by isolating the ovaries from the rest of the internal organs. Specimens were then examined for presence or absence of eggs and then classified by physiological age using a physiological age-grading system developed by Grodowitz et al. (1997) and adapted by Eisenberg (2011). The four physiological age classes (N, P1, P2, P3) were determined by assessing degree of follicular differentiation, maturity of the proximal follicle, quantity and appearance of follicular relics, and cuticular hardness (Table 3.1). The effects of summer or winter conditions on the physiology of *C. salviniae* were compared using Chi-square analyses (JMP®, Version 13).

Table 3.1. Characterization of *Cyrtobagous salviniae* physiological age classes, based on Grodowitz et al. (1997) and Eisenberg (2011).

Age Class	Description
N	Absence of follicles in vitellaria OR developed follicles in vitellaria, but lacking follicular relics. Soft, brown cuticle.
P1	Distinct follicles are present in vitellaria. Follicular relics are pale yellow and occupy a loose area at the bases of the vitellaria. Eggs may be present. Cuticle is hard and black.
P2	Higher number of follicles in vitellaria, in a progressive state of development. Follicular relics are deeper yellow and completely encircle the bases of the vitellaria. Eggs may be present. Cuticle is hard and black.
P3	May contain only few healthy follicles. Follicular relics are amber in color and possess dark inclusions. Cuticle is hard and black.

Fresh weight (FW) was recorded for 20 *C. salviniae* from each treatment to the nearest 0.01 mg with an AX26 DeltaRange Microbalance. Specimens were placed individually into size 3 BEEM embedding capsules and dried in an oven set to 60°C. Dry weight (DW) was recorded after 48 h of drying. Water content (WC) was calculated using the following formula: WC= (FW-DW)*100/FW (Li et al. 2014). Water content of each treatment was analyzed using ANOVA and means were compared using Tukey's HSD (JMP®, Version 13).

Cold Tolerance

The differences in cold tolerance between summer and winter treated *C. salviniae* were determined by the following assays: survival at 0°C, critical thermal minima (CT_{min}), chill coma recovery time, and supercooling point (SCP) (Sinclair et al. 2015).

To assess survival at 0°C, adults were separated into groups of 20 according to treatment and placed inside plastic cups containing 300 mL of reverse osmosis water and host plants. Experimental groups were then exposed to 0°C inside a growth chamber for 3, 6, 9, 12, and 15 d. Following the exposure time, experimental groups were removed from the aforementioned chambers and given 24 h to recover at room temperature (21°C). Mortality was assessed by removing adults from plants and placing them on damp filter paper in a Petri dish. An individual was characterized as dead if it could not right itself after tactile stimulation with forceps (Mukherjee et al. 2014). Data were analyzed in SAS using the Probit function to yield lethal times (LT_{50} and LT_{90}). Non-overlapping 95% confidence intervals signified differences between treatments (Gardner and Altman 1986, Rao 1998, Payton et al. 2003).

The critical thermal minimum (CT_{min}) was assessed for 24 adults per treatment, with each insect representing a replicate. Insects were placed in direct contact with a Peltier cooling plate, separated individually by 3.14 cm^2 PVC rings. Temperature at the surface of the cooling plate was recorded at 30 s intervals using a thermocouple and data logging device. Starting from the treatment temperature (28 or 20°C), insects were cooled at a constant rate of -0.06°/min to 0°C (Sinclair et al. 2015). Insects were observed directly for the duration of cooling, and the temperature at which all movement ceased was recorded as the CT_{min} for that

individual. The CT_{min}s reported for each treatment were analyzed using ANOVA and means were compared using Tukey's HSD (JMP®, Version 13).

To evaluate chill coma recovery time, a comatose-like state was induced in 20 *C. salviniae* adults per treatment by exposure to non-lethal cold temperature inside an ARCTIC SC100-A28 Temperature Controlled Refrigerated Bath containing ethylene glycol diluted with water. Each insect represented a replicate. Experimental groups of 20 were placed in 25 mL graduated tubes and secured inside the refrigerated bath with a horizontal grip rack. This assay was conducted first on the summer-treated populations, and then on the winter-treated populations. Starting from the treatment temperature (28 or 20°C), adults were cooled at a rate of -1°C/min until 0°C, then held at this temperature for 4 h (Mukherjee et al. 2014). Immediately after 4 h, adults were removed from the refrigerated bath and placed on their dorsum inside a Petri dish lined with a damp paper towel at room temperature (21°C). The time required for each individual to recover to a standing position was recorded in minutes and seconds. Recovery times were tested for normality using a Shapiro-Wilk test and analyzed using a Kruskal-Wallis test (JMP®, Version 13). Pairwise comparisons of means were performed using a Wilcoxon rank test (JMP®, Version 13).

The SCP was determined for 20 adults per treatment, with each insect representing a replicate. Individual *C. salviniae* were attached to Type J thermocouples using Down Corning vacuum grease and secured inside 2 mL cryotubes. Cryotubes were placed inside Nalgene Cryo 1°C freezing containers, ensuring a constant cooling rate of -1°C/min. The freezing containers were placed inside a freezer programmed to -25°C and temperature at the surface of the thermocouple was recorded by a DI-1000TC-8 channel data recorder using Windaq Data

Acquisition software. The body temperature of individuals in contact with thermocouples was recorded for 1.25 h. The SCP was designated as the lowest temperature recorded before a steep rebound in temperature, known as the heat of crystallization (Denlinger and Lee 2010). The SCPs of each treatment were tested for normality using a Shapiro-Wilk test and analyzed using a Kruskal-Wallis test (JMP®, Version 13). Pairwise comparisons of means were performed using a Wilcoxon rank test (JMP®, Version 13).

3.2.2 Characterization of physiological changes and population dynamics over time of adult *Cyrtobagous salviniae* collected in the field

Insect collection and processing

Adult *C. salviniae* were collected from field sites in Marrero, LA (29.7908°, -90.1305°) and Lena, LA (31.5184°, -92.7310°) at 30 d intervals between January 2016 and February 2017. Insects were harvested by collecting approximately 10 kg of *S. molesta* on each sampling occasion. Three random samples of *S. molesta*, weighing approximately 300 g, were also collected to monitor the density of *C. salviniae* adults and larvae on each sampling occasion. Samples were immediately transported to the laboratory, where they were desiccated for 24 h using Berlese funnels to extract *C. salviniae* from host plants (Boland and Room 1983).

Characterization of physiological indices

Physiological indices (female reproductive status, female fat body, and water content) were characterized using the methods stated in section 3.2.1- Characterization of physiological indices. Briefly, female reproductive status and fat body were characterized using dissections. Reproductive status was assessed according to presence/absence of eggs and physiological age class (Table 3.1) (Grodowitz et al. 1997, Eisenberg 2011). Fat body was characterized into three

groups (lean, intermediate, and fat) according to the proportion of fat body filling the haemocoel (Eisenberg 2011). The effects of time (date) on the physiology of *C. salviniae* were compared using chi-squared analyses (JMP®, Version 13). Samples measuring the physiological indices from August and September in Marrero were not acquired.

