Bulletin of Insectology ***72** (2): 287-296, 2019*
ISSN 1721-8861 eISSN 2283-0332

Preliminary studies on the cryopreservation of silkworm (*Bombyx mori*) eggs

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Abstract

Developing a technique for the cryopreservation of *Bombyx mori* L. germplasm may provide significant benefits for supplying good strains to farmers and facilitate the conservation of living material in genetic banks. Producing species-specific protocols for the selection of the most apt embryonic stage, dechorionation/permeabilization of eggshells, and the acclimation at low temperature before the treatment of eggs at cryogenic temperature is a prerequisite for cryopreservation of *B. mori* embryos. In this research, non-diapausing eggs of *B. mori* Nistari strain were used to study cold hardiness; the final aim was to select the most apt embryonic development stages, and in addition, reliable permeabilization, and vitrification treatments. Polyhybrid *B. mori* eggs of a diapausing strain were also used for comparison in experiments of embryogenesis, permeability, and acclimation under low temperature before cryopreservation. The Nistari eggs in the stage of 24 hours post-oviposition (h PO) showed a scarce survival rate (0.1%) after their submersion in liquid nitrogen (LN), while the highest survival in loading and dehydration with cryoprotective agents was assessed in 40h PO eggs. Overwintering diapausing eggs preserved for three months at 5 °C and then treated with various vitrification solutions gave rise to good hatching rates. The achieved results show that it is possible to improve the method of cryopreservation of *B. mori* eggs by obtaining a good permeability with cryoprotectants at loading/dehydration steps and by decreasing the toxicity, reducing the treatment time.

Key words: Silkworm, *Bombyx mori*, non-diapausing eggs, polyvoltine strains, embryonic development, cold hardiness.

Introduction

According to the current perspectives in arthropod germplasm preservation (Holmstrup *et al*., 2010), we made a detailed study of the cold hardiness of the eggs laid by the domesticated silkworm, *Bombyx mori* L. (Lepidoptera Bombycidae). Cold hardiness is the capacity of an organism to survive short or long-term exposure to low temperatures (Leopold *et al*., 2001; Banno *et al*., 2013). In case of eggs, it is influenced by the duration of the cooling period and the age of the embryo at the time of exposure to low temperatures (Cho *et al*., 2007) because embryonic development is prolonged under lower temperatures, and the embryonic stages are affected by cold to a significantly varied extent (Yamashita and Yaginuma, 1991).

More than 4000 genotypes of both diapausing and non-diapausing *B. mori* are reared for commercial raw silk production (Nagaraju *et al*., 2000) in the world; additionally silkworm is an interesting model for scientific studies (Sagakuchi, 1978). Therefore, all these genotypes should be maintained as genetic stocks and their preservation represents one of the most important and expensive efforts of the silkworm gene banks (Cappellozza *et al*., 2013). Genetic devolution, genetic drift and contamination are all threats to germplasm stability during mass rearing of many insects (Cohen, 2004). Such problems may be avoided with the development of a long-term preservation method for conservation of stocks of genetic resources. Establishing a repertory of the silkworm genetic resources would ensure preservation of the original genetic makeup, and will permit the study of what genes may have been lost in the selection process.

Cryopreservation has been successfully used for many insect eggs (Sonobe *et al*., 1979; Steponkus and Caldwell, 1993; Leopold, 2000; Nunamaker and Lockwood, 2001; Rajamohan and Leopold, 2007; Roversi *et al*., 2008). For *B. mori*, cryopreservation of gonads has been recently applied to obtain a routine technique to maintain silkworm resources (Kusuda *et al*., 1985; Takemura *et al*., 2000) and eri and ailanthus silkworm (Fukumori *et al*., 2017). The method for long-term maintenance using cryopreservation in liquid nitrogen was successfully applied to silkworm female gonads prepared from fifth-instar larvae and transplanted after storage into larvae of the same sex (Banno *et al*., 2013). However, the required manual skills limit gonad stock preservation of the silkworm on a large scale.

On the other hand, some attempts have been made at storing fertilized eggs for a long time at ultra-low temperatures, but there is no information about any successful preservation. A serious obstacle to cryopreserve the embryo is the low chorion permeability to cryoprotectives (Okada, 1971; Schreuders *et al*., 1996). Furthermore, the main barrier to the cryopreservation of insect eggs is the scarce permeability of external membranes and protective integument layers.

B. mori has a chorion thickness up to 23-25 μm compared to 0.8 μm of *Drosophila melanogaster* L. (Diptera

Drosophilidae); therefore, the permeabilization methods recently established for Dipterans are not applicable to *B. m*ori eggs because of the thick, tough chorion, which does not implement the exchange of cellular water with cryoprotectants. The chorion proteins, which constitute more than 95% of chorion dry mass, have remarkable mechanical and physiochemical properties, forming a protective natural shield for the egg, and defending the developing embryo from a wide range of environmental hazards (Mazur *et al*., 1989).

Recent results obtained with another Lepidopteran (*Galleria mellonella* L.), based on a protocol involving rapid cooling in mechanical freezers (Roversi *et al*., 2008), have prompted a study to assess the possibility of preserving strains of commercial silkworm by vitrification. However, to attain this goal, we require a few basic information, such as an in-depth knowledge of the cold hardiness and chemical resistance of *B. mori* embryos with or without any acclimation at low temperatures (overwintering). Based on that, we identified the most tolerant embryonic stage.

