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Overwintering biology of the carob moth Apomyelois ceratoniae (Lepidoptera: Pyralidae)

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Overwintering Biology of the Pomegranate Fruit Moth *Apomyelois ceratoniae* (Pyralidae: Lepidoptera)

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Abstract

The pomegranate fruit moth, *Apomyelois ceratoniae* (Zeller), is the most important pest of pomegranate orchards in Iran, where infestations lead to 20-80 % fruit loss. *Apomyelois ceratoniae* overwinters as larvae in several instars. The success in overwintering determines the fruit loss in the following season, thus overwintering physiology of *A. ceratoniae* could provide insights into population prediction. To this end, overwintering strategy and some seasonal physiological and biochemical changes were investigated in the field-collected larvae of *A. ceratoniae*. The lowest supercooling point was recorded in November (-14.6 ± 0.91 °C) and the highest in both October and March (-10.2 ± 0.94 °C). The median lethal temperature (LT_{50}) of larvae was higher than supercooling point, suggesting that *A. ceratoniae* is chill-susceptible. In comparison with summer larvae, accumulation of glycerol and sorbitol in overwintering larvae resulted in lower mortality when exposed to sub-zero temperatures. There were no significant seasonal changes in body water content or hemolymph osmolality. Current winter temperatures in Iranian orchards are higher than the cold tolerance thresholds of *A. ceratoniae*, suggesting that overwintering mortality is not a key factor in determining *A. ceratoniae* populations.

Key words: cold hardiness, cryoprotectants, osmolality, supercooling point, water content

INTRODUCTION

The pomegranate fruit moth or carob moth, *Apomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is a polyphagous fruit pest in many tropical and subtropical countries (Gothilf 1984). The larvae attack pomegranate, fig, and pistachio fruit in Iran (Hesami Fesharaki *et al*. 2011), pomegranate in Iraq (Al-Izzi 1985); almond in Australia; fig, carob, mango, date and almond in Mediterranean countries (Armstrong 2007); date in California, United States (Nay 2005); and citrus and macadamia in South Africa (Gothilf 1984). *Apomyelois ceratoniae*, which originates from Mediterranean countries, was first reported in Kashmar (Khorasan, Iran) pomegranate orchards in 1974 and since 1980 it has been the major pest of pomegranate orchards in Iran (Hashemi Fesharaki *et al*. 2011). Eggs are laid on or near the calyx, through which second or third instar larvae enter the fruit, consuming the interior tissue and seeds. This feeding facilitates entrance of saprophytic fungi resulting in fruit decay and leading to a 20-80 % reduction in fruit yield (Hashemi Fesharaki Fesharaki *et al*. 2011). *Apomyelois ceratoniae* has 4-5 generations per year (Shakeri 2004). In Iran, larvae of several instars enter dormancy in mid-autumn (November) (Gothilf 1984; Al Izzi 1985; Shakeri 2004, Karami *et al*. 2011), and overwinter inside fruits remaining on or under the trees, or under the bark of trees (Shakeri 2004). In early spring (mid-April), the larvae pupate and adult emergence coincides with blossoming and fruiting of pomegranate, continuing until the end of June or beginning of July (Shakeri 2004).

Overwintering can comprise half the life cycle of terrestrial organisms in temperate environments (Williams *et al*. 2014). During overwintering, insects must survive low temperature exposure, as well as desiccation and energy drain (Williams *et al*. 2014) that may reduce overall physiological performance and population growth rate (Cárcamo *et al*. 2009). As ectotherms, insects have limited ability to regulate their body temperature; thus, during winter it

is possible for their body fluids to freeze when exposed to freezing temperatures (Tattersall *et al*. 2012). The formation of internal ice can cause damage to tissues, cells and proteins through mechanical damage or osmotic concentration and anoxia. In order to overcome freezing, many temperate and polar insects seasonally enhance their cold-tolerance in preparation for winter (Denlinger & Lee 2010).

Two major strategies, freeze tolerance and freeze avoidance, are adopted by most overwintering insects. The first group withstands the formation of internal ice and maintains a high supercooling point (SCP), while the second die upon freezing and depress the SCP to survive low temperatures. Chill-susceptible insects die within brief exposure to chill at moderate to high sub-zero temperatures (Bale 1996). Both freeze-tolerant and freeze-avoidant insects accumulate cryoprotectants to enhance their cold tolerance (Lee 2010). Accumulation of cryoprotectants increases hemolymph osmolality results in a depression in hemolymph melting point and prevention of ice-crystal formation (Lee 2010). Our knowledge of overwintering biology of *A. ceratoniae* larvae is limited to three field studies in winter. Two of these determined the overall survival of larvae (Khoshamadi & Baghestani 1987; Mehrnejad 1992), reporting 92% and 77% larval survival respectively. Heydari and Izadi (2014) examined low temperature biology of just the last larval instar at an Iranian location that experiences relatively mild winters (Akbarkooh, mean minimum temperature in January: - 1.3 °C), identifying mortality between -5 °C and -10 °C, and suggesting, based on the mortality occurrence at temperature above the SCP that they are chill-intolerant. These authors also observed accumulation of trehalose and myo-inositol in winter (November-February), suggesting that these are the primary cryoprotectants in this species.

