Cryo-Letters 7, 234-245(1986).
Published by Cryo-Letters, 7 Wootton Way, Cambridge CB3 9LX, U.K.

RELATIONSHIP OF ENVIRONMENTAL WATER CONTENT TO GLYCEROL ACCUMULATION IN THE FREEZING TOLERANT LARVAE OF EUROSTA SOLIDAGINIS (FITCH)

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SUMMARY

The third instar larva of Eurosta solidaginis seasonally accumulates glycerol as one component of its cryoprotectant system. Previous studies have shown that glycerol synthesis is temperature-independent (12). Field data reveal a strong correlation between the water content of the plant gall habitat and glycerol accumulation by the larvae, as gall water content drops from 65% to 20% (November and January). During this period glycerol levels increase from 10 to 21 ug/mg, larval water content remains constant at 60-64%, and larval weight falls. Larvae from senescent plants ("dry" galls) have higher glycerol levels than those from "green" galls. Larvae from both northern and southern populations exposed to both high and low relative humidities increased glycerol levels. An approximate two-fold increase occurs at 22°C over a 5-8 day periods while those acclimated to 5°C accumulate substantially lower levels independent of Q_{10} relationships. Larvae lose only water at low humidity conditions but lose dry mass at high humidities.

Key Words: Freezing tolerance, cryoprotectants, insect cold hardiness, water content

INTRODUCTION

Many overwintering insects accumulate carbohydrates and polyols (glycerol, sorbitol, trehalose, etc.) as a component of their low temperature survival strategy (1,11,13,14,15). In freezing intolerant insects these compounds act as antifreeze agents by colligatively depressing the equilibrium freezing point of the body fluids so that extracellular freezing is avoided (14). On the other hand, freezing tolerant insects can

withstand the formation of extracellular ice within their body fluids. Protection is afforded by these agents once freezing occurs. The polyhydroxy nature of cryoprotective agents results in a comparatively reduced ice content following freezing, thereby, lowering the probability of the formation of "lethal" intracellular ice. Cryoprotectants also a attenuate ice crystal growth (2).

Cryoprotectants are frequently produced in response to low temperature exposure and generally lost with warm exposure (5,12). Eurosta solidaginis seasonally accumulates two major cryoprotectants; sorbitol and glycerol (3,4,11). Sorbitol accumulates at temperatures below 10°C with maximum accumulation occurring at 0°C. With warm acclimation sorbitol is rapidly lost (36% reduction in 24 hr and 83% in 48 hr) (12).

Glycerol accumulates early in the season before frost exposures. Baust and Lee (3) and Rojas et al. (12) demonstrated that synthesis was not dependent on chilling. In fact, temperatures below 10°C inhibit glycerol production in both northern and southern populations. This contrasts with the response of other species. For example, in a freezing tolerant beetle Pterostichus brevicornis glycerol synthesis is dependent on exposures to or below 0°C and is irreversibly lost at warm temperatures (5). In view of the apparent temperature-independence of the seasonal pattern of glycerol accumulation in E. solidaginis and the preliminary observation of a correlative association between habitat (dietary) water availability and glycerol accumulation, an evaluation of the possible role of dietary water availability and overall water state was attempted.

MATERIALS AND METHODS

Goldenrod, Solidago canadensis, ball galls containing third-instar larvae of Eurosta solidaginis (Diptera:Tephritidae) were seasonally collected from the Texas coastal plain and the Ohio areas. Larvae were extracted from the gall and quickly frozen (-40°C) for future cryoprotectant analysis. Water content was determined following drying at 80°C (10 larvae) to a constant weight. Larvae, collected during months in which there was a noticeable dichotomy between pre-senesced "green" and senesced "dry" galls, were separated into two groups for cryoprotectant and water content analysis.

