REFRIGERATED STORAGE OF GLASSY-WINGED SHARPSHOOTER EGGS USED FOR PROPAGATION OF THE PARASITOID, *GONATOCERUS ASHMEADI*

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Reporting Period: The results reported here are from work conducted October 1, 2004 to September 15, 2005.

ABSTRACT
The studies investigated the storage of glassy-winged sharpshooter (GWSS) eggs below the temperature threshold for embryonic development and host acceptability and emergence from cold-stored hosts by *Gonatocerus ashmeadi* (*G. ashmeadi*). Our results showed that GWSS embryos failed to hatch after storage at 2˚C for 5 days and 5˚C for 11 days. *G. ashmeadi* parasitized dead *Homalodisca coagulata* (*H. coagulata*) eggs and completed development in hosts killed at 1, 3, 5, and 7 days post oviposition. Host age and length of time in cold storage were factors that influenced host acceptability and progeny production. After exposure to 2˚C for 5 days and storage at 10°C for 10-60 days, parasitism of 1 day old GWSS eggs by *G. ashmeadi* ranged from 95% to 45%. Only 10% of 9 day old GWSS eggs were accepted as hosts by the parasitoids after 10 days and none after 25 days storage. *G. ashmeadi* progeny successfully emerged from 60% of 1 day old host eggs that were stored for 25 days while only about 11% of the 7 day old eggs supported parasitoid development after 25 days storage at 10°C. The parasitoid progeny reared using refrigerated dead GWSS eggs have the same fecundity and lifespan as wasps reared from live hosts that have not been exposed to cold storage.

INTRODUCTION
The egg parasitoid, *G ashmeadi* Girault, is one of the most common natural enemies against the GWSS, *H. coagulata* (Say) in California and southeastern USA (Irvin and Hoddle 2005). The parasitoid may have a considerable potential as an effective biological control agent of the GWSS because it accounts for 80-95% of the observed parasitism on the sharpshooter eggs in California (Phillips 2000). However, the propagation of this parasitoid for an augmentative release program is dependent on sustainable supply of host eggs. Cold storage of insects during the rearing process of insects has proved to be a valuable tool to bio-control when implementing an IPM program (Leopold 1998). Low temperature storage of insects and natural enemies can help synchronize many aspects of the rearing procedure and fill the gaps between parasitoid production and the targeted pest populations when demands become high. During the rearing process, *H. coagulata* eggs may be overproduced and discarded when demands for parasitoids are low or under-produced when demands are high. Therefore, methods for storing host eggs can be useful for improving the production efficiency of a parasitoid to be used later for augmentative release.

Leopold *et al.* (2004) showed that storage of GWSS eggs under a temperature regime that cycled daily had potential for propagating *G. ashmeadi* colonies. However, storage above the temperature threshold for GWSS development resulted in high hatch during the storage period (Leopold et al. 2003). Studies have shown that parasitoids can utilize moribund hosts after cold storage and can complete their development and reproduction normally (Legner 1979, Petersen and Matthews 1984, Rueda and Axtell 1987, Roth et al. 1991, Floate 2002). Further, these results implied that some parasitoids may adjust to the physiological status of their hosts and maximize their fitness under less than ideal conditions.

OBJECTIVES
1. Determine cold tolerance of GWSS eggs stored at a constant temperature below the temperature threshold for development and evaluate survival after cold storage.
2. Determine the suitability and acceptability of dead GWSS eggs as hosts for propagating *G. ashmeadi*.
3. Assess the quality of *G. ashmeadi* progeny reared by using dead GWSS eggs and evaluate progeny fecundity and lifespan.