Apomyelois ceratoniae causes substantial fruit loss, even though there are several pest management strategies such as collecting infested fruit in March, stamen elimination (Sheikali *et al*. 2009), mating disruption (Zolfagharieh *et al*. 2009), biological control (Karami *et al*. 2011) and repellents (Peyrovi *et al*. 2011). Increased knowledge about the physiological performance of different instars of overwintering larvae of *A. ceratoniae* is affected by winter could give the insights in population prediction in spring. The objectives of this study were 1) to determine the overwintering strategy and cold tolerance of all overwintering larval stages of field-collected *A. ceratoniae*, at Chandab village, Semnan, Iran, where low winter temperatures are expected to be important for survival, and 2) to identify some of the biochemical and physiological correlates of seasonal changes in cold hardiness of this population.

MATERIALS AND METHODS

Animals

Apomyelois ceratoniae larvae were collected every month during autumn (October, November, and December), winter (January, February, and March) and summer (June) of 2010-2011 and 2012-2013 by gathering infested pomegranate fruit from orchards located in Chandab village, Semnan, Iran (35°25′N, 51°56′E, 1130 m above sea level). Infested pomegranates were transferred to the Faculty of Agriculture, Tarbiat Modares University, and stored on the ground covered with leaf litter. After one day, the larvae were removed and transferred to artificial diet (bran 100g, yeast 5g, sucrose 20g, water 40ml, glycerol 30ml), and kept at ambient temperature in the shade outdoors until use in experiments. We divided larvae into small (2^{nd} and 3^{rd}) and large (4^{th} and 5^{th}) instars and kept them separately. Larvae were used in experiments within one day of transfer to artificial diet.

Cold tolerance

To determine the SCP (n=13-23 per month), larvae were individually attached to NiCr-Ni thermocouples (Type K, diameter 1.5 mm) using adhesive tape (Jason, Ariana Packing co. Tehran, Iran), attached to a transparent plastic sheet and placed inside a programmable refrigerated test chamber (Model MK53, Binder GmbH Bergstr., Tuttlingen, Germany). The temperature of the test chamber was decreased from 15 °C to -30 °C at 0.5 °C/min. The temperature of each insect was recorded every 30 s with a four-channel data logger (Testo Model 177-T4; Mehrkanaz Sanat Co.Tehran, Iran) and monitored using comsoft4 software (ComSoft

110 Basic Software; Mehrkanaz Sanat Co., Tehran, Iran). The SCP was recorded as the lowest
111 temperature prior to the start of the exotherm indicating the initiation of freezing.

112 We determined survival of low temperatures each month in both years by measuring survival of
113 field-collected larvae after 3 h exposure to -7, -10 and -12 °C. For each of the temperature
114 treatments, three replicates of seven larvae were placed in glass Petri dishes (100 mm ×15 mm),
115 the bottom of which was covered by dry tissue paper. The Petri dishes were then placed in the
116 programmable refrigerator and cooled at 0.5 °C/min from 10 °C to the test temperature (-7, -10
117 or -12 °C). After 3 h, the larvae were rewarmed at 0.5 °C/min until reaching 10 °C, and survival
118 (ability to walk in a coordinated fashion) was assessed after 24 h.
119

120 Haemolymph composition and body water content

121 Haemolymph was collected from 20 individuals for osmometry. Ten microliters of hemolymph
122 was collected with a pipette after cutting the 3rd leg of a larva. Hemolymph was frozen at -80 °C
123 in microcentrifuge tubes (the cap of which was sealed with parafilm) until osmometry was
124 performed. Measurements of hemolymph osmolality were performed with a nanolitre osmometer
125 (Clifton Technical Physics, Hartford, NY; Koštál *et al*. 2011). To measure water content of the
126 larvae, the whole fresh body (FW) was weighed to the nearest 0.1 µg and then dried at -40 °C in
127 a freeze dryer (48 h) to constant mass (DW). Water content (WC) was expressed as mg mg^{-1} DW
128 (Koštál & Simek 2000).
129

130 Cryoprotectants

131 Van Handel's (1965) method was performed to extract the sugars and polyols from whole larvae.
132 Each larva (n=3/month) was weighed and homogenized in a few drops of methanol and 0.05 ml

of saturated sodium sulfate solution (for details see Van Handel 1965). The extracts were dried at 35 °C in vacuum drying oven (model VO400, Germany) and resuspended in 200 µl of high performance liquid chromatography (HPLC) grade water. The samples were filtered using a 0.45 µm syringe filter (Millex, Tokyo, Japan) and 30 µl of each sample was injected into an HPLC (Waters 600 Controller, Milford, MA, USA) and separated on a carbohydrate column (Supelcolgel ^{TM}, Ca HPLC column) with 9 µm particle size (300 mm long × 7.8 mm ID). The solvent was water and flow rate was kept constant at 0.5 ml/min. Separation was performed at room temperature and all data were acquired and processed with Empower chromatography software (Waters) compounds were identified and quantified from retention time of carbohydrate standards (Fulka, Bush, Switzerland).