Experimental desiccation: Larvae were simultaneously exposed to different relative humidities and acclimation temperatures. Groups of four larvae were weighed to obtain an initial wet weight. They were then placed in individual plastic weighing trays and set in glass desiccating chambers adjusted to obtain a specific relative humidity: 100% R.H. over distilled water, 75% R.H. over a saturated NaCl solution, 50% by a saturated lithium

nitrate solution and 0-5% over calcium carbonate (Drierite). Humidities were confirmed by hygrometry. The desiccators were held at 22°C and 0°C (Texas) or 22°C and 5°C (Ohio). Fasted larvae were sampled on days, 1 3 and 5 (Texas) and on days 2, 5 and 8 (Ohio) for cryoprotectants and water content. These intervals were selected based upon studies (4,12) which demonstrated that maximum rates of sorbitol synthesis would be induced with little or no change in glycerol levels.

Cryoprotectant Analysis: Sugars and sugar alcohols were analyzed by high performance liquid chromatography according to Hendrix et al. (8). Approximately 4 larvae (150 mg wet weight) were used for each analysis. The tissue was homogenized in 3.0 ml of 80% ethanol and decanted. The homogenizer residue was washed with 3.0 ml 80% ethanol and the homogenates combined. The homogenate was next heated to 80°C for 5 minutes and then centrifuged (90 rpm) for 15 minutes to remove precipitants. The resulting supernatant was pipetted into a separate test tube, the precipitate washed twice with 1 ml 80% ethanol and the washes combined with the supernatant.

The supernatant was evaporated to dryness under air at 60°C. The resulting residue was resuspended in 2.0 ml distilled water and partitioned against 3.0 ml of chloroform: methanol (2:1) (V/V). This partition mixture was centrifuged (5,400 rpm, 4°C, 30 min). The surface aqueous layer was removed by pipetting followed by a 2.0 ml wash of the organic phase and evaporated to dryness at 60°C. The resulting residue was resuspended in 0.5 ml water, filtered (0.2 um) and analyzed by reverse phase high performance liquid chromatography.

Cryoprotectant levels are expressed as ug per mg of wet weight tissue. Larvae that were acclimated to differing relative humidities had levels expressed as ug per initial wet weight to negate any concentration effects due to water loss.

Glycogen analysis: The precipitate of the 80% ethanol extract was used for glycogen analysis. Five ml of 5% trichloracetic acid (TCA) was added to the precipitate and heated for 10 min at 100°C, chilled and centrifuged. The supernatants were combined following a double wash of precipitates with 5% TCA. Glycogen levels were estimated by the anthrone colorimetric method using combined supernatants.

RESULTS

Field Data: Field data suggest a high correlation between gall (larval habitat) water content and glycerol accumulation, as plant water content dropped glycerol accumulated in the larvae (Fig. 1). However, larval water content remained relatively constant throughout the year in the southern population (60-64%) ($y = 0.02x + 61.4$; $r = 0.19$). Gall water content

decreased gradually from 65.5% ± 1.6 in mid-November to 20.5% ± 0.8 in late January. Morrissey and Baust (11) noted that in a northern population the gall water content dropped between autumn and winter from 63% to 17%. Glycerol levels rose during this interval from 9.5 ± 0.6 ug/mg to 21.2 ± 0.7 ug/mg (Fig. 1).