RESULTS AND CONCLUSIONS
Survival of host eggs
To determine the effect of storage temperature on the survival of *H. coagulata,* egg masses (< 24 hrs old) deposited on plant cuttings (*Euonymus japonica*) were placed into incubators that were set at constant temperatures at 2°C, 5°C, and 10°C under an 8L:16D photoperiod. Leopold *et al.* (2003, 2004) had previously studied the influences of constant temperatures above developmental threshold and cycled temperature regimes on survival of the GWSS eggs and their acceptability by *G. ashmeadi*. Here, we mainly report on the suitability and acceptability of GWSS eggs for parasitoid propagation after storage at temperatures below the developmental temperature threshold over time. Our results show that hatching of *H. coagulata* eggs after low temperature exposure varied significantly with temperature over storage time (Table 1). Hatching of GWSS eggs did not occur after storage at 2°C for 5 days or 5°C for 11 days. After 1 day of storage, <50% of GWSS eggs at 2°C

failed to hatch, whereas approximately 95% eggs successfully hatched at 10°C and 5°C. After 3 days of storage, 68% and 45% of the eggs stored at 10°C and 5°C hatched, respectively. After storage at 10°C for 7 days, hatching was about twice that at 5°C (50% vs. 26%). After 11 days at 10°C, the hatching percentage sharply dropped to 20% (Table 1).

A two-way ANOVA analysis, using storage time and temperature as factors, showed that the hatching percentage of the GWSS eggs was not only significantly influenced by storage temperature ($F = 34.28$, df = 2,274, $P < 0.0001$) and storage time ($F = 38.52$, df = 5,274, $P < 0.0001$), but by the interaction of temperature X storage time ($F = 4.07$, df = 8,274, $P = 0.0003$). Therefore, exposure to temperatures below the embryonic developmental temperature threshold for a sufficient length of time causes developmental arrest and death of the GWSS eggs.

Table 1. Hatching percentage of *H. coagulata* eggs at three temperatures over storage time (days).

Storage temp.	**Hatch percentage (means ± S.E.)**[a]					
	1 d[b]	**3d**[b]	**5d**[b]	**7d**[c]	**9d**[c]	**11d**[c]
10°C	95.7 ± 1.3 Aa	67.8 ± 4.3 Aab	68.1 ± 6.9 Aab	50.1± 4.5*bc	47.1 ± 6.7*c	19.8 ± 6.9 *d
5°C	94.5 ± 1.5 Aa	44.6 ± 7.2 Ab	29.2 ± 6.9 Bbc	25.9± 9.2c	9.4 ± 6.5d	0.0 ± 0.0 d
2°C	49.9 ± 4.3 Ba	9.8 ± 2.9 Bb	0.0 ± 0.0 Cc			
F	57.18	22.85	36.86	$t = 2.73$	$t = 3.82$	$t = 3.33$
df	2,48	2,52	2,47	22	36	15
P	< 0.0001	< 0.0001	< 0.0001	0.0123	0.0005	0.0046

[a]Hatching percentages were log-transformed before analysis to meet the assumptions of normality and homogeneity of variances because the size of the egg masses was not constant. [b]A one-way ANOVA followed by the LSD test ($P < 0.05$) (PROC GLM, SAS) was used to determine if there were significant differences in hatch percent. Means in the same column followed by a different capital letter and in same row followed by a different small letter are significantly different. [c]The T-test was used to determine if there was a significant difference between two sets of treatments (PROC TTEST, SAS). Asterisks indicate the percentages between the two treatments were significantly different ($P < 0.05$).

Host acceptability by *G. ashmeadi*

Host acceptability (parasitism) was assessed by microscopically examining each GWSS egg within an egg mass for presence of a developing parasitoid. We found that the GWSS eggs killed by chilling were still utilized by *G. ashmeadi* as egg hosts under no-choice conditions. However, extending the holding time at the killing temperature was found to be detrimental. For example, approximately 18% of the dead host eggs caused by exposure to 5°C for 11 days were parasitized as opposed to >90% of those eggs killed by exposure to 2°C for only 5 days. Further, within these moribund GWSS eggs, the developing parasitoids successfully complete development to adulthood. To further determine the effectiveness of using moribund hosts in propagation of *G. ashmeadi*, GWSS eggs deposited on euonymus cuttings were killed by placing at 2°C for 5 days after holding for 1, 3, 5, 7 or 9 days post oviposition. They were then stored in an incubator set at 10°C with a photoperiod of 8L:16D. Next, these eggs were exposed to colonies (about 200 individuals/ cage) for 2 days at room temperature. After removal from the parasitoid cages, the acceptability of the dead GWSS eggs was evaluated by assessing the incidence of parasitism by physically examining each host for presence of a parasitoid.