Statistics

Mean osmolality and cryoprotectants were compared among months by one-way analysis of variance (ANOVA) followed by Tukey's (differences were considered significant at $P < 0.05$). Water content was compared with ANCOVA (with dry mass as covariate). A two-way mixed-model ANOVA followed by Tukey's *post hoc* test was performed to compare the differences among SCP in both years. The lethal temperature at which 50% of the population died after a 3 h exposure to subzero temperatures (LT_{50}) was determined using binary logistic regression (Berkvens *et al*. 2010). Comparisons of LT_{50} values were based on non-overlapping 95% confidence intervals. Because neither SCP nor cold tolerance was affected by larval instar ($P > 0.05$) the data for both small (2^{nd} and 3^{rd} instars) and large (4^{th} and 5^{th} instars) larvae were pooled. Analyses were conducted using SPSS for Windows (v. 20.0; IBM, Armonk, NY, USA)

RESULTS

Supercooling point and lethal temperatures were compared to determine the cold tolerance strategy. SCP was measured in a total of 250 larvae in 2010-2011 and 2012-2013. The distribution of SCP values in different months (Fig. 2) and among small (2^{nd} and 3^{rd} instars) and large larvae (4^{th} and 5^{th} instars; Fig. 3) was unimodal. The lowest absolute and mean SCP of *A. ceratoniae* larvae was recorded in November 2010 (-21.4 °C and -14.6 ± 0.91 °C respectively), whereas the highest SCP was recorded in March 2013 (-6 °C). The highest mean SCPs of overwintering larvae (-10.2 ± 0.94 °C) were recorded in October 2010 and March 2013 (Fig. 1). There was no significant month × year interaction ($F_{5,238} = 1.344$, $P = 0.24$), however there was a significant main effect of month in both years ($F_{5,235} = 4.712$, $P < 0.001$). Seasonal fluctuations in SCP pattern in different month was equal in followed the same pattern in both years. None of the larvae survived freezing at the SCP.

Overwintering individuals were more resistant to sub-zero temperatures than summer-collected larvae. Overwintering individuals survived 3 h exposures to sub-zero temperatures as low as -12 °C, whereas summer-collected individuals only survived 3 h exposures as low as -7 °C (Fig. 5). As exposure temperature to sub-zero temperatures decreased, survival of the overwintering larvae decreased (Fig. 4). Cold tolerance did not differ among months during autumn and winter in either year (2010-2011: $F_{5,83} = 1.18$, $P = 0.327$; 2012-2013: $F_{5,63} = 1.008$, $P = 0.420$).

The median lethal temperature (LT_{50}) was higher than the SCP of overwintering larvae during sampling months. In 2010-2011 the highest and the lowest LT_{50} were observed in March (-8.9 °C) and February (-12.4 °C) respectively. In 2012-2013, however, the highest and lowest LT_{50} occurred in January (-8.6 °C) and December (-10.7 °C) (Fig. 1). The LT_{50} of larvae in each

month was higher than their corresponding SCP (Fig. 1), which suggests the larvae are chill-susceptible. There was no correlation between SCP of overwintering larvae and their respective LT_{50} in sampling months (r_5=0.096, P=0.081).

There was no significant difference between the water content of overwintering (1.73 ± 0.18 mg water/mg DW) and summer-collected larvae (1.87 ± 0.13 Water/mg DW; $F_{7,70}$ = 1.913, P=0.098). Hemolymph osmolality ranged from 413 mOsmol kg^{-1} in March to 443 mOsmol kg^{-1} in January, but there were no significant differences across the year (Fig. 6 A). Glycerol, glucose and sorbitol were detected by HPLC. Glucose levels did not change during the year (0.025-0.03 mg/g fresh weight); whereas glycerol increased steadily during autumn, peaked in January (2.5 mg/g fresh weight), declined in February and reached its lowest point (0.02 mg/g fresh weight) in March. Sorbitol went up in November (2 mg/g fresh weight) decreased to 1 mg/g fresh weight in February and reached its lowest point in March (Fig. 6 B).