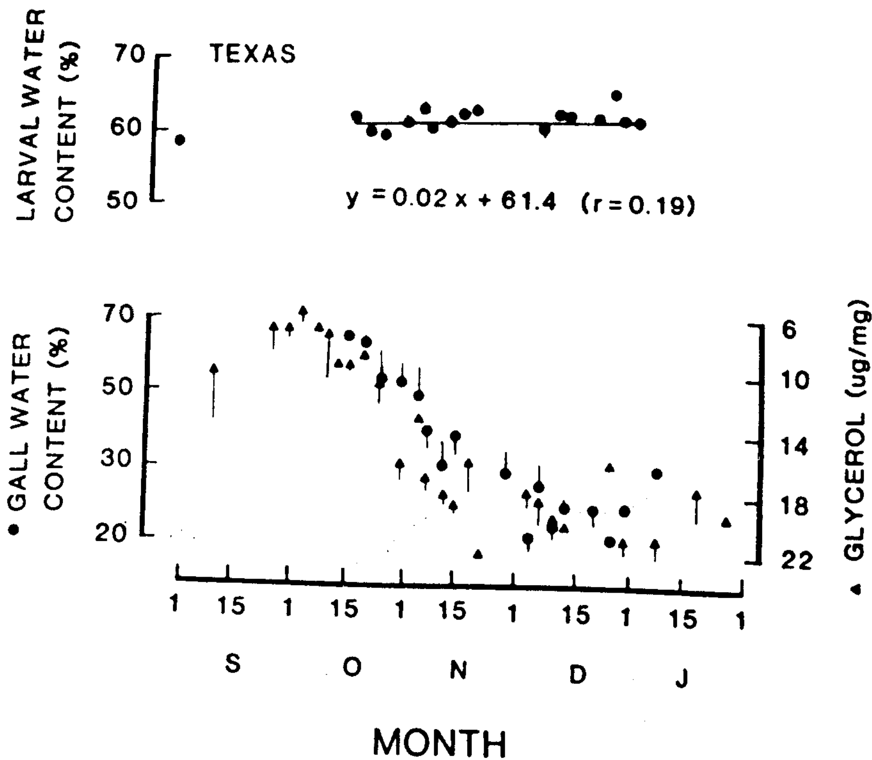

Fig. 1. Seasonal variations in *Eurosta solidaginis* (third instar larvae) glycerol and water contents and goldenrod (*Solidago* sp.) gall water content. Values are ± SEM.

Though larval water content remained constant throughout the season, the larval weight decreased by nearly 50%. The larval weight loss is correlated to the gall water content drop (Fig. 2).

During the second year of study it was noted that a certain proportion of the goldenrod plants began "winter" senescence in October rather than December. Senescence is characterized in part by a change in stem appearance (green to brown), seed maturation and a decrease in water content below 60%. Galls were sorted according to plant water content ("green" vs. "dry") and larval glycerol (Fig. 3) and glycogen levels (Fig. 4) measured. Dry gall larvae had higher glycerol levels than wet gall larvae (7.5 ug/mg vs 4.6 ug/mg) ($P \leq 0.05$). Sorbitol levels were not related to changes in gall water content. Glycogen levels were lower in the dry gall larvae (Fig. 4) while trehalose concentrations remained higher.

These data led to the hypothesis that glycerol synthesis is triggered by changes in water availability caused by drying of the larval habitat. Parallel models exist in both Artemia (6) and nematodes (10). Accordingly, if water state is important in regulating glycerol synthesis, desiccation studies might serve to test this hypothesis.

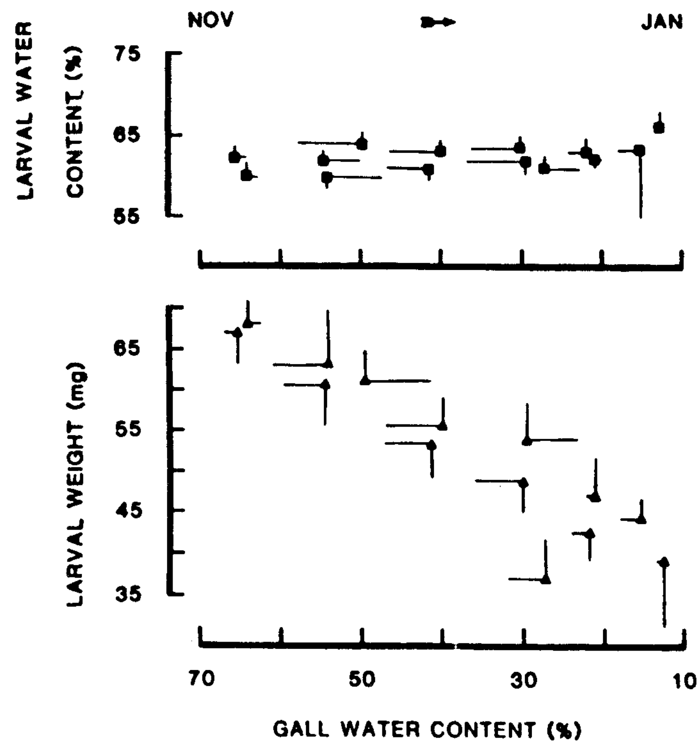

Fig. 2. Seasonal variations in larval (third instar) water content and weight vs. gall tissue water content in Eurosta solidaginis. Values are ± SEM.