A two-way ANOVA analysis, using host age and storage time as factors, showed that percentage parasitism varied significantly with storage time ($F = 10.59$, df = 6, 474, $P < 0.0001$) and host age ($F = 84.53$, df = 4,474, $P < 0.0001$). Also, the interaction between storage time and host age also significantly influenced the incidence of parasitism ($F = 2.13$, df = 16,474, $P = 0.0066$) (Table 2).

After storage at 10°C for 10-25 days, parasitism of dead 1 day old GWSS eggs by *G. ashmeadi* was statistically similar, ranging from 65-95%. Also, there were no differences in parasitism among 1 day old eggs having a storage time between 30-60 days. This range was 45-55% parasitism. For 3 day old hosts, only after storage for 30 days did the parasitism significantly decrease. After storage of 60 days, only 25% of 3 day old GWSS eggs were parasitized. The 5 day old eggs had a similar incidence of parasitism when stored for 10-20 days. The parasitism for 5 day old eggs stored 25 days was significantly lower than those having a 10-day storage period. For 7 day old eggs, parasitism also decreased significantly after storage for 25 days. After storage for 10 days, only 11% of dead 9 day old eggs were parasitized by *G. ashmeadi* (Table 2). At 25 days of storage at 10°C, 9 day old eggs displayed no parasitism by the wasps, even under the no-choice conditions.

Host age significantly affected acceptance by *G. ashmead*i of dead GWSS eggs over a storage time of 10-60 days (Table 2). After storage for 10 days, 95% of 1 day old eggs were parasitized, significantly higher than 57% and 10% of 7 and 9 day old eggs, respectively ($F = 36.56$, df = 4,94, $P < 0.0001$). There were no differences in parasitism among 1, 3 and 5day old

embryos. After storage for 15 days at 10°C, 86% of dead 1 day old eggs were successfully parasitized by the wasps, and 74, 62, 51 and 5% for 3, 5, 7, and 9 day old eggs. There were significant differences in parasitism between 7 and 9 day old and 1 day old eggs (F = 16.95, df = 4,90, *P* <0.0001). *G. ashmeadi* had a similar parasitism of 1,3 and 5 day old eggs that were stored for 20-25 days (Table 2). Percentage parasitism of 7 and 9day old eggs significantly decreased (20days, F = 29.93, df = 4,83, *P* <0.0001; 25 days, F = 21.27, df = 4, 74, *P* <0.0001). *G. ashmeadi* displayed 47-57% parasitism of 1, 3 and 5 day old eggs stored for 30 days, 45-56%, and 24-45% of 1 and 3 day old eggs for 50 and 60 days, respectively. There were no differences in parasitism among refrigerated 1, 3 and 5 day old eggs after storage for 30 days (F= 0.23, df = 2,61, P = 0.80), and between 1and 3 day old eggs after storage between 50 and 60 days (Table 2).

Table 2. Effect of the age of refrigerated *H. coagulata* eggs on parasitism by *G. ashmeadi* over time (days).

Host Age (d)	Percentage parasitism over storage time[a] (means ± S. E.) [b]					
	10	**20**	**25**	**30**	**50**	**60**
1	94.5 ± 1.6Aa	78.3 ± 4.9Aa	65.4 ± 6.3Aab	48.1 ± 6.1Abc	55.8 ± 6.6Abc	45.2 ± 10.5Ac
3	83.7 ± 4.0ABa	73.4 ± 7.7Aab	60.6 ± 6.8Aab	57.2 ± 8.0Aab	45.7 ± 4.9Ab	23.5 ± 8.1Ac
5	64.7 ± 7.6BCa	62.0 ± 5.9Aab	52.2 ± 8.4Ab	47.3 ± 6.7Ab	Not available	Not available
7	57.1 ± 6.3Ca	48.6 ± 8.0Ba	19.8 ± 8.2Bb	Not available	Not available	Not available
9	10.8 ± 7.6Da	3.0 ± 2.1Ca	0 Ca			

[a] Storage time is represented by the days the time was 10°C. [b] A one-way ANOVA followed by the LSD test (*P* < 0.05) (PROC GLM, SAS) was used to determine significant differences. Means in the same column followed by a different capital letter and in same row followed by a different small letter are significantly different. Percentage parasitism was log transformed before statistical analysis to meet the assumptions of normality and homogeneity of variances because the size of egg masses was not constant.