DISCUSSION

The larvae of *A. ceratoniae* consistently died at temperatures above the supercooling point, suggesting that they are chill-susceptible. In addition, the lack of relationship between SCP and cold hardiness confirms that SCP is not indicative of cold hardiness in this species. Heydari and Izadi (2014) similarly concluded that another (Akbarkooh, Iran) population of *A. ceratoniae* are chill-intolerant (a synonym of chill-susceptibility, Denlinger & Lee 2010), however they did not expose the larvae to temperatures lower than -10 °C. Chill-susceptibility has also been reported for overwintering adults of *Alphitobius diaperinus* (Panzer) (Colinet *et al*. 2011), larvae of *Drosophila melanogaster* (Koštál *et al*. 2012) pupae of *Helicoverpa zea* (Boddie) (Morey *et*

al. 2012), and the larvae of *Thaumatotibia leucotreta* (Meyrick) (Boardman *et al.* 2012). We

found that the lower lethal temperature and LT_{50} of both small and large overwintering *A. ceratoniae* larvae decreased in November 2010 (-11.8 °C), February 2011 (-12.4 °C) and December 2012 (-10.7 °C). This winter decrease in lower thermal limits was previously reported in last instar *A. ceratoniae* (Heydari & Izadi 2014), and is typical of insects overwintering in temperate regions (Khani & Moharramipour 2007; Denlinger & Lee 2010; Crosthwaite *et al*. 2011). However LT_{50} of *A. ceratoniae* was not determined in Akbarkooh population.

We found that *A. ceratoniae* accumulated glycerol and sorbitol during the dormant period, and it appears that this accounts for the observed increase in cold tolerance in overwintering larvae. Glycerol is the most widely-distributed metabolite reported in cold-hardy (Denlinger & Lee 2010) insects (e.g.; chill-susceptible bark beetle *Pityogenes chalcographus* (L.); Koštál *et al*. 2014 while sorbitol is a common secondary cryoprotectant in other overwintering insects (Khani *et al*. 2007; Williams & Lee 2011). By contrast, Heydari and Izadi (2014) reported accumulations of trehalose and myo-inositol (but not glycerol) in the last instar larvae. This discrepancy could be because of differences in population as divergent selective pressure in local environments can result in differentiation in thermal biology (Sinclair *et al*. 2012). The difference in the cryoprotectants may reflect differences in the cryoprotectant physiology of the populations studied, since the present study was conducted in Chandab where the duration of sub-zero temperatures is longer in winter, so the colder weather could have triggered sorbitol production (e.g. *Eurosta solidaginis* produces sorbitol only when exposed in temperatures below 5 °C (Storey & Storey 1990)). Further exploration and direct comparison will be necessary to determine the underlying cause of the apparently divergent cryoprotectant strategies in these two populations.

Although partial dehydration may increase cold tolerance (Ring and Danks 1994; Block 1996); insects can rapidly change their cuticular permeability and resist dehydration (Bazinet *et al*. 2010). In the case of *A. ceratoniae,* the difference in water content between overwintering and active larvae (c. 6 %) was probably not biologically-significant. Furthermore hemolymph osmolality (October-March) was similar or only slightly higher in the January. *Apomyelois ceratoniae* larvae overwinter inside the fruit, a protected site that buffers against low ambient temperatures, reduces the risk of ice damage, and likely protects the larvae from water loss.

Although overwintering larvae of *A. ceratotinae* are chill-susceptible, the minimum air temperature in pomegranate-growing areas of Iran normally does not decrease below -10 °C, and -12 °C was the lowest temperature recorded (http://www.irimo.ir/); otherwise the pomegranate tree encounters serious injuries. Thus, low temperatures are unlikely to cause significant overwinter mortality, allowing a substantial population to persist through to the following season. However we studied the effect of only a single short time exposure to cold and only measured survival. Nevertheless during winter *A. ceratoniae* encounters prolonged and repeated cold exposures with sub-lethal effects on reproductive fitness as they might invest energy to repair chilling injuries as well as changing pathways to produce heat shock proteins (Marshall & Sinclair 2012). The first pomegranate flowers (which lead to the highest-quality fruit) emerge on late April (Shakeri 2008); and seem to be the host for the first generation of *A. ceratoniae*. Thus, future work understanding the timing of cold exposure (and cold tolerance of non-dormant life stages) and termination of dormancy may improve the timing of integrated pest management interventions aimed at preventing egg-laying by this first generation.

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354

355

Figure captions

Figure 1 The median lethal temperature (LT_{50}) and supercooling point (SCP) (mean ± SE) of overwintering and summer larvae of *Apomyelois ceratoniae* in 2010-2011 and 2012-2013. In October (2010) and February (2013), the number of larvae was too small to calculate LT_{50}. Means with the same letter are not significantly different (Tukey's *post-hoc* tests, $P \leq 0.05$).