Experimental desiccation of larvae:

1. Texas Year 1: Fig. 5 illustrates the effect of desiccation on November larvae. Glycerol levels were elevated by day 5 over initial levels for all conditions. The greatest accumulation (2x) occurred in the warm acclimation groups (22°C) at both 0% and 75% R.H.. The least accumulation occurred in the cold (0°C). Storey et al. (17) reported that glycerol synthesis in this species is inhibited at temperatures below 10°C.

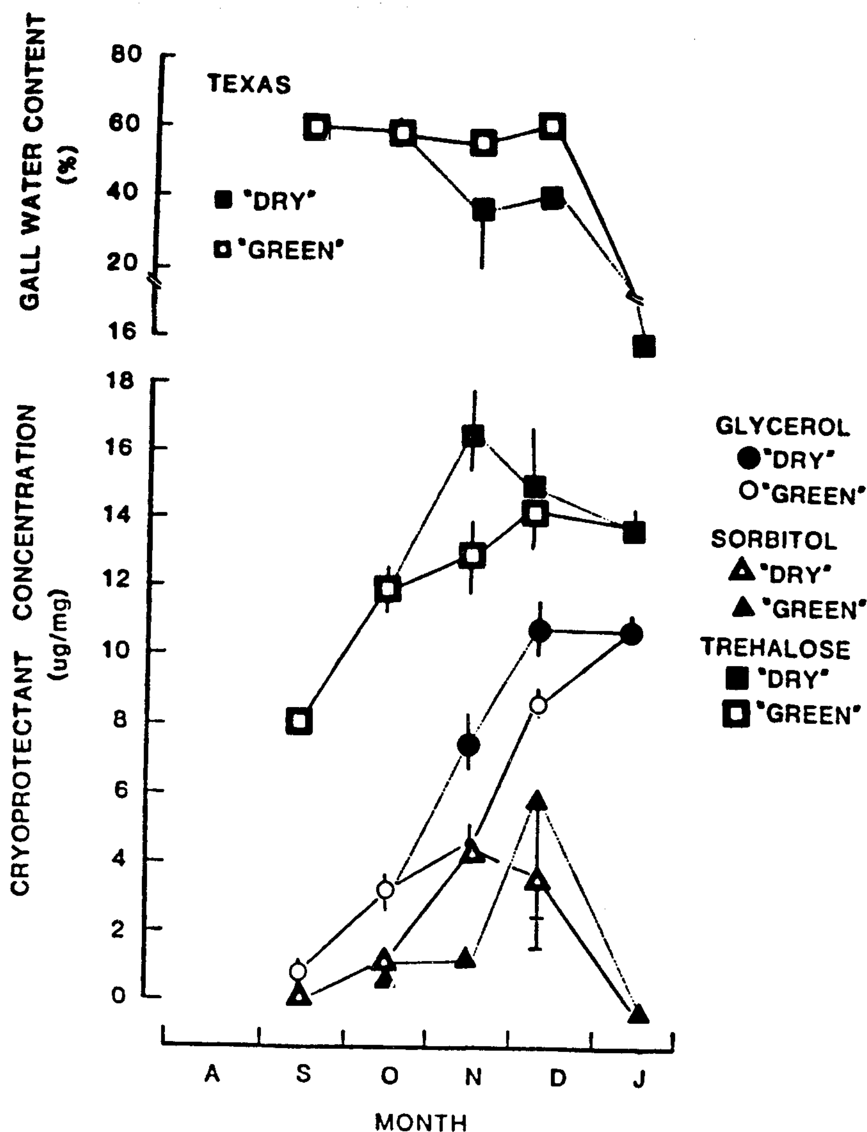

Fig. 3. Seasonal variation in Eurosta solidaginis (third instar larvae) cryoprotectant (glycerol, sorbitol and trehalose) levels in larvae taken from "green" galls and "dry" galls. Values are ± SEM.