Materials and methods

Chemicals

Sodium hypochlorite, SH (NaOCl) (7 ± 2% Cl active) and sodium thiosulfate ($Na_2S_2O_3$) were purchased from Carlo Erba (Milano, Italy). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Isopropyl alcohol (IPA) and heptane (HP) were HPLC grade. The TMN-FH medium (pH 6.1, 360 mOsm) consisted of a supplemented Grace's medium.

Rearing of the silkworm strains and production of egg batches

B. mori larvae of the non-diapausing polyvoltine Nistari strain, and a diapausing four-way polyhybrid (70 × 129) (124 × 64) (the numbers refer to parental classification in the germplasm bank of CREA-AA, sericulture laboratory), were fed on an artificial diet containing 40% mulberry leaves on a dry weight basis (Cappellozza *et al*., 2005).

Staging and embryonic development studies

The morphology of dissected embryos of *B. mori* (diapausing or non-diapausing) developed in untreated eggs were examined under a microscope (Nikon SMZ 1500) to identify the embryonic stage. The eggs of subsequent developmental stages were examined under differential interference contrast (DIC) microscope (Nikon Eclipse TE2000-U) and were dechorionated according to Pang-chuan and Da-chuang (1992). This dechorionation procedure, quite strong and fast, was used to evaluate the embryo development only, without any caution for its survival. The age of tested embryos at the various PO times was calculated from the midpoint of the oviposition period as reported by Leopold (2000). On the other hand, the egg surface of *B. mori* after dechorionation and permeabilization (D/P) (Wang *et al*., 2000) was observed under a scanning electron microscope (Philips 515 SEM) to evaluate possible damages to the chorion structure. In this case the dechorionation procedure was that explained in the below section, entitled "Cryopreservation".

Chilling sensitivity

After the oviposition of polyvoltine Nistari eggs and incubation for different periods under the same conditions (25 ± 1 °C, 80 ± 5% relative humidity, RH), embryonic developmental stages (20, 22, 24, 38, 40, and 42h PO) were kept for 1 and 2 months at two different temperatures: 5 ± 0.5 °C and 0 ± 0.5 °C.

We focused on these stages because previous unpublished experiments gave unsuccessful hatching results for later developmental hours in time. The 5 °C temperature was achieved with a Samsung refrigerator, while the 0 °C temperature was achieved with an ice water bath kept in the refrigerator (5 ± 0.5 °C). After the chilling treatments, the eggs were incubated in a humidified chamber at 25 ± 1 °C until hatching to calculate the survival rate. The effects of short exposure (one and two months) to low temperatures during the development of *B. mori* eggs were quantified as hatching percentages. White shells of eggs from which larvae had hatched were easily distinguished from yellow desiccated non-fertilized eggs and from pigmented eggs with dead embryos inside. Each treatment was repeated three times.

Cryopreservation

Dechorionation and permeabilization

For this experiment we used both non-diapausing and diapausing strains after overcoming the diapause period; in fact, we assumed and experimentally confirmed that actively developing eggs have comparable behaviours. For both strains, eggs were removed from the deposition paper by dipping them for some minutes in sterile distilled water and by using a brush. The dechorionation solution ingredients were SH plus 0.04% Tween80 in all trials; in the different experiments, we maintained identical treatment steps by only varying the SH concentration and the egg exposure time to it. Then, the procedure comprised: rinsing the eggs in 150 ml of distilled and sterile water (DSW), followed by rinsing them in 5% sodium thiosulfate ($Na_2S_2O_3$) for 7-10 seconds to remove residual chlorine (Okada, 1971) and again rinsing with 150 ml of DSW. Afterwards, permeabilization steps were represented by washing them with IPA for 30 seconds to remove residual water, and then by treatment with hexane (HX) for 90 seconds.

The first test was conducted with two embryonic stages of diapausing eggs, after diapause overcoming (hours indicate the time elapsed under incubation conditions 25 ± 1 °C, 80 ± 5% RH from the egg extraction from the refrigerator); here they were kept at 2.5 ± 1 °C to avoid embryo development, after diapause overcoming. The dechorionation procedure consisted of the gentle agitation of eggs (120 hours: A-D and 168 hours: E-H) in the following solutions:

2.5% SH for 5 minutes (A) and 10 minutes (min) (B).
1.25% SH for 10 minutes (C) and 15 min (D).
2.5% SH for 5 minutes (E) and 10 min (F).
1.25% SH for 10 minutes (G) and 15 min (H).

The second test was conducted with two embryonic stages of non-diapausing Nistari eggs (40 and 60 hours old); in this case the dechorionation procedure consisted of gently agitating the eggs in 3% SH for 2, 4 and 6 minutes.

Permeability assessment

The evaluation of a successful permeabilization treatment is usually carried out through two visual assessment mechanisms. Firstly, the shrinkage and re-swelling with a high molecular weight (MW) non-permeating agent like sucrose followed by a low MW permeating agent like ethylene glycol (EG) are evaluated. Subsequently, the easy access into the treated eggs of colour dyes, like Rhodamine B (0.1%), is estimated. In our experiment, for the first observation, eggs were examined under a stereomicroscope. To implement the second analysis, eggs were observed under a DIC microscope.