Population dynamics

Adult and larval densities were monitored to characterize population dynamics at monthly sampling occasions from January 2016 to February 2017. As previously mentioned, three 300 g samples were collected on each sampling occasion for this purpose. The fresh weight of each sample was recorded with an electronic balance, and then desiccated for 24 h using Berlese funnels. *Cyrtobagous salviniae* were collected and preserved in 95% ethanol. The number of adults and larvae in each sample were counted using a dissection microscope, and the density of adult and larvae per kg of *S. molesta* was calculated by dividing the number of adult or larvae in the sample by the fresh weight of the sample.

3.3 Results

3.3.1 Effects of summer and winter conditions on physiology and cold tolerance

The frequency of gravid females from all treatments (Lena and Grand Chenier, winter and summer) was 0%. The distribution of physiological age class did not change significantly from the baseline conditions for each site (Grand Chenier: $\chi^2 = 0.86$, $P = 0.9296$, Lena: $\chi^2 = 0.36$, $P = 0.9992$). In Grand Chenier, the baseline age class distribution was 40% N, 50% P1, and 10% P2; the summer age class distribution was 34% N, 58% P1, and 8% P2; and the winter age class distribution was 25% N, 58% P1, and 17% P2. In Lena, the baseline age class distribution was 26% N, 58% P1, 8% P2, and 8% P3; the summer age class distribution was 27% N, 53% P1, 13%

P2, and 7% P3; and the winter age class distribution was 21% N, 57% P1, 14% P2, and 7% P3. The age class distributions between sites were also not significantly different (χ^2 = 4.20, *P* = 0.9997).

Fat body content did not change significantly from the baseline conditions for each site (Grand Chenier: χ^2 = 2.50, *P* = 0.6441, Lena: χ^2 = 2.14, *P* = 0.7098). In Grand Chenier, the frequency of fat body was 10% lean, 40% intermediate, and 50% fat for the baseline conditions; 8% lean, 42% intermediate, and 50% fat for summer conditions; and 0% lean, 25% intermediate, 75% fat for winter conditions. In Lena, the frequency of fat body was 0% lean, 50% intermediate, 50% fat for baseline conditions; 7% lean, 53% intermediate, and 40% fat for summer conditions; and 14% lean, 43% intermediate, and 43% fat for winter conditions. Fat body did not differ significantly between sites (χ^2 = 6.40, *P* = 0.7810).

Water content in the Lena treatments decreased significantly from the baseline conditions (χ^2 = 12.89, *P* = 0.0016), however no significant difference was found between the summer and winter treatments (*P* = 0.1629). Water content in the Grand Chenier treatments did not change significantly in comparison to the baseline conditions (χ^2 = 0.27, *P* = 0.8716).

Survival at 0°C increased by 1.8- and 1.7-times in the winter exposed Lena and Grand Chenier treatments, respectively (Table 3.2). The LT_{50}s for the Lena population were significantly different (6.9 d-winter vs. 3.9 d-summer). However, the LT_{90}s for the Lena population were different (10.4 d-winter vs. 6.7 d-summer), but not significant due to overlapping 95% CI. Similarly, the LT_{50} for the Grand Chenier winter treatment was significantly greater than the summer treatment (6.6 d-winter vs. 4.0 d-summer), whereas the LT_{90}s for the

Grand Chenier population were not significant (9.7 d-winter vs. 6.4 d-summer). Additionally, the LT50s and 90s were not significantly different between populations for either treatment.

Table 3.2. LT_{50} and LT_{90} at 0°C of *Cyrtobagous salviniae* populations exposed to summer or winter conditions. LT_{50} and LT_{90} values represent the time (d) required to kill 50 and 90% of the population at 0°C. Differences in treatments are indicated by non-overlapping 95% confidence intervals (CI). χ^2 is the Wald test for the hypothesis that the slope parameter is zero.

Treatment	n	Slope ± SE	LT_{50} (95% CI) d	LT_{90} (95% CI) d	χ^2
Lena, LA- winter	100	0.63 ±0.13	6.9 (5.8 – 8.1)	10.4 (8.9 – 13.1)	24.7
Lena, LA- summer	53	0.79 ±0.24	3.9 (2.2 – 5.6)	6.7 (5.1 – 11.7)	10.49
Grand Chenier, LA- winter	106	0.71 ±0.14	6.6 (5.5 – 7.7)	9.7 (8.4 – 12.1)	24.52
Grand Chenier, LA- summer	71	0.94 ±0.26	4.0 (2.8 – 5.3)	6.4 (5.1 – 9.8)	13.01

The critical thermal minimum (CT_{min}) differed significantly between treatments ($F_{3,92}$= 58.37, $P < 0.0001$) (Figure 3.1). The CT_{min} for the Lena winter treatment (4.8 ± 0.1°C, mean ± SE) was 1.2-times lower than the CT_{min} for the Lena summer treatment (6.0 ± 0.1°C). The CT_{min} for the Grand Chenier winter treatment (5.0 ± 0.1°C) was 1.3-times lower than the Grand Chenier summer treatment (6.3 ± 0.1°C), but did not differ significantly from the Lena winter treatment.