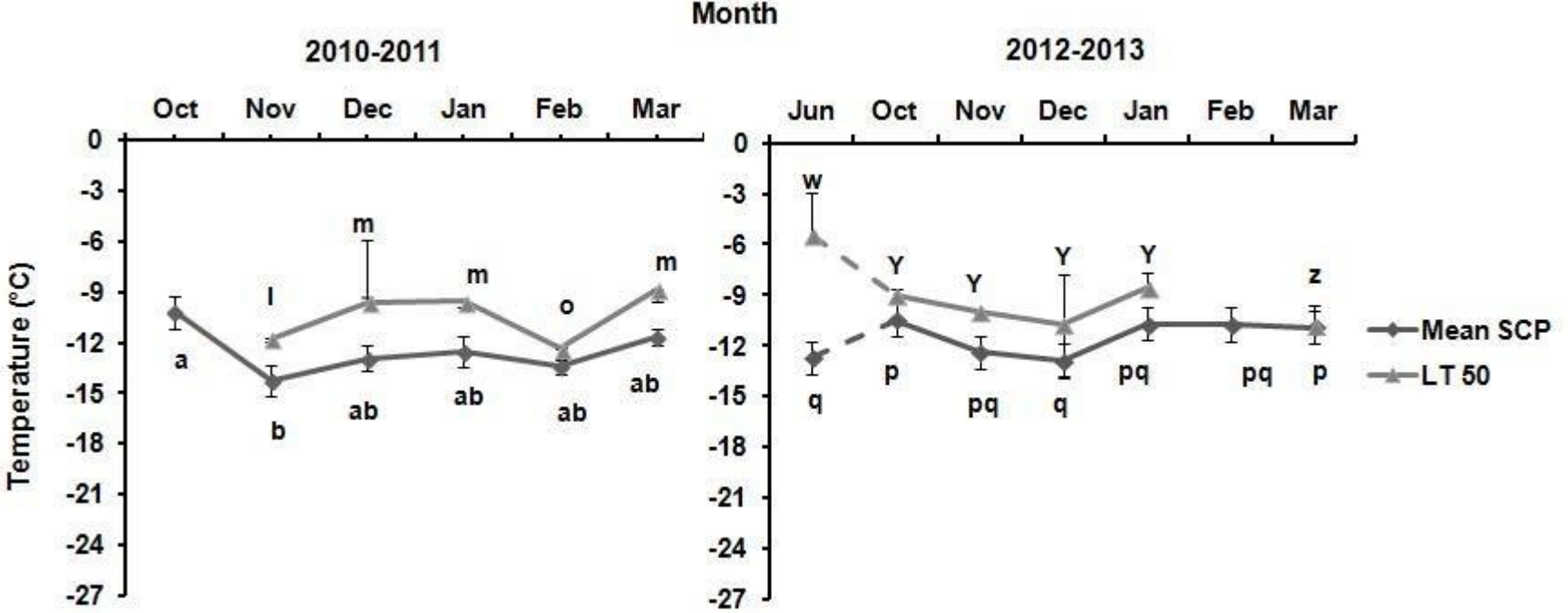
Figure 2 Frequency of supercooling point (SCP) in overwintering larvae of *Apomyelois ceratoniae* in autumn and winter 2010-2011and 2011-2012.

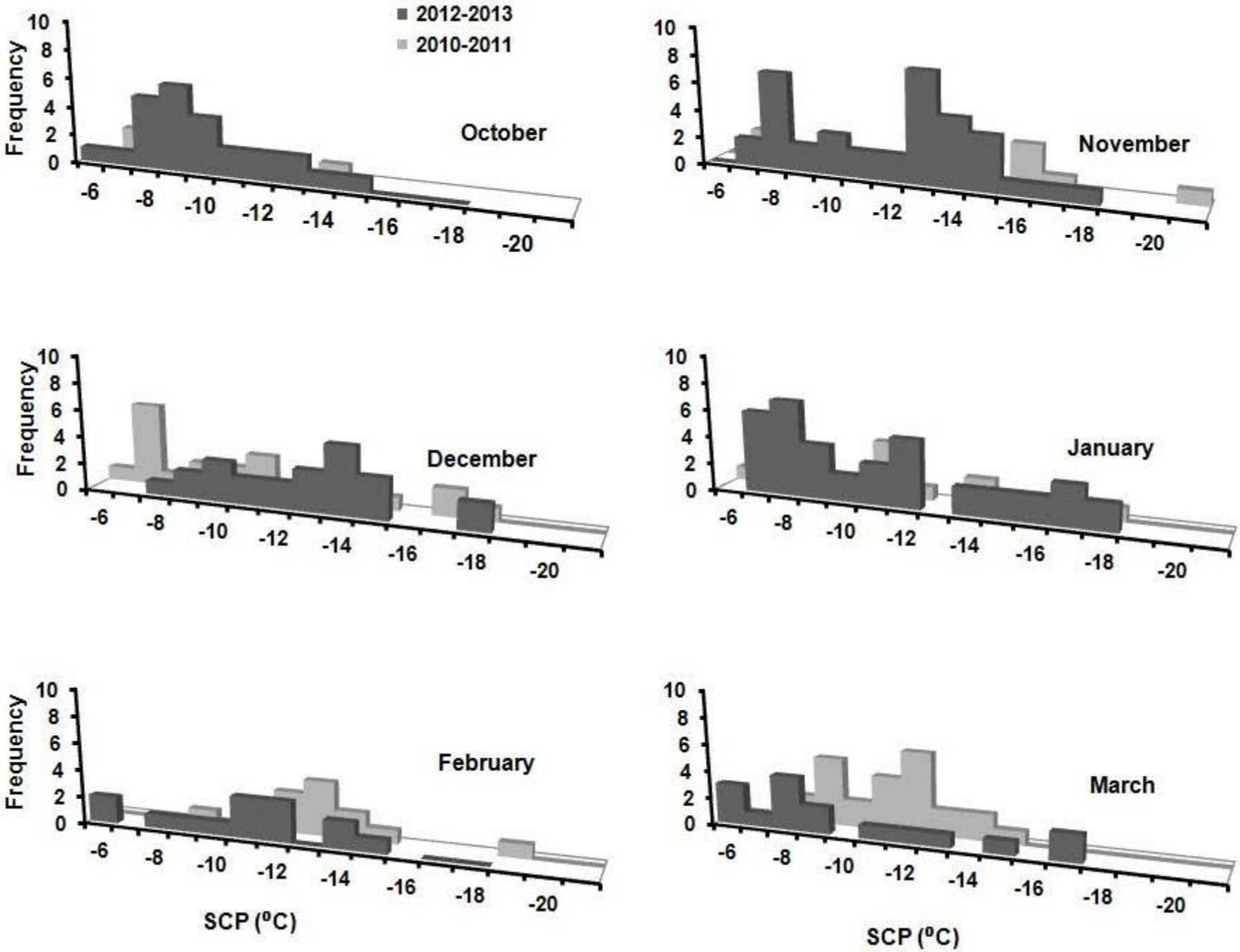
Figure 3 Frequency of supercooling point (SCP) in small (2^{nd} and 3^{rd} instars) and large (4^{th} and 5^{th} instars) larvae of *Apomyelois ceratoniae.*