Vitrification tests

In the third test, polyvoltine Nistari eggs in two embryonic developmental stages (24 and 40h PO), were treated with cryopreservants [dechorionation, permeabilization, loading, and dehydration treatments required before immersion in liquid nitrogen (LN)]. The early development stage 24h PO was chosen on the basis of some previous observations in the eggs of the greater wax moth, *G. mellonella* (Roversi *et al.*, 2008; Cosi *et al.*, 2010). These results suggested to test an early embryonic stage before the development and formation of the pre-final cuticle. A germ band with amnion and primary serosa membrane is characteristic of this moment in time, before the development and formation of the pre-final cuticle. Furthermore, the stage of 40h PO was selected, when monovoltine eggs block their growth and enter diapause; in this stage they have already started the pigmentation of the serosa membrane. The results of chilling tests and microscopy observations also contributed to encourage the selection of these stages.

To dechorionate them, eggs were treated with 2% SH + 0.04% Tween80 for 4 min, and washed in 5% $Na_2S_2O_3$ for 7 sec, then subsequently rinsed in 500 ml DSW. After this, the permeabilization steps described above (IPA and HX) were applied. Due to the low hatching rate of 24h PO eggs previously obtained with this protocol, they were exposed to half of the usual exposure time of organic solvents (IPA and HX): 15 and 45 sec, respectively. Before the loading/dehydration (L/D) steps, HX was removed by washing three times with a TMN-FH medium (Insect cell culture tested with L-glutamine, without sodium bicarbonate) + 10% Fetal Bovine Serum (FBS), and then the eggs were incubated for 15 min in 1M sucrose dissolved in TMN-FH medium, for further removal of HX before beginning the loading step.

The eggs were subsequently loaded with 10% ethylene glycol (EG) at room temperature for 60 min. Then, they were transferred to the dehydration solution [40% EG + 10% Trehalose + 5% polyethylene glycol (PEG) + 10% methanol (MeOH)], on ice for 15 min. The eggs were distributed on a nylon membrane (Roche Diagnostics GmbH, Germany), which was dried on filter paper and quickly dipped into LN (−196 °C) for 5-10 min. In the following warming step, the eggs underwent a quick return to room temperature in a warming solution (0.5 M Trehalose + TMN-FH) for 2 min and were repeatedly washed in lower concentrations of Trehalose in TMN-FH (0.25 M and 0.1M) for 10 min. The final washing was at 10% FBS + TMN-FH for 10 min, followed by a quick washing in DWS to remove any medium excess.

In order to calculate the hatching rate of embryos, the eggs were placed into 60 mm plastic Petri dishes containing 1.4% agar dissolved in a lepidopteran ringer solution (Matsumoto *et al.*, 1995) in order to keep the eggs moist, and incubated in a growth chamber (Binder GmbH, Germany) at 25 °C at 85% RH.

Overwintering diapausing eggs

To study the effects of polyols inside the eggs before the vitrification steps, diapausing eggs, which had just overcome their diapause period at 5 °C, were treated. After egg laying, the embryos were incubated to continue their development to the desired stage of 40h PO. Eggs were subsequently exposed to cold at 5 °C to overcome the diapause period (three months) then were treated with cryopreservation steps. Cold preservation was used to show the effect of polyols production on the following treatment steps preceding cryopreservation. Post-permeabilization treatments were initiated with loading with 10% EG for 60 min, and dehydration of the eggs was carried out for 30 min in three different types of vitrification solutions containing the following: 1) standard solution (40% EG + 10% Trehalose + 5% PEG); 2) standard solution + 10% dimethyl sulfoxide (DMSO); 3) standard solution + 10% MeOH. After dehydration, immersion of the eggs in LN and warming steps were completed.

Statistical analysis

The hatching percentage of control and treated embryos was calculated as the number of hatched larvae out of the total number of eggs. A minimum of three replicates for each experiment was established, which were represented by three different egg batches. The number of eggs collected in each oviposition period varied; however, a minimum number of 20 eggs per replication was tested. Data were normalized by arcsine transformation prior to analysis, and were processed by one, two or three-way analysis of variance (ANOVA) for evaluating the interactions among variables. The Tukey post-hoc test was used to determine intergroup differences. The probability level of 95% was accepted as statistically significant. All statistical analyses were conducted by using SPSS 11.0 software.

Results

Staging and embryonic development studies

We performed microscopy examination with the aim of relating cold sensitivity to a precise embryonic developmental stage. We succeeded in identifying thirteen successive embryonic stages in Nistari eggs according

to literature references (Sagakuchi, 1978).

In addition, we observed what is already known; i.e., that between *B. mori* diapausing and non-diapausing eggs there are no morphological differences in development from oogenesis to early embryogenesis, apart for the absence of diapausing and hibernating stages (8 stages) in polyvoltine eggs; diapausing and hibernating stages usually range from the 24 to the 40h PO (Sagakuchi, 1978). We could assess that, in monovoltine eggs, after the end of the diapause period, the embryonic development continues in the same way as in 40h PO eggs of non-diapausing polyvoltine strains (figure 1). This behaviour verified our first hypothesis: that diapausing and non-diapausing eggs can be equally used to carry out experiments on cold tolerance; in fact, from the overcoming of the quiescence period (after hibernation), the embryonic development is identical irrespectively on voltinism.