2. Ohio Year 1: Larvae collected in Ohio in September increased glycerol levels at all conditions: 5° and 22°C x 5% and 75% R.H. (Fig. 6). The greatest increase was observed in larvae acclimated to 22°C, 5% RH and secondly in the 22°C, 75% R.H. The least accumulation was observed in the 5°C acclimated larvae. Storey et al. (17) reported that glycerol synthesis in E. solidaginis ceases below 5°C. Storey and Storey (16) reports that upon switching larvae from 13 to 3°C there is a negative cross-over at the pyruvate kinase locus suggesting an inhibition of glycolytic carbon flux. This suggests an inhibition of glycolytic

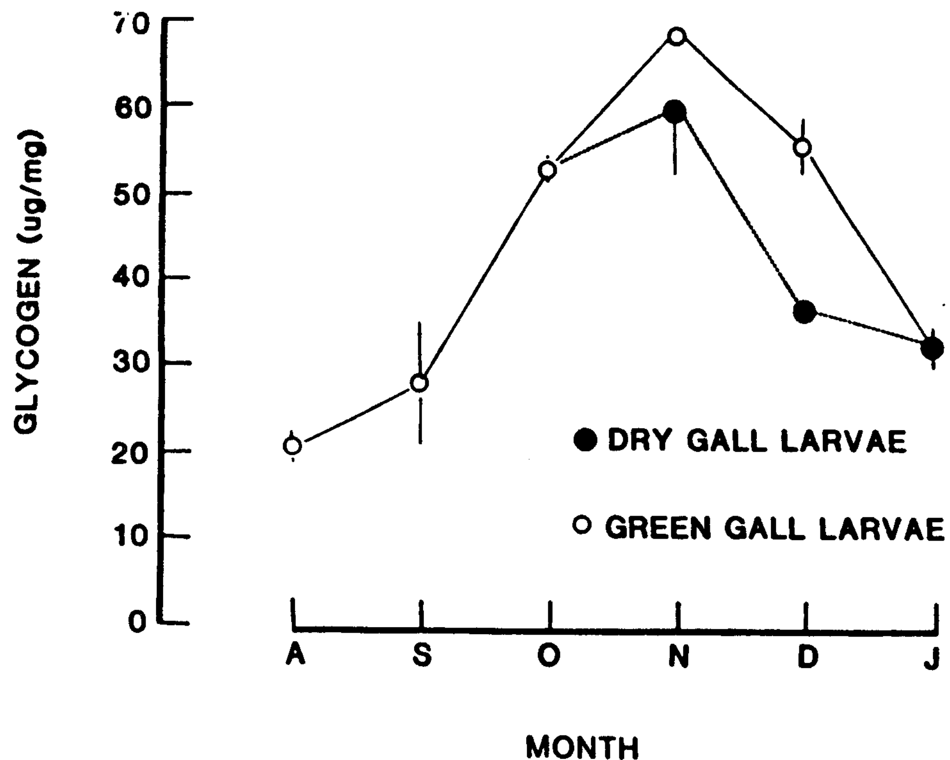

Fig. 4. Comparison of seasonal variation in Eurosta solidaginis (third instar larvae) glycogen levels between larvae from "green" galls vs. "dry" galls. Values are ± SEM.

flux past the PFK locus; carbon would have to pass through this locus in order for glycerol to be synthesized. Hence, a greatly reduced rate of glycerol accumulation at the lower temperatures (5°C). Those larvae kept in the gall at 22°C had very variable levels of glycerol.

As expected, the larvae maintained at the 5% RH (22°C and 5°C) lost the most water over the 8 day period. Larvae at all conditions lost wet weight from time 0. After 8 days the 22°C, 5% R.H. lost 36% of their initial weight; the 22°C, 75% R.H. lost 19%; the 5°C, 5% R.H. lost 17% and the 5°C, 75% R.H. lost 10%.