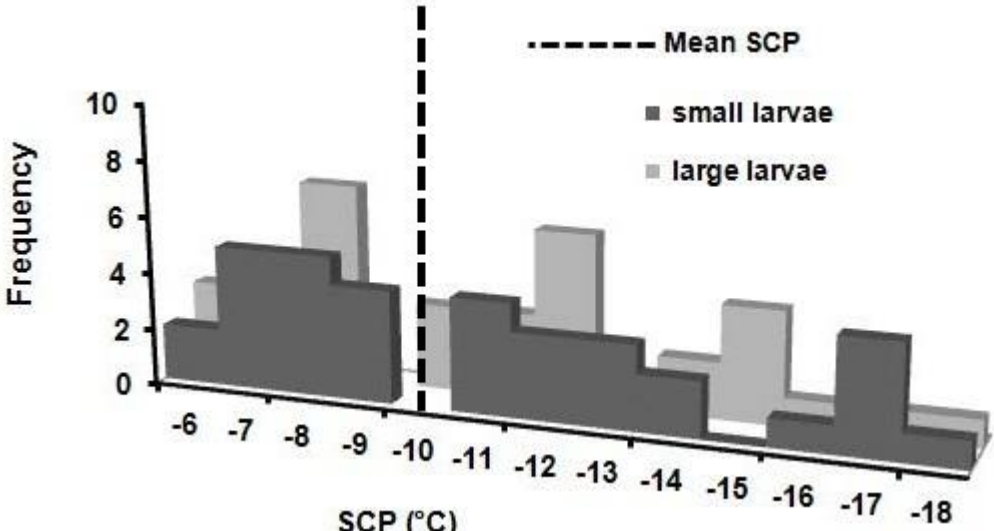
Figure 4 Mortality and cumulative supercooling point (SCP) in overwintering larvae of *Apomyelois ceratoniae* in different sub-zero temperatures in autumn and winter 2010-2011 and 2012-2013. In October (2010) and February (2013), the number of larvae was too small to calculate mortality.