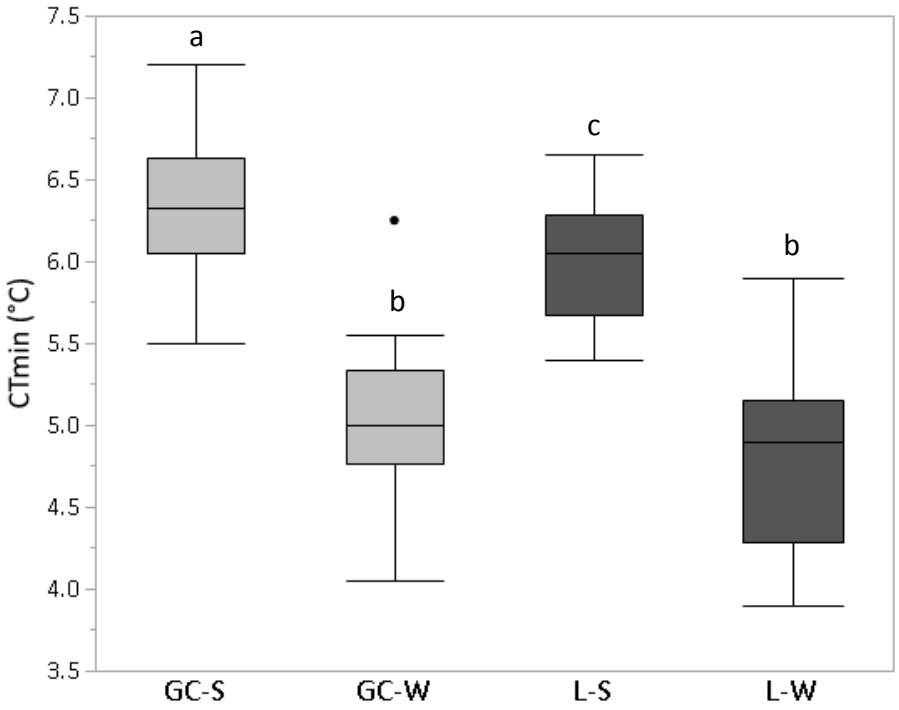

Figure 3.1. Critical thermal minimum (°C) for summer and winter treated *Cyrtobagous salviniae* populations from Grand Chenier and Lena, LA. GC-S= Grand Chenier summer, GC-W= Grand Chenier winter, L-S= Lena summer, L-W= Lena winter. Bars with different letters are statistically significant ($P < 0.05$).

The chill coma recovery times differed significantly between treatments ($\chi^2 = 23.09$, $P < 0.0001$) (Figure 3.2). Recovery times were 2.7- and 1.5-times faster in the winter treatments compared to summer treatments for Lena and Grand Chenier, respectively. The Lena winter treatment recovered the fastest (10.4 min ± 1.1, mean ± SE), followed by Grand Chenier winter (12.94 ± 1.13 min), Grand Chenier summer (19.58 ± 3.18 min), and Lena summer (27.73 ± 3.78 min).

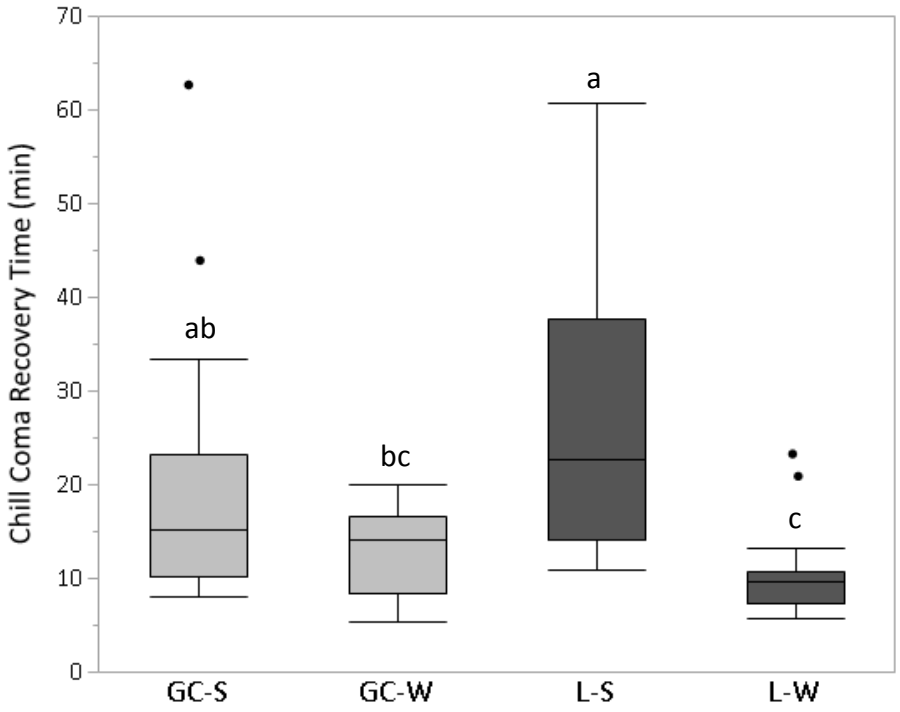

Figure 3.2. Chill coma recovery time (min) for summer and winter treated *Cyrtobagous salviniae* populations from Grand Chenier and Lena, LA. GC-S= Grand Chenier summer, GC-W= Grand Chenier winter, L-S= Lena summer, L-W= Lena winter. Bars with different letters are statistically significant ($P < 0.05$).

The supercooling point (SCP) did not differ significantly between treatments ($\chi^2 = 5.54$, $P = 0.1362$) (Figure 3.3). Mean SCPs were similar across population and treatment (GC-S= -14.1, GC-W= -14.4, L-S= -15.5, L-W= -15.1) with a difference of 1.4°C between the highest (GC-S) and lowest (L-S) SCP.

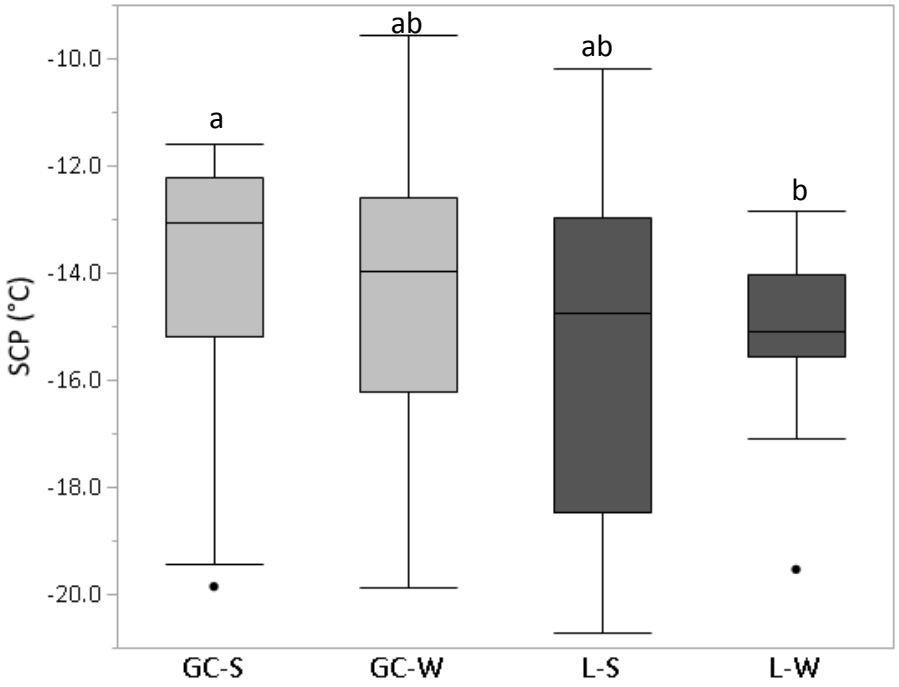

Figure 3.3. Supercooling point (°C) for summer and winter treated *Cyrtobagous salviniae* populations from southern and central Louisiana. GC-S= Grand Chenier summer, GC-W= Grand Chenier winter, L-S= Lena summer, L-W= Lena winter. Bars with different letters are statistically significant ($P < 0.05$).