In this experiment, we could not observe the embryonic membranes formation (serosa, amnion) because our experimental procedure permitted to study organogenesis stages after the diapause termination only.

Chilling sensitivity

In table 1, differences among average hatching rates of embryos of the Nistari strain exposed at 0 °C and 5 °C for 1 or 2 months are reported. Three-way ANOVA was performed (factors: temperature, different preservation periods, and different developmental stages).

The control eggs of polyvoltine Nistari strain started hatching at about 9.5 days PO (at 228h PO).

For the same periods of cold storage, the two examined chilling temperatures of 5 and 0 °C differed very significantly ($F_{2,119}$ = 497.63; P < 0.001); a highly significant difference was also recorded for the length of the periods of cold storage of one and two months at the same temperature ($F_{1,119}$ = 224.525; P < 0.001), for the developmental stages ($F_{5,119}$ = 5.688; P < 0.001), for the interaction temperature × time ($F_{2,119}$ = 35.140, P < 0.001) and stage × temperature ($F_{10,119}$ = 3.186; P < 0.001), and for stage × temperature × time ($F_{10,119}$ = 6.028; P < 0.001), while the interaction stage × time resulted as not significant.

The significant interaction of stages × temperature confirms that the resistance of the various developmental

Table 1. Hatching percentages (%) of Nistari eggs after exposition at 5 and 0 °C for 1 month (T1) and 2 months (T2). The eggs were exposed to chilling temperatures at different developmental steps to assess their resistance according to the embryo stage. Different letters indicate significant differences among means (Tukey test, P < 0.05, post-hoc test, following three-way ANOVA). h PO = hours post oviposition.

h PO	5 °C - T1 (%)		0 °C - T1 (%)		5 °C - T2 (%)		0 °C - T2 (%)		Control (%)	
20	29.7 ± 7.1	cdefghi	20.6 ± 12.1	fghil	0	l	0.5 ± 0.5	h l	94.3 ± 1.6	a
22	75.3 ± 7.5	abcd	41.9 ± 17.1	cdeg	0	l	0	l	93.3 ± 1.6	a
24	47.6 ± 13.6	bcdefg	10.0 ± 4.1	f hil	0.8 ± 0.8	h l	0	l	92.9 ± 1.3	ab
38	35.3 ± 4.9	cdefg i	77.5 ± 6.9	abc	28.3 ± 4.2	defg i	0	l	93.6 ± 1.5	a
40	38.7 ± 17.5	cdefg i	62.6 ± 7.4	cde	20.3 ± 4.1	efghil	0.5 ± 0.5	h l	92.4 ± 1.8	ab
42	35.2 ± 9.7	cdefg i	49.8 ± 3.7	cde	0	l	8.9 ± 8.9	hil	94.6 ± 1.5	a

Figure 1. (see next page) Embryonic stages of a polyhybrid diapausing strain of *B. mori* after the termination of the diapause period: (1) (stage C1) Late period of cold storage: the cephalic lobe is bigger than the caudal one. It is quite rounded and smooth; segments are not well-differentiated. At this point the eggs are put into incubation, corresponding to about 40h PO in the Nistari polyvoltine strain (2) (stage C2): Elongation of the germ band; head lobe (protocephalon-gnathal parts) largely develops, about 24h after incubation beginning, corresponding to about 48h PO in the Nistari strain; (3) (stage D1-D2) Complete segmentation of the mesoderm; neural groove and body segments can be clearly observed; corresponding to about 72h PO in the Nistari strain; (4) (stage E1-E2) Development of the appendages at protocephalon (labial and antennary), gnathal (mandibles, maxillae, labium) and thoracic region occurs, then differentiation of the appendages at the abdominal region occurs. Invagination of the proctodeum and the trachea, and differentiation of protocephalon and gnathal and thoracic segments, where the head lobe becomes shorter, take place; four anterior segments start movement to form the head (morphogenetic movement of cephalo-gnathal region begins to form the head) corresponding to 96h PO in the Nistari strain; (5a, b) (stage E3; F1;) the body is greatly shortened and enlarged. Beginning of blastokinesis movement (a). Abdominal end flexes to reach the level of the prothorax and blastokinesis is completed (b); corresponding to 120h PO in the Nistari strain (6) (stage F2) External processes are formed: head parts as mouth, antennae, eyes; and thoracic legs are segmented and pointed. Abdominal appendages and telson are completed, the alimentary canal is formed; corresponding to 144h PO in the Nistari strain (7-8) (stage F3) Taenidium in spiral band is formed in the tracheal tube; trachea are coloured. Corresponding to 168h PO in Nistari strain (9) (stage F4) The egg turns deep brown and black spots may be seen through the eggshells. Amnion, serosa and vitelline are swallowed by the embryo itself, together with the yolk; therefore, there are some pigments in the foregut, but the embryo surface is not coloured yet. Corresponding to 192h PO in the Nistari strain. (10 not shown) (stage F4) The larva is formed; the whole body is coloured. Corresponding to 216h PO in the Nistari strain. Scale Bar 20 µm. h PO = hours post oviposition.