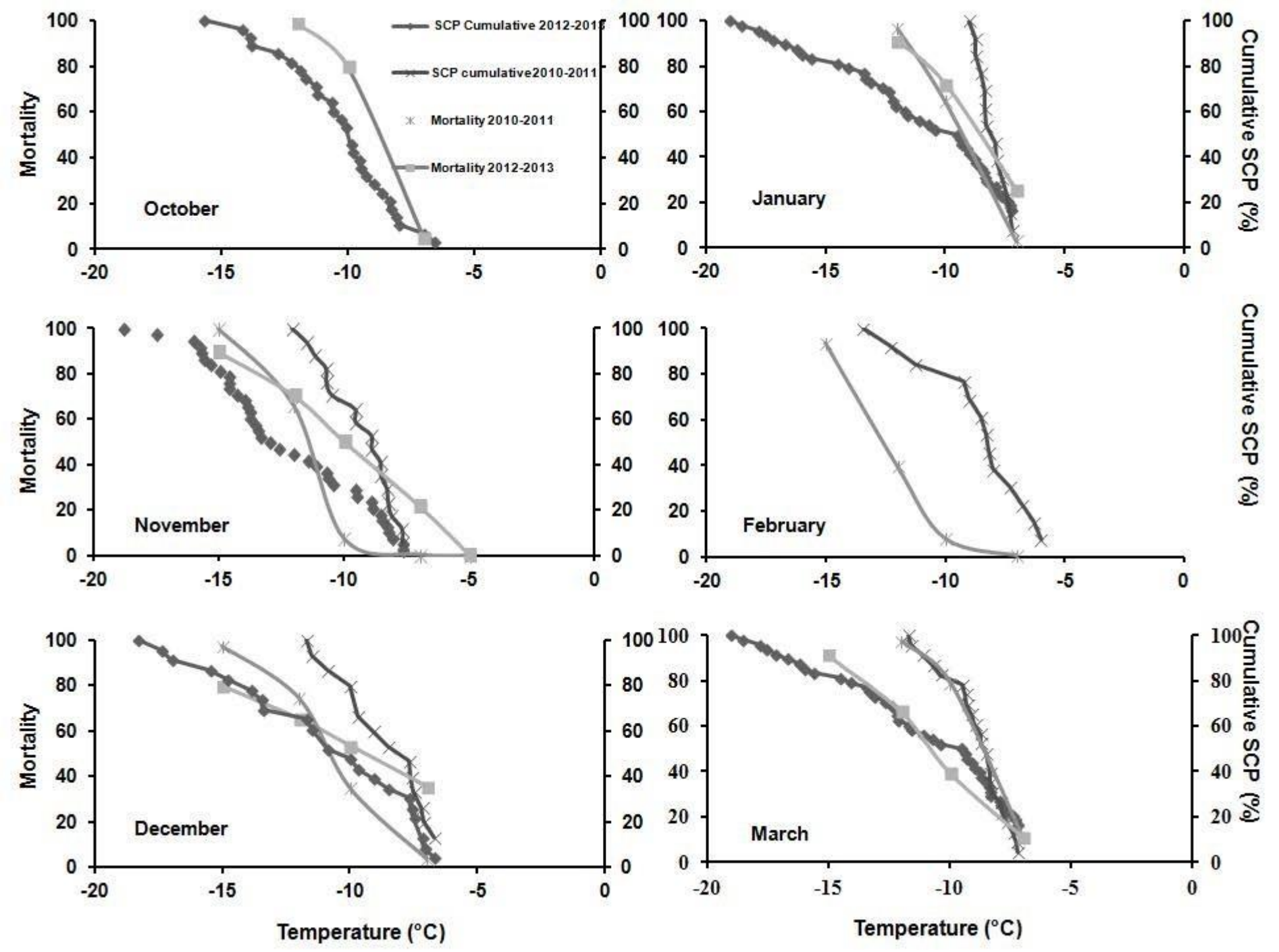
Figure 5 Mortality and cumulative supercooling point (SCP) of larvae of *Apomyelois ceratoniae* in different sub-zero temperatures in June 2012.

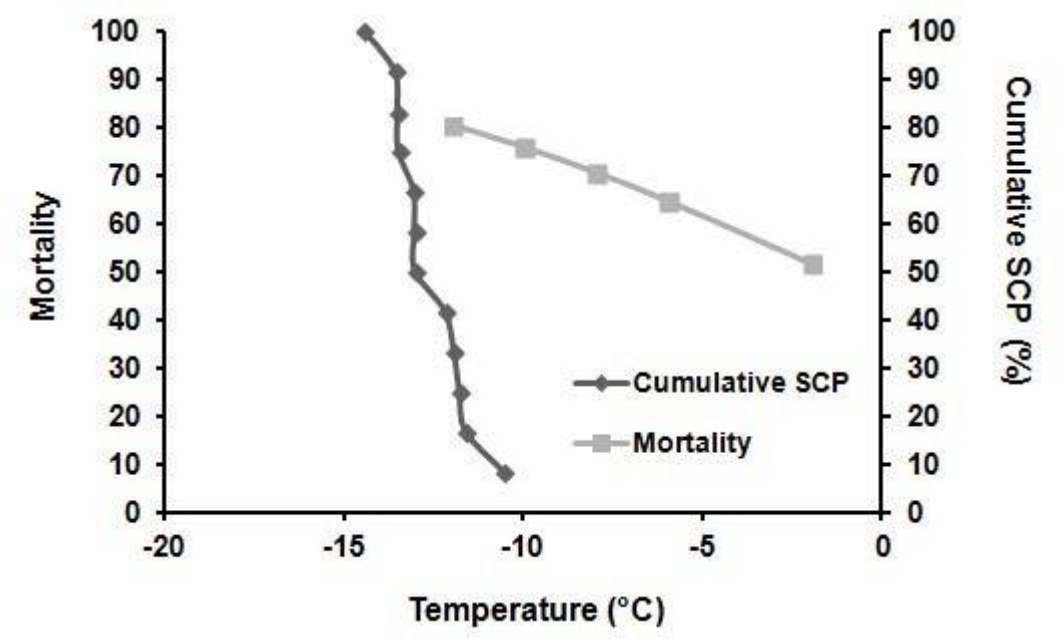
Figure 6 Hemolymph osmolality (A) and whole-body cryoprotectants (B) of field-collected overwintering larvae of *Apomyelois ceratoniae* in 2010-2011.