3.3.2 Characterization of physiological changes and population dynamics over time of adult *Cyrtobagous salviniae* collected in the field

In both sites (Marrero and Lena), the frequency of gravid females changed significantly throughout time (Marrero: $\chi^2 = 51.13$, $P < 0.0001$, Lena: $\chi^2 = 52.04$, $P < 0.0001$) (Figure 3.4). In Marrero, the frequency of gravid females varied from 6% at its lowest in October to 67% at its highest in May (Figure 3.4). In Lena, frequencies ranged from 0% in February to 75% in April (Figure 3.4).

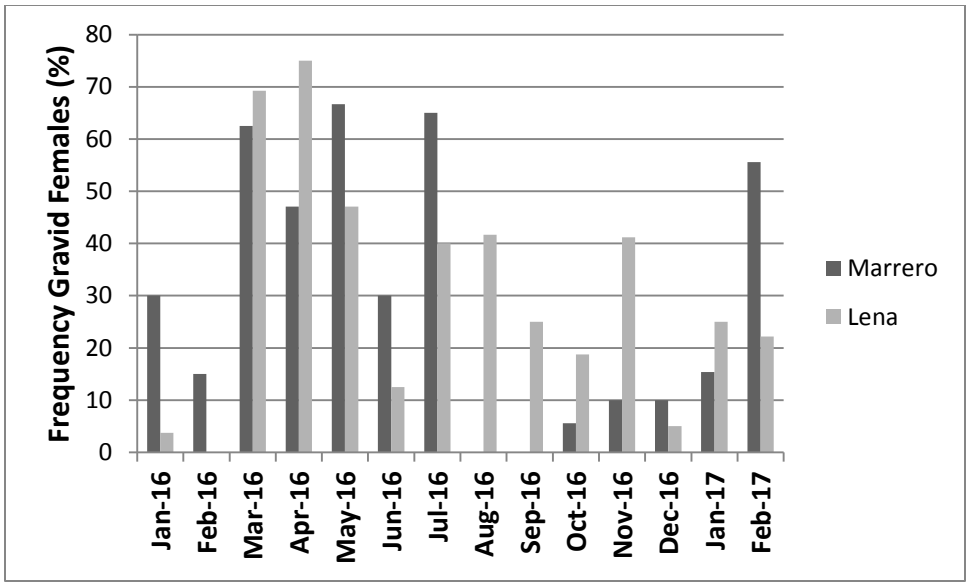

Figure 3.4. Frequency of gravid females (female with eggs in ovaries) of *Cyrtobagous salviniae* from Marrero and Lena, LA.

Weevil age class distribution changed over time at both sites (Marrero: $\chi^2 = 77.28$, $P < 0.0001$, Lena: $\chi^2 = 85.81$, $P < 0.0001$). Nonparous (N) females were not present in samples from both populations until March, and were most abundant in June (Marrero: 50%, Lena: 62.5%). Higher frequencies of N females were found in the summer months, whereas higher frequencies of P2/P3 adults were seen in winter months (Table A.2). Fat body content of both populations also changed over time (Marrero: $\chi^2 = 46.30$, $P = 0.0018$, Lena: $\chi^2 = 57.82$, $P = 0.0003$). The highest frequencies in fat individuals occurred during the summer and fall months (Table A.3). Water content differed throughout time in both the Marrero ($F_{10,184} = 5.20$, $P < 0.0001$) and Lena ($F_{12,162} = 4.34$, $P < 0.0001$) populations (Figure 3.5). Water content was lowest in April in Marrero (44%) and November in Lena (46%).

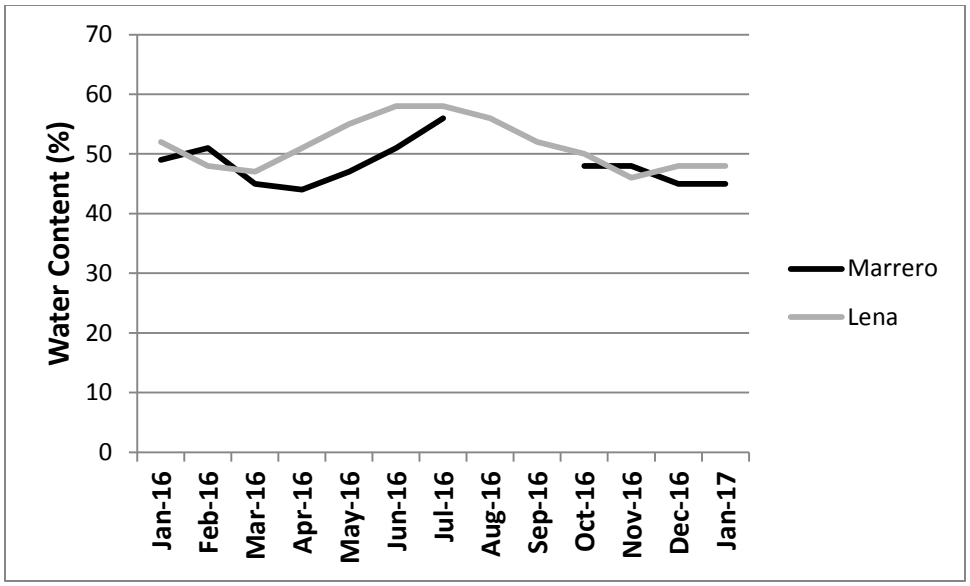

Figure 3.5. Body water content of *Cyrtobagous salviniae* from Marrero and Lena, LA.

Adult and larval densities varied in both populations throughout the year (Figures 3.6, 3.7). In Marrero, adults were present in samples throughout the year, with densities increasing gradually from summer to late fall/winter, peaking at 91/kg (Figure 3.6). A population crash occurred between January and February, and adult densities were lowest in spring. Larvae were not detected in samples for the months of January, February, November, and December in Marrero (Figure 3.6). Despite appearing in samples in March, densities remained relatively low until a rapid increase between May-June. Densities remained fairly stable in the summer before stiffly dropping off in November. In Lena, the adult population crashed in April to 3.4/kg, peaked again in May at 71.9/kg, and then crashed again in June, remaining close to 20/kg for the remainder of the study (Figure 3.7). Larvae were not detected in samples for the months of January or February (Figure 3.7). Larval densities initially increased between March and May to 24.7/kg, before crashing to 3.2/kg in June. Larval densities declined steadily from July to

January. Adult densities were generally lowest in the spring for both populations, whereas larval densities were lowest in the winter.