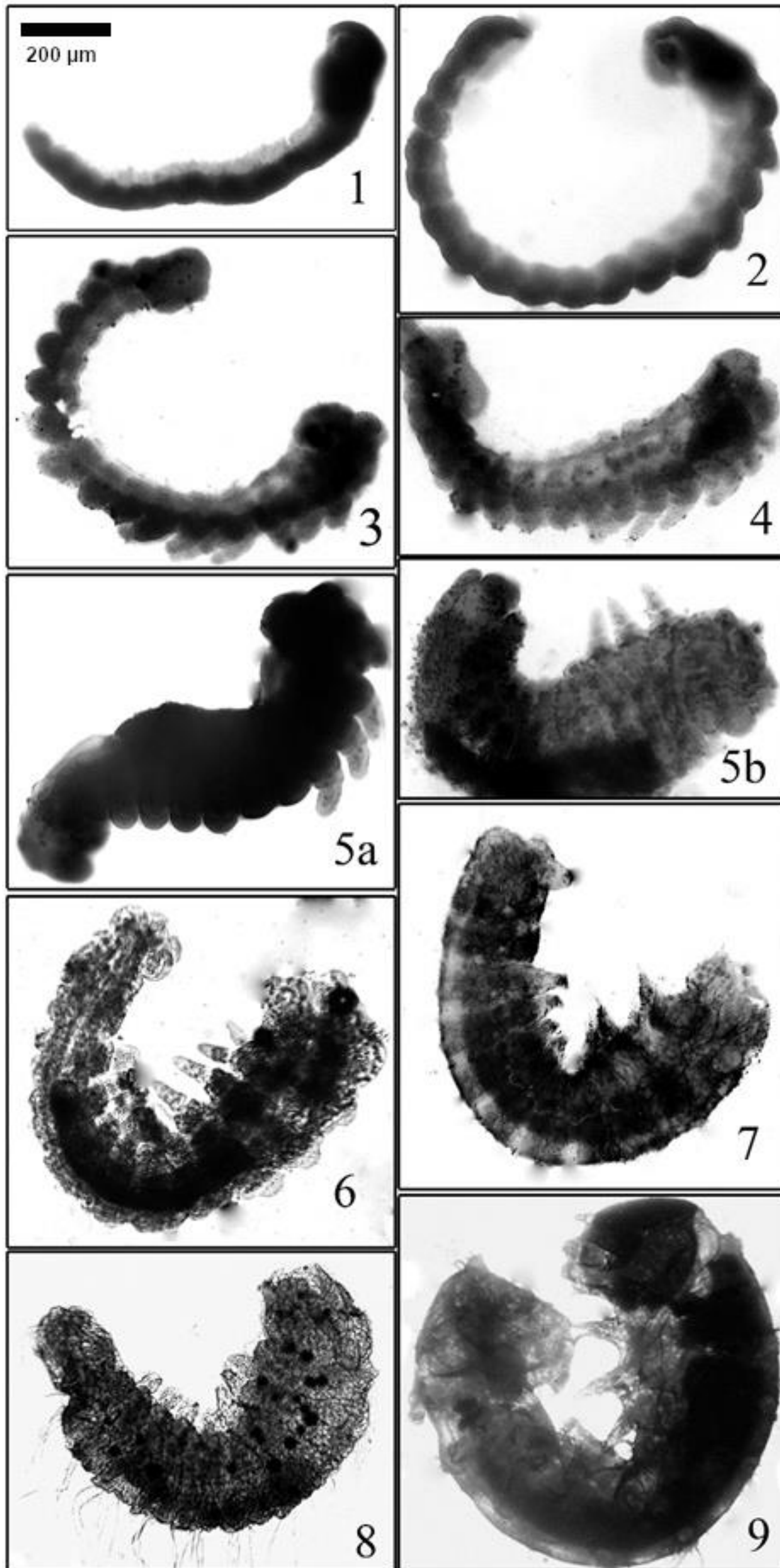

Figure 1. (see previous page for caption).

stages is not the same for the different temperatures; in fact, as it is possible to observe in table 1, the 22nd h PO corresponds to the best developmental stage to preserve embryos at 5 °C (i.e. just before the formation of the germ band), while the best period to preserve eggs at 0 °C is between the 38th and the 40th h PO, corresponding to the entrance in diapause of the monovoltine strains. We recorded the same behaviour in the case of the prolongation of the period of preservation: with two month preservation, the best hatching rate is obtained in cases where the chilling begins between the 38th and the 40th h PO (table 1).

Cryopreservation

Dechorionation and permeabilization

In table 2 data that have been separately analysed for the different SH concentrations and have undergone two-way ANOVA are reported. The analysis demonstrated that the treatments were significantly different in terms of concentrations, treatment durations and embryo stages. In addition, the interaction between the factor "hrs" and the factor "t" was significant for both the concentrations ($F_{2,12}$ = 9.747, P < 0.001 for SH = 2.5%; $F_{2,12}$ = 14.315, P < 0.001 for SH = 1.25%).