Texas Year 2: Larvae collected in October were exposed to 100% RH and 50% RH at 20°C. Both RH groups increased glycerol levels by day 10 from approx. 6 ug/mg to 10 ug/mg (Fig. 7). Larval water content remained relatively constant in the 100% R.H. group at 60% but fell in the 50% R.H. group to 48%. Both groups lost wet weight over the 10 day

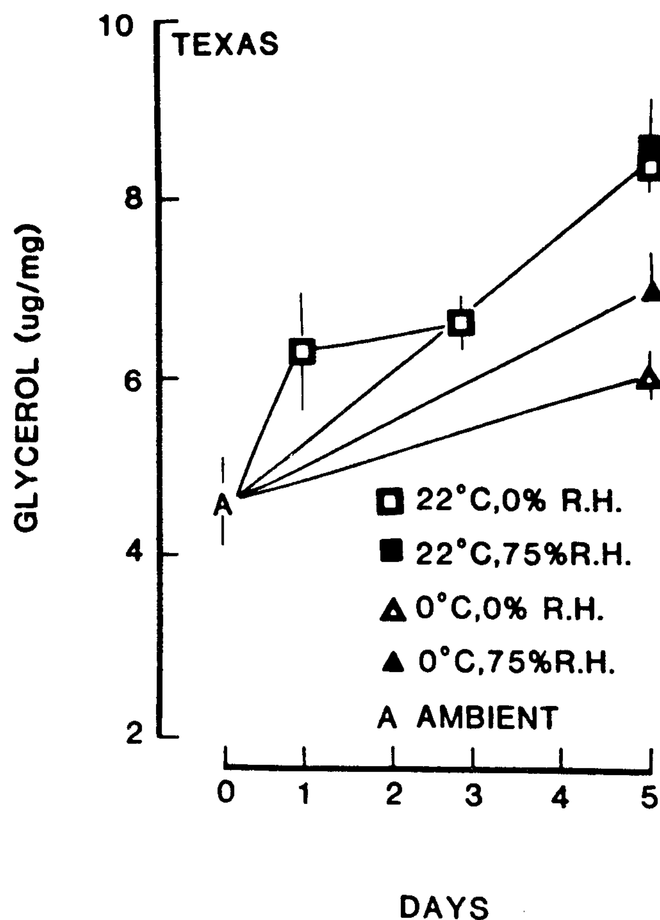

Fig. 5. Glycerol levels in southern (Texas) Eurosta solidaginis (third instar larvae) exposed to high relative humidity (75% r.h.) and low relative humidity (0% r.h.) and to 22°C and 0°C. Values are ± SEM. Larvae were collected in November.

period. Wet weight losses were higher in the 50% group. The 100% R.H. group is a more accurate mimic to field conditions; where larval water content remains constant while larval weight drops and glycerol is accumulated.

DISCUSSION

The availability of environmental water was considered a possible endogenous trigger to glycerol synthesis when a strong correlation between gall water content and glycerol accumulation was observed. Glycerol accumulation in *E. solidaginis* occurs before first frost exposures and is independent of temperature (4,12). The goldenrod plant undergoes senescence