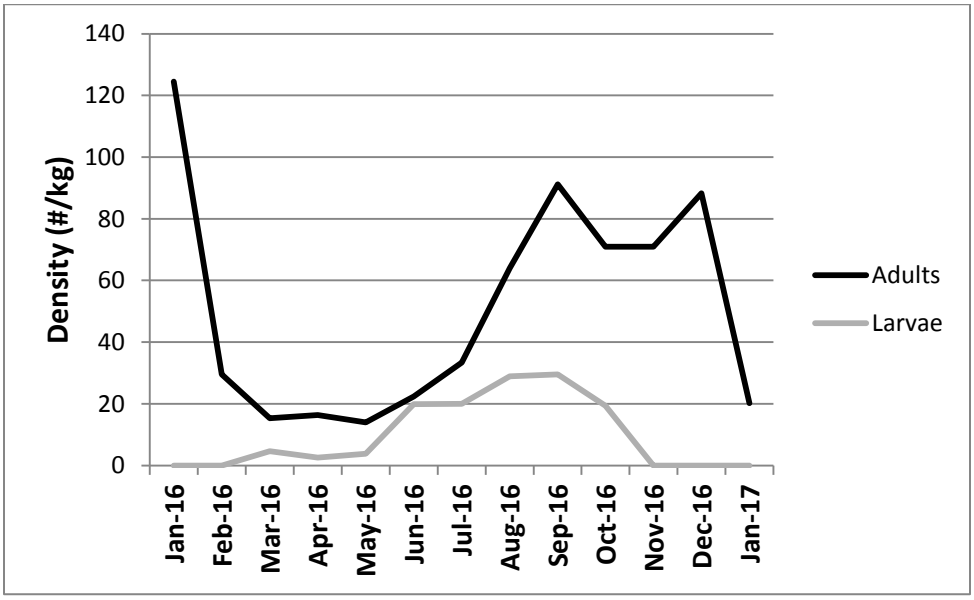

Figure 3.6. Adult and larval densities over time of a *Cyrtobagous salviniae* population from Marrero, LA.

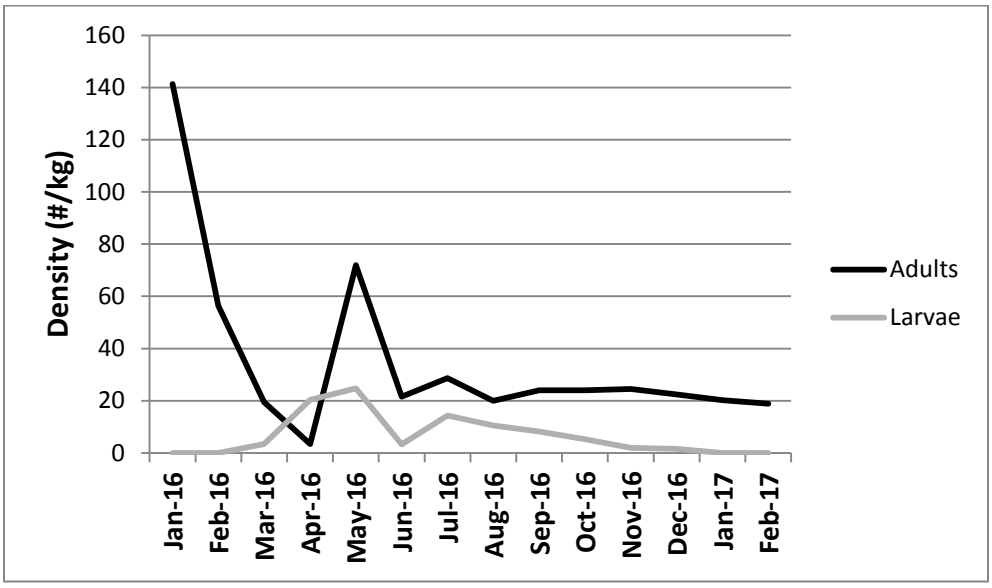

Figure 3.7. Adult and larval densities over time of a *Cyrtobagous salviniae* population from Lena, LA.

3.4 Discussion

Differences in cold tolerance observed within the populations of this study suggest phenotypic plasticity in cold tolerance of Louisiana *C. salviniae* (Denlinger and Lee 2010). Compared to those from tropical environments, organisms from a variable, temperate environment are expected to exhibit a higher degree of phenotypic plasticity, due to diurnal and seasonal temperature variation in the latter (Levins 1968, Overgaard et al. 2011). For example, in a common garden study on the acclimation responses between temperate and tropical species of *Drosophila*, Overgaard et al. (2011) demonstrated that temperate species showed a greater phenotypic response to cold acclimation, compare to the tropical species. Results from this study demonstrate the ability of a species from a sub-tropical origin to acclimate to cold temperatures. In an analysis of temperature data at the study cite in central Louisiana (Lena), diurnal temperature variations within the month of December differed between 1.1 (04 December) and 19.5 (16 December)°C. This substantial variation within and between daily temperatures demonstrates that *C. salviniae* in Louisiana are subjected to fluctuating thermal regimes, which has been known to increase insect cold tolerance (Colinet et al. 2016). The ability of *C. salviniae* populations from Louisiana to acclimate to cold temperatures, as demonstrated by this study, suggests that these populations possess cold hardening adaptations.

Despite differences in cold tolerance, no significant differences in reproductive status, fat body, or water content were observed in the laboratory portion of this study. Because nitrogen levels in the plant tissues are known to affect reproduction and development in *C. salviniae* (Forno et al. 1983, Sands et al. 1983, Sands et al. 1986), I speculate that compromised

chamber conditions, such as poor light intensity and air flow, negatively impacted host plant quality. Reproductively active *C. salviniae* may have undergone oosorption (reabsorption of oocytes) to conserve energy and nutrition (Bell and Bohm 1975), which could explain the lack of gravid females found in both the summer and winter treatments. The lack of changes in distribution of physiological age class means that negligible ovipositions occurred during the experiment, further suggesting arrested reproduction. Given the sensitive nature of *C. salviniae* performance under artificial conditions, studying the physiological changes of *C. salviniae* may require improving chamber settings.

In the field portion of this study, seasonal trends were observed in the physiological changes of *C. salviniae* from both sites. First, female reproduction was suppressed in the winter months, as shown by lower frequencies of gravid and nonparous females. These results are consistent with a population dynamics study by Mukherjee et al. (2017), which demonstrated that *C. salviniae* reproduction ceased during the winter at a site in northern Texas. In this study, lower frequencies of gravid females in the winter were observed at the Lena site, compared to the Marrero site. In an analysis of temperatures recorded from weather stations located in the vicinity of our study sites, daily maximum temperatures remained below the thermal limit for oviposition (19°C) (Forno et al. 1983, Hennecke and Postle 2006) for 71 d in Marrero, compared to 135 d in Lena for the duration of this study (424 d). I speculate that the lower frequency of gravid females was due to temperatures persisting below the thermal oviposition limit for longer periods. The frequencies of *C. salviniae* from both sites with full fat body were highest in the summer and fall. In a previous study of the relationship between fat body and age class in *C. salviniae*, nonparous (N) females were found to contain the highest

levels of fat body (Eisenberg 2011). I speculate that the increase in fatty individuals in the summer is due to the higher frequency of nonparous adults (Grodowitz 1997, Eisenberg 2011). Furthermore, the high frequency of fatty individuals in the fall could be due to accumulation of energy reserves in parous females preparing to overwinter (Tauber et al. 1986). To explain the changes in fat body, I suggest that further analysis of the lipids and fatty acids of *C. salviniae* over time should be conducted to elicit the types of fatty acids present at different time points. Mean water content changed over time, decreasing approx. 10% in winter months. Seasonal water loss could be used as a mechanism to increase supercooling capacity (Denlinger and Lee 2010), or a symptom of declining food reserves (Block et al. 1990).