The permeabilization treatment, when applied at 1.25% SH concentration and to the embryonic stage of 120h PO in diapausing eggs, caused a remarkable decrease in hatching in relation to an increase of the treatment time (from 83% to 55% hatching rate respectively, for 10 and 15 min) (table 2). The same treatment carried out at a later embryonic stage of 168h PO did not vary the hatching rate in the case of 10 min, while reduced considerably the hatching rate to 15% in the case of 15 min treatment and increased the hole size (table 2). A higher concentration of NaOCl in the permeabilization solution from 1.25% to 2.5%, led to an increased mortality calculated as a reduction of the hatching percentage. The hatching rate at 120h PO resulted in 0 and 69.33% for 10 and 5 min treatment time respectively (table 2), while at the embryonic stage of 168h PO, the same duration of treatments resulted in a hatching rate of 0 and 38.33% respectively (table 2). For this experiment, carried out on monovoltine diapausing strains, developmental hours were chosen towards the end of the development because it was carried out as a first exploration activity.

As shown in table 2 and confirmed by the Tukey's test, for both the SH concentrations, the increase in the age of the embryo was significant in terms of worsening of the hatching rate; on the other hand, we recorded an adverse effect with the enhancement of the treatment time, as it could be expected.

On the basis of the previous results, the non-diapausing Nistari eggs, were treated at the two embryonic stages of 40 and 60h PO with 3% SH for 2 min (figure 2A and 2D), 4 min (figure 2B and 2E), and 6 min (figure 2C and 2F); hatching rates are reported in figure 3 and analysed by means of two-way ANOVA (main factors: "developmental stage" and "exposition time to SH"). Only the main factor, "exposition time to SH", exhibited significant differences ($F_{3,24}$ = 68.148, P < 0.001) while the differences among "developmental stages", and the interaction between the two main factors were not significant.

Toxicity of cryoprotectants during vitrification

Cryopreservation procedure was applied to the embryonic stages of 24 and 40h PO (figure 4) in the polyvoltine eggs. The two different stages were treated with IPA and HX, for 15 and 45 seconds respectively, as described in materials and methods.

The difference between the two tested embryonic stages was highly significant ($F_{1,56}$ = 277.312, P < 0.001). Cryopreservation treatments were also significantly different ($F_{3,56}$ = 1186.181, P < 0.001) (figure 4). The interaction between the embryonic stages and cryo-treatments was highly significant ($F_{3,56}$ = 95.176, P < 0.001).

There was a significant difference between the control and treated eggs; no significant differences were recorded between D/P and L/D treatments within the same embryonic developmental stage, but there was between the two treatments.

Data showed a significant difference among stages, in particular the embryos of 40h PO had a higher hatching rate than those of 24h PO; nevertheless, surprisingly, a very low but encouraging embryo survival after LN (0.1%) treatment was obtained for 24h PO eggs treated under a longer exposure time for the permeabilization procedure.

Table 2. Hatching percentages (%) of monovoltine polyhybrid *B. mori* eggs after the dechorionation and permeabilization treatment. Polyhybrid eggs (at stages corresponding to 120 and 168h PO in polyvoltine strains) have been exposed to SH at different concentrations for a period of 5, 10 or 15 minutes (t) and at two SH concentrations. The size of the holes produced by the treatments is also reported. Different letters indicate significant differences among means according to Tukey's post-hoc test, P < 0.05. h PO = hours post oviposition; SH = sodium hypochlorite; CTRL = control; Ø = hole diameter; t = exposition time to SH; hatch. = hatching; SE = standard error.

SH concentration (%)							
2.5				1.25			
h PO	t (min.)	hatch. ± SE (%)	Ø ± SE (µm)	h PO	t (min.)	hatch. ± SE (%)	Ø ± SE (µm)
120	5	69.33 ± 6.39 b	1.27 ± 0.07 c	120	10	83.00 ± 4.62 a	1.21 ± 0.06 c
	10	0.00 ± 0.00 d	1.35 ± 0.08 c		15	55.00 ± 4.04 b	1.91 ± 0.08 b
	CTRL	89.16 ± 4.47 a	-		CTRL	89.16 ± 4.47 a	-
168	5	38.33 ± 1.76 c	1.72 ± 0.03 b	168	10	83.33 ± 2.60 a	1.30 ± 0.01 c
	10	0.00 ± 0.00 d	2.27 ± 0.09 a		15	15.67 ± 0.88 b	2.25 ± 0.07 a
	CTRL	95.06 ± 1.57 a	-		CTRL	95.06 ± 1.57 a	-

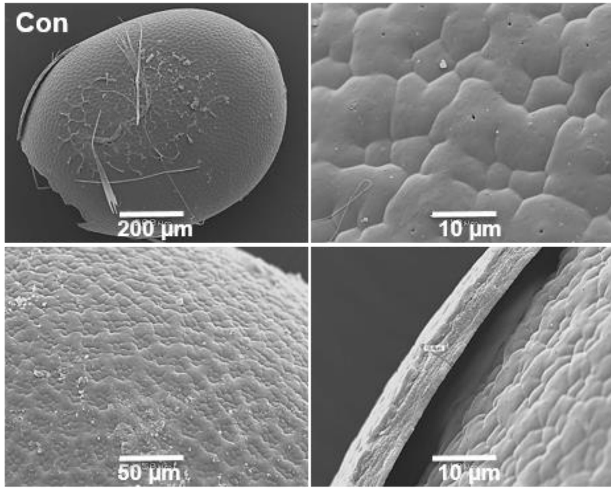

Figure 2. Scanning electronic microscopic images of the eggs of a polyhybrid diapausing strain in *B. mori*, which underwent SH treatment. SH caused enlarging of the holes at the aeropyle level; different concentrations of SH and various egg exposure times resulted in a different hole size of aeropyles as shown in table 2.
Scale Bar: 200, 50, and 10 µm. Letters refer to different egg treatments (see materials and methods); Con = Control; SH = sodium hypochlorite.