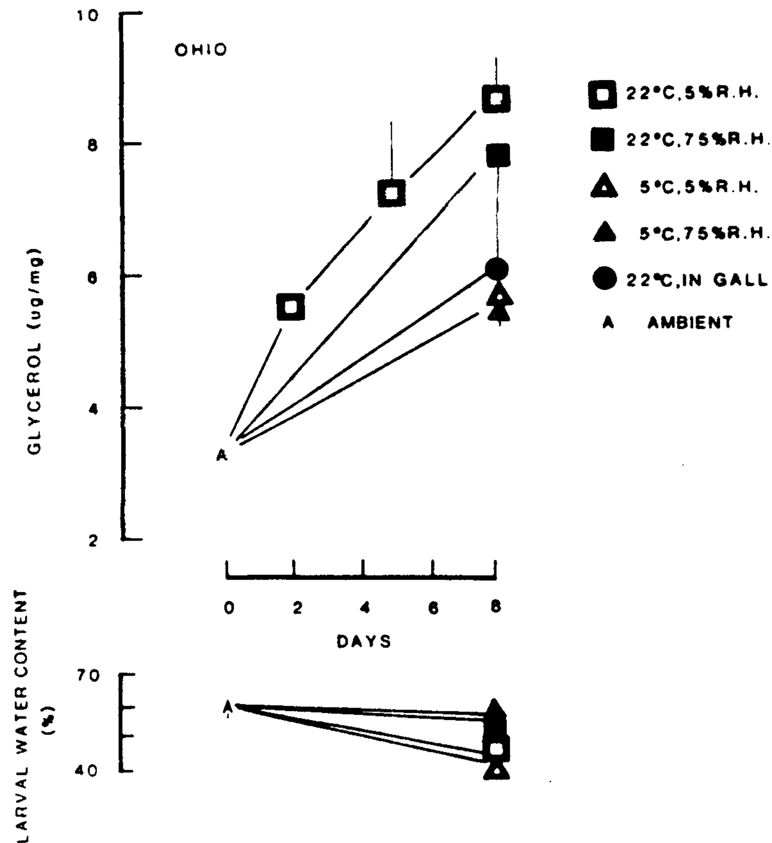

Fig. 6. Glycerol levels in northern (Ohio) Eurosta solidaginis (third instar larvae) exposed to high relative humidity (75% R.H.) and low relative humidity (5% R.H.) and to 22°C and 5°C.

between autumn and winter and loses water (60% to 17%). During this time the larvae accumulate glycerol. Larval water content remains constant throughout the year at 65%; however, larval weight falls. One popular hypothesis suggests that larvae accumulate glycerol in order to cope with a water stress. Glycerol due to its hydrophilic nature would act to prevent further loss of water and prevent protein and membrane denaturation (9). However, under laboratory conditions glycerol is accumulated at both low and high relative humidities with a simultaneous weight loss.

It has been hypothesized that gall desiccation may serve as an indirect

trigger to glycerol accumulation. Larvae may be unable to feed effectviely on dry galls or must consume a nutritionally altered food source during plant senescence. Glycogen levels rise in the developing larvae and peak in November. Glycogen levels are lower in larvae from dry galls suggesting that there is possibly a difference in nutritional states between green gall vs. dry gall larvae. Larvae from dry galls start to build their exit tunnel before those from green galls (general observation). These observations suggest that the larval nutrient source is altered. Therefore,

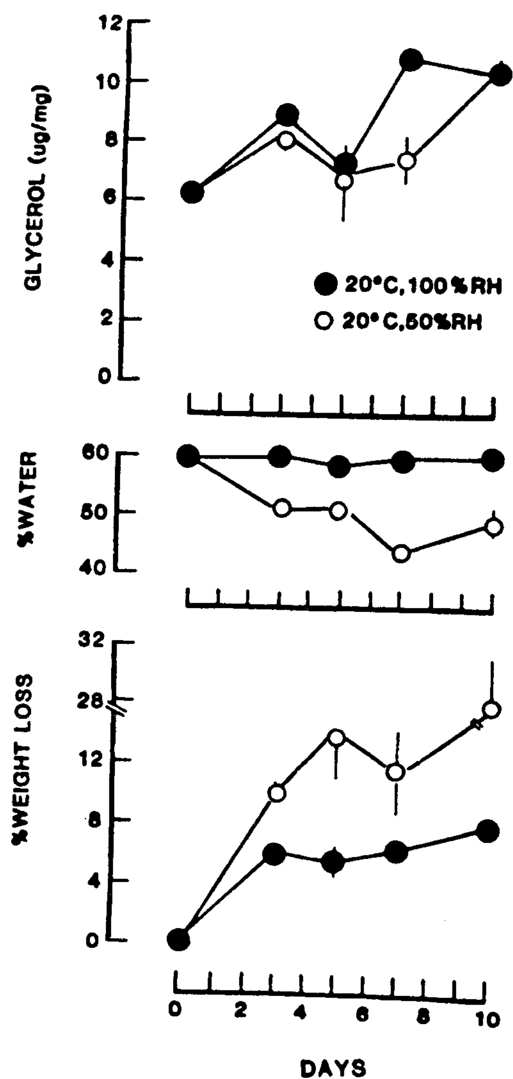

Fig. 7. Glycerol levels in southern (Texas) *Eurosta solidaginis* (third instar larvae) exposed to 100% R.H. and 50% R.H. at 22°C. Values are ± SEM.