Emergence

A two-way ANOVA analysis, using host age and storage time as factors, showed that the percentage emergence of *G. ashmeadi* not only varied significantly with host age (F = 80.35, df = 4, 474, *P* <0.0001) and storage time (F = 14.50, df = 6, 474, *P* <0.0001), but also with the interaction between storage time and host age (F = 1.84, df = 16, 474, *P* = 0.0243) (Table 3). When storage was for 10 and 15 days before parasitism, percentage wasp emergences from 1 and 3 day old eggs were significantly greater than that for 5 or 9 day old hosts (10 days, F = 75.43, df = 4, 94, *P* <0.0001; 15 days, F = 25.05, df = 4, 90, *P* <0.0001). About 90 and 80% of *G. ashmeadi* adults emerged from 1 and 3 day old eggs stored for 10 days and 71 and 66% for 15 days, respectively (Table 3). After storage for 20 days, percentage emergence of the parasitoids from 1 day old eggs was significantly higher than that from 3 to 9 day old eggs (F = 20.85, df = 4, 83, *P* < 0.0001). About 60 and 51% of the parasitoids successfully emerged from refrigerated 1 and 3 day old eggs, significantly more than that from 7 and 9 day old eggs (F = 16.91, df = 4,74, *P* < 0.0001). After storage for 30-60 days, no significant differences were found among 1, 3, and 5 day old eggs (30 days, F = 0.49, df = 2,61, P = 0.62), or between 1- and 3-d-old eggs (50days, *P* = 0.08; 60 days, *P* = 0.21).

Storage time at 10°C also significantly affected the percentage emergence of the wasp progeny from the dead GWSS eggs. The maximum emergence occurred for 1 day old parasitized eggs that were previously stored for 10 days (Table 3). For 1 day old eggs, the percentage wasp emergence remained statistically similar when they were stored for 10-25 days and it was significantly higher than that for 30-60 days (F = 8.21, df = 6,145, *P* < 0.0001). Only 28% of *G. ashmeadi* emerged from 1 day old eggs stored for 60 days. For 3 day old eggs, the wasps had similar percentage emergences after storage for 10-20 days and they were also significantly higher than those for 30-60 days (F = 5.56, df = 6,114, *P* < 0.0001). For 5 day old eggs, the emergence percentages remained stable when they were stored for 10-30 days (F = 0.88, df = 4,89, P =0.48), ranging from 38 to 58%. Percentage emergence from 7 day old eggs stored for 10-20 days was 37-46%, and only 11% for 25 days. No *G. ashmeadi* emerged from stored 9 day old hosts.

Table 3. Effect of age of refrigerated *H. coagulata* eggs on emergence of *G. ashmeadi* over storage time [a] *(days).*

Age (d)	Percentage emergence over storage time in days (means ± S. E.) [b]						
	10 d	15 d	20 d	25 d	30 d	50 d	60 d
1	90.4 ± 2.2Aa	75.7 ± 5.1Aa	70.6 ± 5.2Aa	60.2 ± 5.6Aa	40.6 ± 8.1Ab	48.7 ± 5.2Ab	27.8 ± 8.9Ac
3	79.9 ± 4.3Aa	66.0 ± 9.5Aab	59.2 ± 8.4ABab	51.2 ± 5.8ABbc	43.8 ± 8.9Abc	26.0 ± 5.5Acd	19.0 ± 8.5Ad
5	56.7 ± 7.2Ba	48.8 ± 8.4Ba	45.7 ± 7.6ABa	46.9 ± 5.6Ba	37.6 ± 6.4Aa	N.A.	N.A.
7	46.3 ± 5.5Ba	38.5 ± 7.1Ba	37.4 ± 6.4Ba	11.3 ± 6.1Cb	N.A.		
9	0.8 ± 0.8Ca	0 Ca	0 Ca	0 Da			

[a] Storage time represented the duration in the refrigerator set at 10°C. [b] A one-way ANOVA followed by the LSD test ($P < 0.05$) (PROC GLM, SAS) was used to determine if there were significant differences in percentage parasitism. Means in the same column followed by a different capital letter and in same row followed by a different small letter are significantly different. Percentage parasitism was log-transformed before analysis to meet the assumptions of normality and homogeneity of variances because the size of the egg masses was not constant.