This study presents the first attempt to characterize the reproductive status and fat body content through dissections of *C. salviniae* collected from the field. Additionally, this study is one of the first comprehensive investigations of the seasonal population dynamics in *C. salviniae* (Mukherjee et al. 2017). Results indicate that adult *C. salviniae* successfully overwintered at both study sites. The presence of reproductively active females at all sampling points throughout the year suggests that *C. salviniae* females do not enter reproductive diapause (Tauber et al. 1986), and therefore are capable of oviposition during winter months when temperatures exceed 19°C (Mukherjee et al. 2017). Adults were present in all samples throughout the year, whereas larvae were not detected until March at both sites, indicating that temperatures were not suitable for oviposition and larval development until March. Populations at both sites experienced steep adult crashes in the late winter, and were most vulnerable in early spring. This is likely due to a combination of low temperatures and poor plant quality. However, another adult and larval crash was observed in the summer at the Lena

site, when average temperatures were well above the developmental threshold. Photographic evidence from the sampling during this time period shows that the *S. molesta* population was crowded out by other aquatic weeds, and therefore crashed along with *C. salviniae* populations.

Results from this study have implications towards the seasonal management of *C. salviniae*. Since *C. salviniae* females do not enter reproductive diapause, the frequency of reproductive females may vary from year to year, depending on winter temperatures. It is therefore important to consider seasonal temperature, female reproductive status, and larval densities before making management decisions. For example, when winter temperatures are exceptionally low, managers should consider delaying spring *C. salviniae* releases until female reproduction and larval densities have recovered to sustainable levels in rearing ponds. Moreover, management in late winter/spring should focus on the reproductive and fat body status of populations, when high frequencies of P3 (old) and lean females make populations vulnerable to changing environmental conditions.

In conclusion, *C. salviniae* populations from Louisiana demonstrate seasonal changes in reproductive status, fat body, and water content. Results from laboratory assays indicate phenotypic plasticity in *C. salviniae* cold tolerance when acclimated to winter conditions. Temperature, female reproductive status, fat body, and larval densities should be incorporated into seasonal management strategies to improve the management of *C. salviniae* in Louisiana.

3.5 References

Allen, J.L., Clusella-Trullas, S., Chown, S.L., 2014. Thermal tolerance of *Cyrtobagous salviniae*: a biocontrol agent in a changing world. BioControl 59, 357-366.

Bell, W.J., Bohm, M.K., 1975. Oosorption in insects. Biol. Rev. 50, 373-396.

Block, W., Baust, J.G., Franks, F., Johnston, I.A., Bale, J., 1990. Cold tolerance of insects and other arthropods [and discussion]. Philos. T. Roy. Soc. B. 326, 613-633.

Boland, N.P., Room, P.M., 1983. Estimating population density of a *Cyrtobagous* sp. (Coleoptera: Curculionidae) on the floating weed salvinia using Berlese funnels. Aust. J. Entomol. 22:353-354.

Chown, S.L., Nicolson, S., 2004. Insect physiology and ecology: mechanisms and patterns. Oxford University Press, Oxford.

Cilliers, C.J., 1991. Biological control of water fern, *Salvinia molesta* (Salviniaceae), in South Africa. Agr. Ecosyst. Environ. 37, 219-224.

Coetzee, J.A., Byrne, M.J., Hill, M.P., 2007. Predicting the distribution of *Eccritotarsus catarinensis*, a natural enemy released on water hyacinth in South Africa. Entomol. Exp. Appl. 125, 237-247.

Colinet, H., Renault, D., Javal, M., Berková, P., Šimekc, P., Koštálc, V., 2016. Uncovering the benefits of fluctuating thermal regimes on cold tolerance of drosophila flies by combines metabolomics and lipidomic approach. BBA-MOL. Cell. Res. 1861, 1736-1745.

Denlinger, D. L., Lee, R.E., 2010. Low Temperature Biology of Insects. Cambridge University Press, Cambridge.

Eisenberg, L., 2011. The reproductive morphology and physiological age-grading of female *Cyrtobagous salviniae*, the salvinia weevil. LSU Master's Thesis. 4299. http://digitalcommons.lsu.edu/gradschool_theses/4299.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243-282.

Forno, I.W., Sands, D.P.A., Sexton, W., 1983. Distribution, biology and host specificity of *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae) for the biological control of *Salvinia molesta*. B. Entomol. Res. 73, 85-95.

Gardner, M.J., Altman, D.G., 1986. Confidence intervals rather than P values: estimation rather than hypothesis testing. Brit. Med. J. 292, 746-750.

Grodowitz, M.J., Center, T.D., Freedman, J.E., 1997. A physiological age-grading system for *Neochetina eichhorniae* (Warner) (Coleoptera: Curculionidae), a biological control agent of water hyacinth, *Eichhornia crassipes* (Mart.) Solms. Biol. Control 9, 89-105.

Hayes, E.J., Wall, R., 1999. Age-grading adult insects: a review of techniques. Physiol. Entomol. 24. 1-10.

Hennecke, B., Postle, L,. 2006. The key to success: an investigation into oviposition of the salvinia weevil in cool climate regions, in: C. Preston, et al., 15th Australian Weeds Conference, Papers and Proceedings, September 2006, Adelaide, South Australia.

JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989-2007.

Julien M.H., Hill, M.P., Tipping, P.W., 2009. *Salvinia molesta* D.S. Mitchell (Salviniaceae), in: Muniappan, R., Reddy, G.V.P., Raman, A. Biological control of tropical weeds using arthropods. Cambridge University Press, Cambridge, pp. 378–407.

Lee, R.E., 1989. Insect cold-hardiness: To freeze or not to freeze. BioScience 39, 308-313.

Levins, R., 1968. Evolution in changing environments. Princeton University Press, Princeton.

Li, J., Shi, J., Xue, Y., Mao, H., Luo, Y., 2014. Major physiological adjustments in freezing-tolerant grey tiger longicorn beetle (*Xylotrechus rusticus*) during overwintering period. J. Forest. Res. 25, 653-659.