larvae may be relying on stored glycogen reserves once the gall becomes nutritionally inadequate either due to plant senescence or to inability of larvae to feed on it. Storey et al. (17) showed that at temperatures above +5°C glycerol is accumulated at the expense of glycogen and once accumulated, levels are maintained. Storey and Storey (16) attribute this to a lack of the glycerol kinase which is necessary for glycerol turnover.

The desiccation experiments present a two-fold problem in trying to mimic field conditions. First, larvae may be experiencing a non-physiological water loss. Desiccation in the field is more gradual and may allow for metabolic adjustments to maintain a constant water content. Laboratory desiccation may be too rapid for such adjustments to take place. Second larvae are fasted while in the laboratory and may experience simultaneous "energy" and desiccation stresses.

There is one other report of desiccation as a trigger to glycerol synthesis in the literature. Young and Block (18) showed that in *Alaskozetes antarcticus* glycerol induction was influenced by low temperature, short photoperiod and low relative humidity (40% and 60% r.h.).

CONCLUSION

This study was designed to test the hypothesis suggesting that glycerol accumulation in *E. solidaginis* was dependent on water relationships. Data obtained from field collections provide a strong correlation in support of this hypothesis. Acclimation studies were, however, less conclusive. Since low temperature appears not to be the exogenous cue responsible for induction of glycerol synthesis and since desiccation experiments yield inconclusive data, it appears likely that other factors (i.e., diet, developmental timing, etc.) may play a more direct role in the regulations of glycerol production.

ACKNOWLEDGEMENT

This study was supported by grants from the National Science Foundation (PCM 81-10327) and the University of Houston Coastal Center to JGB.

REFERENCES

1 E. Asahina, Adv. Insect. Physiol. 6, 1-49 (1969).

2 J.G. Baust, Cryobiology 10, 197-205 (1973).

3 J.G. Baust, and R.E. Lee, Jr., J. Insect Physiol. 27, 485-490 (1981).

4 J.G. Baust, and R.E. Lee, Jr., J. Insect Physiol. 28, 431-436 (1982).

5 J.G. Baust, and L.K. Miller, J. Insect Physiol. 18, 1935-1947 (1972).

6 J.S. Clegg, Comp. Biochem. Physiol. 14, 135-143 (1965).

7 F. Franks, Cryo-Letters 2, 27-31 (1981).

8 D.L. Hendrix, R.E. Lee, Jr. and J.G. Baust, J. Chromatog. 210, 45-53 (1981).

9 K. Gekko, and T. Morikawa, J. Biochem. 90, 39-50 (1981).

10 K.A.C. Madin, and J.H. Crowe, J. Exp. Zool. 193, 335-342 (1975).

11 R.E. Morrissey, and J.G. Baust, J. Insect Physiol. 22, 365-372 (1976).

12 R.R. Rojas, R.E. Lee, Jr., T.-A. Luu, and J.G. Baust, J. Insect Physiol. 29, 865-869 (1983).

13 R.W. Salt, Can. Entomologist, 89, 491-494 (1957).

14 R.W. Salt, Can. J. Zool., 37, 59-69 (1959).

15 L. Somme, Comp. Biochem. Physiol. 73, 519-543 (1982).

16 K.B. Storey, and J.M. Storey, J. Comp. Physiol. 144, 183-190 (1981).

17 K.B. Storey, J.G. Baust, and J. Storey, J. Comp. Physiol. 144, 183-190 (1981).

18 S.R. Young, and W. Block, J. Insect Physiol. 26, 189-200 (1980).