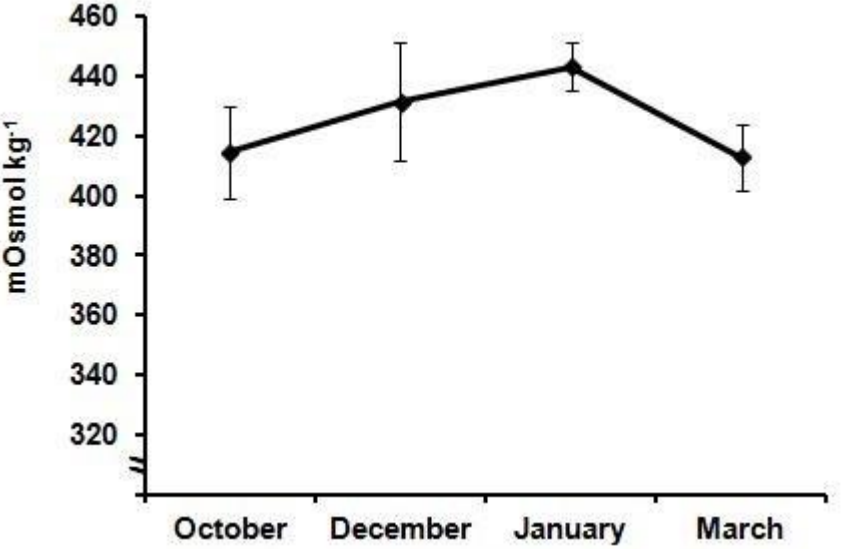

Month
2010-2011
2012-2013
Oct
Nov
Dec
Jan
Feb
Mar
Jun
0
-3
-6
-9
-12
-15
-18
-21
-24
-27
Temperature (°C)
a
b
ab
ab
ab
ab
l
m
m
o
m
w
Y
Y
Y
Y
z
q
p
pq
q
pq
pq
p
Mean SCP
LT 50

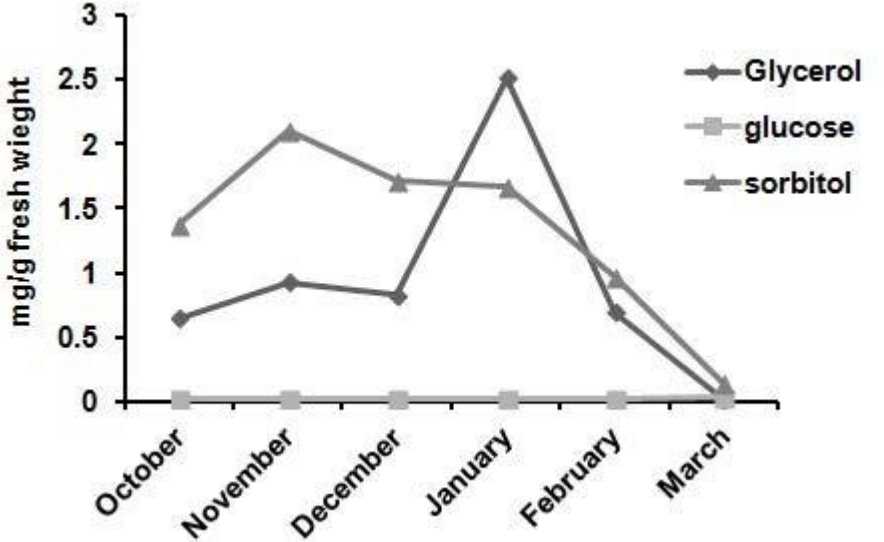

2012-2013
2010-2011
Frequency
October
November
December
January
February
March
SCP (°C)
SCP (°C)


Mean SCP
small larvae
large larvae
Frequency
10
8
6
4
2
0
-6
-7
-8
-9
-10
-11
-12
-13
-14
-15
-16
-17
-18
SCP (°C)


SCP Cumulative 2012-2013
SCP cumulative2010-2011
Mortality 2010-2011
Mortality 2012-2013
October
January
November
February
December
March
Mortality
Cumulative SCP (%)
Temperature (°C)
100
80
60
40
20
0
-20
-15
-10
-5
0


Mortality
Cumulative SCP (%)
Temperature (°C)
Cumulative SCP
Mortality
100
90
80
70
60
50
40
30
20
10
0
-20
-15
-10
-5
0


460
440
420
400
380
360
340
320
mOsmol kg-1
October
December
January
March


3
2.5
2
1.5
1
0.5
0
mg/g fresh wieght
Glycerol
glucose
sorbitol
October
November
December
January
February
March