May, B., Coetzee, J., 2013. Comparisons of the thermal physiology of water hyacinth biological control agents: predicting establishment and distribution pre- and post-release. Entomol. Exp. Appl. 147, 241-250.

Mukherjee, A., Knutson, A., Hahn, D.A., Heinz, K.M., 2014. Biological control of giant salvinia (*Salvinia molesta*) in a temperate region: cold tolerance and low temperature oviposition of *Cyrtobagous salviniae*. BioControl 59, 781-790.

Mukherjee, A., Knutson, A., Heinz, K., 2017. Biological control of *Salvinia molesta*: population dynamics of *Cyrtobagous salviniae* in Lake B.A. Steinhagen. J. Aquat. Plant. Manage. 55, 46-49.

Nation, J.L., 2008. Insect physiology and biochemistry. CRC Press, Boca Raton.

NOAA, 2016. Climate Data Online. https://www.ncdc.noaa.gov/cdo-web/. (Accessed 12.08.16).

Overgaard, J., Kristensen, T.N., Mitchell, K.A., Hoffmann, A.A., 2011. Thermal tolerance in widespread and tropical *Drosophila* species: Does phenotypic plasticity increase with latitude? Am. Nat. 178, S80-S96.

Payton, M.E., Greenstone, M.H., Schenker, N., 2003. Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? J. Insect Sci. 3, 34 http://dx.doi.org/10.1093/jis/3.1.34.

Rao, P.V., 1998. Statistical research methods in the life sciences. International Thompson Publising, Inc., Pacific Grove.

Raymond, J.A., DeVries, A.L., 1977. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. P. Natl. Acad. Sci. USA 74, 2589-2593.

Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A.L., Denlinger, D.L., 2007. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. PNAS 104, 11130-11137.

Room, P.M., Harley, K.L.S., Forno, L.W., Sands, D.P.A., 1981. Successful biological control of the floating weed salvinia. Nature 294, 78-80.

Russell, A., Johnson, S., Cibils, X., McKay, F., Moshman, L., Madeira, P., Blair, Z., Diaz, R., 2017. Surveys in Argentina and Uruguay reveal *Cyrtobagous salviniae* (Coleoptera: Curculionidae) populations adapted to survive temperate climates in southeastern USA. Biol. Control 107, 41-49.

Salt, R.W., 1961. Principles of insect cold-hardiness. Annu. Rev. Entomol. 6, 55-74.

Sands, D.P.A., Schotz, M., Bourne, A.S., 1983. The feeding characteristics and development of larvae of a salvinia weevil *Cyrtobagous* sp. Entomol. Exp. Appl. 34, 291-296.

Sands, D.P.A., Schotz, M., Bourne, A.S., 1986. A comparative study on the intrinsic rates of increase of *Cyrtobagous singularis* and *C. salviniae* on the water weed *Salvinia molesta*. Entomol. Exp. Appl. 42, 231-237.

Sinclair, B.J., Coello Alvarado, L.E., Ferguson, L.V., 2015. An invitation to measure insect cold tolerance: Methods, approaches, and workflow. J. Therm. Biol. 53, 180-197.

Sinensky, M., 1974. Homeoviscous adaptation- a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. P. Natl. Acad. Sci. USA 71, 522-525.

Sullivan, P.R., Postle, L.A., 2010. Low temperature reproduction of *Cyrtobagous salviniae*: good news for biological control of salvinia in temperate climate. J. Aquat. Plant. Manage. 48, 92-96.

Tauber, M.J., Tauber, C.A., Masaki, S., 1986. Seasonal adaptations of insects. Oxford University Press, New York.

Teets, N.M., Denlinger, D.L., 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol. Entomol. 38, 105-116.

Tipping, P.W., Center, T.D., 2003. *Cyrtobagous salviniae* (Coleoptera: Curculionidae) successfully overwinters in Texas and Louisiana. Fla. Entomol. 86, 92-93.

Zachariassen, K.E., 1985. Physiology of cold tolerance in insects. Physiol. Rev. 65, 799-832.

Zhao, L., Jia, D., Yuan, X., Guo, Y., Zhou, W., Ma, R., 2015. Cold hardiness of the biological control agent, *Agasicles hygrophila*, and implications for its potential distribution. Biol. Control 87, 1-5.

CHAPTER 4. SUMMARY AND CONCLUSION

Cyrtobagous salviniae Calder and Sand (Coleoptera: Curculionidae) is an important species used for the biological control of *Salvinia molesta* Mitchell in Louisiana. Despite successful establishment at southern latitudes, *C. salviniae* populations have failed to establish at northern latitudes. Understanding the thermal biology of this species can assist predictions on successful establishment, and ultimately improve the management of *S. molesta* in northern Louisiana.

This study sought to overcome inconsistent control of *S. molesta* in northern Louisiana by searching for *C. salviniae* populations adapted to freezing temperatures in the temperate native range. Surveys for cold tolerant populations in the Lower Paraná-Uruguay Delta were prioritized based on results from the climate matching model, MaxEnt. Results from these surveys revealed the most southern distribution of *C. salviniae* in the native range known to date, and resulted in the first record of this species in Uruguay. When compared to a *C. salviniae* population from central Louisiana, the Lower Paraná-Uruguay Delta population was significantly more cold tolerant, presumably due to greater genetic diversity and longer time exposed to conditions selecting for cold tolerant genes. This study concludes that *C. salviniae* from the Lower Paraná-Uruguay Delta should be considered to improve management of *S. molesta* in northern Louisiana. However, host range and cross breeding tests should be conducted on the Lower Paraná-Uruguay Delta population before releases, to determine host specificity and life histories of this population.

Despite improvements in the knowledge of *C. salviniae* cold tolerance, fewer studies have focused on the mechanisms that confer cold tolerance and seasonal population dynamics

in this species. The second chapter of this study sought to evaluate the seasonal changes in physiology and population dynamics of *C. salviniae* populations from south and central Louisiana to improve management in northern Louisiana. Laboratory assays demonstrated phenotypic plasticity in cold tolerance of populations from both sites when acclimated to winter conditions. The field portion of this study revealed seasonal changes in reproductive status, fat body, and adult/larval densities. Further studies assessing the effect of plant quality on seasonal physiology and population dynamics are suggested. This study concludes that seasonal assessments of population health through the characterization of reproductive status, fat body, and adult/larval densities should be conducted to improve the management of *C. salviniae* in Louisiana.