Quality assessment of *G. ashmeadi* progeny

There is no significant difference in the number of GWSS eggs deposited by *G. ashmeadi* females that were reared using untreated eggs (control) and those reared from 1 day old eggs stored for 50 days ($t = 2.2$, $P = 0.068$), or 7 day old eggs stored for 10 days ($t = 2.5$, $P = 0.066$) (Figure 1). Dissections of parasitized GWWS eggs showed that within the first three days single females deposited from 50-58 eggs. Also, there is no difference in the female or male lifespan of parasitoids reared from untreated host eggs compared to that of wasps reared from 1 day old host eggs previously stored for 50 days at 10°C ($P > 0.05$) (Figure 2). In the laboratory, the female lifespan was about 18 days and that of males, 15 days.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the USDA Agricultural Research Service.

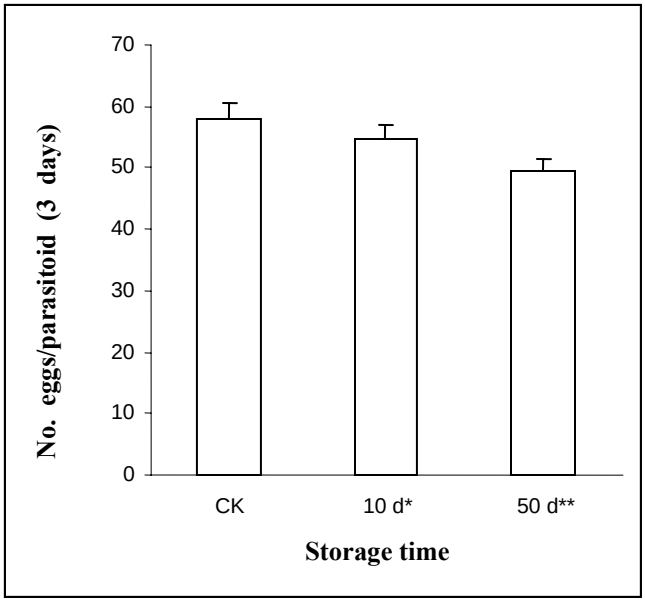

Figure 1. The fecundity of *G. ashmeadi* females that had emerged from 7 day old* *H. coagulata* eggs after storage at 10°C for 10 days and from 1 day old** eggs stored 50 days compared to females emerged from untreated eggs. The number of parasitoid eggs was collected within first three days. The T-test was used to determine that there were no significant differences in fecundity of *G. ashmeadi* between the groups ($P < 0.05$, PROC TTEST, SAS).

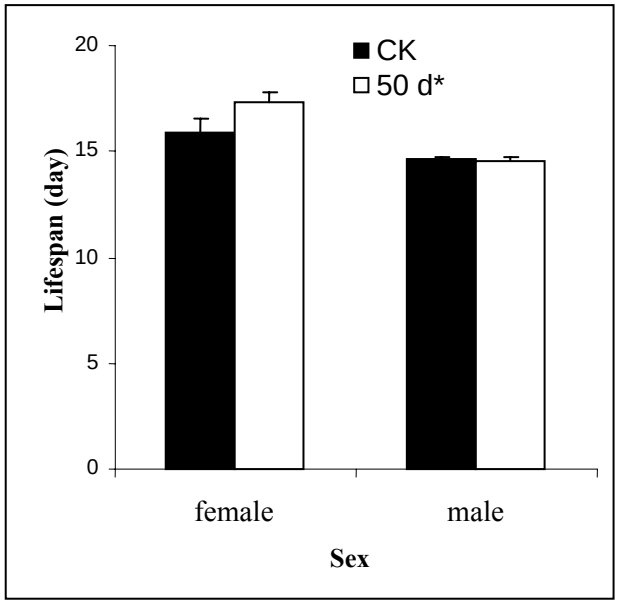

Figure 2. The lifespan of *G. ashmeadi* females and males having emerged from untreated eggs (black bar) compared to 1 day old *H. coagulata* eggs (white bar) after storage at 10°C for 50 days. The T-test was used to determine that there were no significant differences in lifespan between the treated and the control groups ($P < 0.05$, PROC TTEST, SAS).