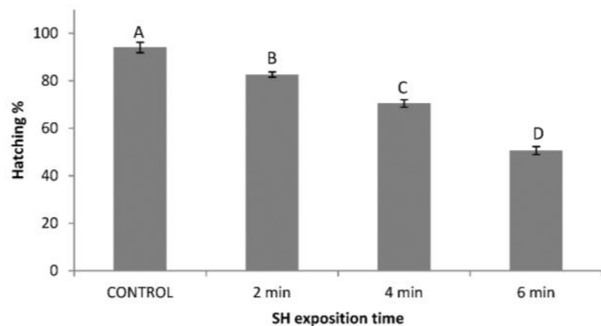

Figure 3. Polyvoltine Nistari eggs hatching percentages (mean ± SE) after treatment with SH (sodium hypochlorite). Eggs at two developmental stages (40 and 60h PO = hours post oviposition) were treated with 3% SH to achieve dechorionation. Different letters indicate significant differences among means according to Tukey's test with $P < 0.05$.

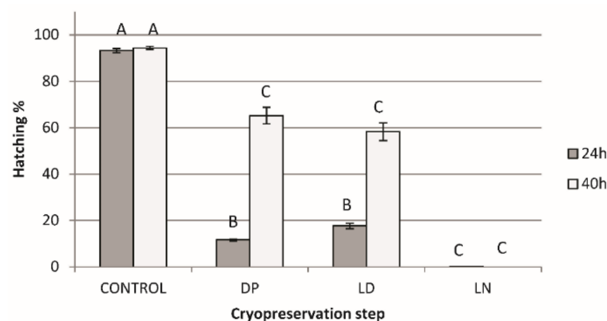

Figure 4. Hatching percentages of *B. mori* polyvoltine Nistari eggs at different steps of the cryopreservation procedure (mean ± SE). Cryopreservation steps were applied to eggs at two different developmental stages (24 and 40 hours). Two-way ANOVA highlighted significant differences for main factors and for the interaction between them (hours and cryopreservation steps). Different letters indicate significant differences among means according to Tukey post-hoc test with $P < 0.05$. DP: dechorionation/permeabilization step, LD: loading/dehydration step, LN: immersion in liquid nitrogen.

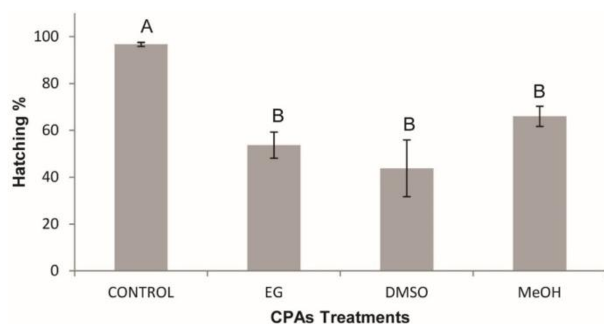

Figure 5. Hatching percentages (mean ± SE) of monovoltine *B. mori* eggs after overwintering and overcoming diapause following treatments with different cryoprotectant agents (CPAs) (EG = ethylene glycol, DMSO = dimethylsulfoxide, MeOH = methanol). ANOVA highlights that the only significant difference is in comparison to the control.

Overwintering effect

Overwintering diapausing *B. mori* eggs were related to the loading and dehydration procedures in the experiment (figure 5). ANOVA highlighted a significant difference between the control and the treatments ($F_{3,32}$ = 13.367; $P < 0.001$), which showed no differences among them. If we compare figure 4 and 5, it is very clear that there are no differences between the effect of cryoprotectants on polyvoltine eggs at the 40h PO and on the monovoltine ones, after diapause overcoming.

Discussion and conclusions

Chilling sensitivity is one of the most important indices to identify tolerance of embryonic stage to cold exposure. Usually, the rate of chill injury increases dramatically with the decrease in the environmental temperature, as shown in honey bee embryos (Collins and Mazur, 2006).

In the first 24-30 hours of embryonic growth, the silkworm development is identical in diapausing and non-diapausing eggs. At around 30-40h PO, the eggs of monovoltine strains enter pre-diapause, and their metabolic pathway differs from that of polyvoltine eggs because of their need to survive in low winter temperatures. Nevertheless, our experiments demonstrated that polyvoltine eggs could also cope with low temperatures to a certain extent. The resistance of the Nistari eggs to 5 °C was not surprising, although they are usually reared in tropical and sub-tropical zones; in fact, when the expected date of hatching must be postponed, it is customary that experienced silkworm rearers cold-store the eggs at 5 °C even for two weeks or more.