APPENDIX 1. LETTERS OF PERMISSION

On February 13, 2017, I called the Elsevier Permissions Helpdesk Manager, Hop Wechsler, and left a voicemail message asking for permission to include an article written by myself in my thesis. I received the following written response via email:

RE: Alana Russell, Seth Johnson, Ximena Cibils, Fernando McKay, Lori Moshman, Paul Madeira, Zizah Blair, Rodrigo Diaz, Surveys in Argentina and Uruguay reveal Cyrtobagous salviniae (Coleoptera: Curculionidae) populations adapted to survive temperate climates in southeastern USA, *Biological Control*, Volume 107, April 2017, Pages 41-49, ISSN 1049-9644, http://dx.doi.org/10.1016/j.biocontrol.2017.01.010

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If I may be of further assistance, please let me know.

Best of luck with your thesis and best regards,
Hop

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APPENDIX 2. SUPPLEMENTARY DATA

Table A.1. *Salvinia* species and *Cyrtobagous salviniae* locations surveyed in Argentina and Uruguay, South America during 2015 and 2016.

Population and collection site	Coordinates (lat., long.)	Collection dates and season	*Salvinia* spp.	*C. salviniae* (Y/N)
San Francisco: Treinta y Tres, UY	-33.1876°, -54.1036°	17 Nov. 2015 (spring)	*S. minima*	N
Rio Branco: Rio Branco, UY	-32.6125°, -53.3669°	17 Nov. 2015 (spring)	*S. minima*	N
Laguna Merín: Lago Merín, UY	-32.7327°, -53.2764°	17 Nov. 2015 (spring)	*S. biloba*	N
Canal: Rio Branco, UY	-32.6102°, -53.3694°	17 Nov. 2015 (spring)	*S. minima*	N
Canova: Rocha, UY	-33.8630°, -54.2547°	18 Nov. 2015 (spring)	*S. biloba*	N
Canal H: Rocha, UY	-33.8580°, -54.1858°	18 Nov. 2015 (spring)	*S. biloba*	N
Ana Paula: Lascano, UY	-33.7304°, -54.1365°	18 Nov. 2015 (spring)	unknown	N
Rt. 1 Canal: Ciudad del Plata, UY	-34.7629°, -56.4103°	22 Mar. 2016 (fall), 20 June 2016	*S. biloba*	N
Rio Negro: Villa Soriano, UY	-33.3897°, -58.3193°	23 Mar. 2016 (fall), 20 June 2016	*S. minima*	N
Cantera Ferrando: Colonia, UY	-34.4741°, -57.8264°	24 Mar. 2016 (fall), 21 June 2016	*S. biloba*	Y
Rt. 21: Nueva Palmira, UY	-33.8588°, -58.3910°	23 June 2016	*S. biloba*	N
Puente: Colonia, UY	-34.4418°, -57.8656°	24 June 2016	*S. biloba*	Y
Riachuelo: Colonia, UY	-34.4452°, -57.7278°	24 June 2016	*S. biloba*	Y
Ciudad Vieja: Colonia, UY	-34.4738°, -57.8509°	27 June 2016	*S. biloba*	Y
Feria Artesanal: Colonia, UY	-34.4683°, -57.8475°	27 June 2016	*S. biloba*	N
Sitio 1: Entre Ríos, AR	-33.8977°, -58.8706°	24 Feb. 2016 (fall)	*S. biloba*	Y
Ceibas: Entre Ríos, AR	-33.4387°, -58.7919°	2 Mar. 2016 (fall)	*S. biloba*	Y
Rosario: Santa Fe, AR	-32.8580°, -60.6150°	Feb. 2016 (fall)	*S. biloba*	Y
Punta Lara: Buenos Aires, AR	-34.8435°, -57.9597°	18 Nov. 2015 (spring)	*S. biloba*	Y
Dique Luján: Buenos Aires, AR	-34.3733°, -58.7265°	20 Nov. 2015 (spring)	*S. biloba* & *S. minima*	Y

Table A.2. Frequencies (percent) of age class in *Cyrtobagous salviniae* populations from Marrero and Lena, LA over time.

Month	N	P1	P2	P3
January 2016				
Marrero	0	55	45	0
Lena	0	74	19	7
February 2016				
Marrero	0	50	45	5
Lena	0	69	19	12
March 2016				
Marrero	12.5	50	25	12.5
Lena	15	46	31	8
April 2016				
Marrero	1	37	37	21
Lena	20	40	25	15
May 2016				
Marrero	8	50	25	17
Lena	35	41	12	12
June 2016				
Marrero	50	40	10	0
Lena	62.5	12.5	12.5	12.5
July 2016				
Marrero	35	55	10	0
Lena	30	50	20	0
August 2016				
Marrero*	N/A	N/A	N/A	N/A
Lena	33	50	17	0
September 2016				
Marrero*	N/A	N/A	N/A	N/A
Lena	42	50	8	0
October 2016				
Marrero	33	28	22	17
Lena	50	44	6	0
November 2016				
Marrero	15	20	45	20
Lena	12	47	23	18
December 2016				
Marrero	10	25	40	25
Lena	55	15	30	0
January 2017				
Marrero	8	16	38	38
Lena	0	40	40	20
February 2017				
Marrero	0	22	33	45
Lena	0	11	56	33

*Data not obtained

Table A.3. Frequency (percent) of fat body in *Cyrtobagous salviniae* populations from Marrero and Lena, LA over time.

Month	Lean	Intermediate	Fat
January 2016			
Marrero	15	45	40
Lena	11	48	41
February 2016			
Marrero	10	10	80
Lena	6	94	0
March 2016			
Marrero	12.5	37.5	50
Lena	24	46	30
April 2016			
Marrero	5	32	63
Lena	40	25	35
May 2016			
Marrero	8	25	67
Lena	35	53	12
June 2016			
Marrero	40	35	25
Lena	75	12.5	12.5
July 2016			
Marrero	25	30	45
Lena	30	30	40
August 2016			
Marrero*	N/A	N/A	N/A
Lena	33	25	42
September 2016			
Marrero*	N/A	N/A	N/A
Lena	42	33	25
October 2016			
Marrero	28	55	17
Lena	50	25	25
November 2016			
Marrero	45	35	20
Lena	29	53	18
December 2016			
Marrero	45	35	20
Lena	55	35	10
January 2017			
Marrero	8	38	54
Lena	20	55	25
February 2017			
Marrero	23	44	33
Lena	11	22	67

*Data not obtained

VITA

Alana Russell was born and raised in a small town in southern Rhode Island. She received a Bachelor of Science degree in Natural Resources with a concentration in Wildlife Conservation from the University of Connecticut (UCONN) in 2013. In her last year at UCONN she spent 3 months in South Africa studying rhinoceros conservation and working at the world's first rhino orphanage. However, a lifelong interest in integrated pest management inspired her to pursue a master's degree in the Department of Entomology at Louisiana State University. She is a candidate to graduate with a master's degree in Entomology in May 2017 and plans to pursue a career in the biological control of insect pests and weeds.