The results of table 1 indicate that the polyvoltine Nistari eggs are very sensitive to low temperature at the very beginning of their developmental process. The 2nd hour after laying is a critical point with regard to cold hardiness. In fact, the fertilization occurs about two hours after oviposition, with the fusion of the pronuclei and immediate initiation of mitosis; this process is repeated about 10 times in the first 10 hours after oviposition (Ohtsuki and Murakami, 1968). The cellularization process of the blastoderm stage is probably damaged by a low temperature, according to the observations performed in *Musca domestica* L. (Strong-Gunderson and Leopold, 1989; Leopold, 2000). Low temperature affects spindle fibre development, and, therefore, chromosomes form masses of irregular shape during the early embryonic mitosis (Kawamura, 1978). In addition, very early stages are unable to systematize the regulation of oxygen flux and the water balance during low temperature conditions because they have neither the sensory organs for monitoring water and oxygen status, nor the physiological systems for exerting control over them (Woods, 2010). The tolerance rate in later stages of the first days of development may be due to sorbitol, which can be found in the early embryonic stages of non-diapausing silkworm eggs only (Takahashi *et al.*, 1971).

On the other hand, our previous research (data not shown) has proven that later stages than those tested in

this experiment suffered more than the developmental stages considered in table 1 for cold preservation (20-42h PO).

In our experiments, there is a clear relationship between the number of days of cold treatment and the mortality rate (table 1). Nevertheless, it is possible to observe a hatching rate higher than 75% in certain stages of the polyvoltine silkworm eggs maintained at a temperature ranging from 0 to 5 °C for a month, which is quite good for a strain of *B. mori* adapted to a warm environment. It is noteworthy in this regard that non-diapausing eggs subjected to chilling from the first day after oviposition until stages even later than that corresponding to entry into diapause (i.e. 72h PO), showed a significant accumulation of sorbitol and glycerol in comparison to eggs maintained at 25 °C and 20 °C (Furusawa *et al*., 1987).

The cryopreservation experiment showed a significant difference between the two stages (24 and 40h PO embryos) mostly due to the different results between D/P and L/D treatments; in fact, 40h PO embryos resulted in being more resistant to chemical toxicity. However, the survival rate after LN treatment was zero; surprisingly the 24h PO stage treated with a high permeabilization time recorded 0.1% survival. The difficulty of removing even partially the thick layer of chorion (about 20 µm) was the main factor that prevented sufficient permeability. Therefore, to make the eggshell partially permeable is the prerequisite to obtain hatching after cryopreservation, and this seems to be more important even than chemical toxicity by cryoprotectants.

We think that acclimation of non-diapausing eggs might lower their supercooling point and decrease the possibility of ice formation at freezing temperatures. In addition, a cold-acclimation period of non-diapausing eggs before treatments may help polyol content to increase (Kageyama and Ohnishi, 1973); in this case, however, there is a difference between diapausing and non-diapausing eggs in the biochemical adaptation mechanism against low temperature, especially during chilling at 1 °C and below 10 °C respectively (Adachi *et al*., 1999). Acclimation was successfully used in eggs of many species, such as the *Ochlerotatus albifasciatus* Macquart (Campos, 2008), *Simulium ornatum* Meigen (Goll *et al*., 1989), and the migratory locust *Locusta migratoria* L. (Jing and Kang, 2003).

Nevertheless, if acclimation might be a resource to vary the chemical composition in non-diapausing eggs, a big problem arises for monovoltine eggs. In fact, when we treat monovoltine eggs with cryoprotectants at their diapause entry, they should remain for at least three months at a temperature of about 5 °C; this period is necessary to overcome their diapause period, before accomplishing their further development. It is clear that the damage caused to the chorion by the chemical agents could make it impossible for the embryos to survive for three months in cold storage, and then to resume their growth until hatching.

The test performed on the monovoltine eggs at the diapauses termination showed good hatching results after the cryopreservation treatments, confirming the data previously obtained with polyvoltine eggs. As hatching results are comparable it means that we did not obtain any improvement after a three-month storage at 5 °C (acclimation period). In fact, no eggs survived the LN treatment. This is very clearly based on the consideration that from the diapause termination, the behaviour of monovoltine and polyvoltine eggs is the same.

Thus, even though the mechanisms involved in rapid cold hardening are still partly unknown (Bale, 2002), the negative effects at the later stages of embryogenesis seem to be induced by weakening of the embryos rather than to be due to specific freezing damages to their tissues and organs. In fact, during the later stages, the eggs consume more oxygen for their morphogenesis, and with the development of the tracheal system, a remarkable metabolic progress appears, which is highly affected by the exposure to low temperatures. Chaturvedi and Upadhyay (1990) found that the hatchability of eggs at various embryonic stages from 48 hours after oviposition gradually decreased when they were cold-stored for more than five days. On the other hand, it is likely that in 24h PO embryos the metabolic activity of oxygen uptake is at minimum levels, so that the exposure to low temperature does not affect eggs development (Yaginuma and Yamashita, 1999).

Whatever the physiological and biochemical mechanisms involved in the rapid cold hardening are, the resistance to 0 °C and 5 °C of polyvoltine eggs in our experiments was remarkable; in fact, from the best tolerant embryonic stage (24h PO), high-performing adults developed (data not shown) as in the case of cold exposure to 5 °C of 3h house fly embryos (Leopold, 2000). Therefore, the results of our research, although being considered only preliminary, represent one of the most reasonable approaches to the cryopreservation technique of *B. mori*.

Acknowledgements

The authors are grateful to Jacopo de Silva (CREA-DC, Florence) for assistance in performing the statistical analysis of the data. They also acknowledge Claudia Benvenuti (CREA-DC, Florence) for assistance in performing the photos under the electronic microscope SEM.

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Received February 6, 2019. Accepted September 30, 2019.