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Effects of chilling and cryoprotectants on glycans in shrimp embryos

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ABSTRACT

Glycans are carbohydrates present in every organism that bind to specific molecules such as lectins, a diverse group of proteins. Glycans are vital to cell proliferation and protein trafficking. In addition, embryogenesis is a critical phase in the development of marine organisms. This study investigated the effects of chilling and cryoprotective agents (CPAs) on glycans in the embryos of *Stenopus hispidus*. The glycan profiles of embryos of *S. hispidus* at the heartbeat stage were analyzed using lectin arrays. The results of analyses revealed that mannose was the most abundant glycan in the *S. hispidus* embryos; mannose is crucial to cell proliferation, providing the energy required for embryonic growth. Additionally, the results reveled that chilling altered the content of several glycans, including fucose and Gla-GlcNAc. Chilling may promote monosaccharide accumulation, facilitating osmotic regulation of cells and signal molecules to aid *S. hispidus* embryos in adapting to cold conditions. Changes were also observed in the lectins NPA, orysata, PALa, ASA, discoidin II, discoidin I, UDA, PA-IIL, and PHA-P after the samples were treated with different CPAs. DMSO may minimize cell damage during exposure to chilling by preserving cell structures, membrane properties, and functions. The present study is the first to investigate the profiles and functions of glycans in shrimp embryos subjected to low-temperature injuries. This study enhances the understanding of cell reproduction during embryogenesis and provides valuable information for the study of glycans in embryos.

1. Introduction

Chilling involves exposure to temperatures above the freezing point that are low enough to cool a sample [24]. However, cooling at low temperatures applied too rapidly or slowly may lead to chilling injury or cold shock [31,41]. Cell structures and biochemical processes are highly sensitive to low temperatures, which can adversely affect membranes, selective permeability, transport processes, metabolic activities, and protein denaturation [28,50,79]. Various species have been studied with respect to their chilling sensitivity, including the embryos of carp (*Cyprinus carpio* [11]), red drums (*Sciaenopus ocellatus* [18]), goldfish (*Carassius auratus* [36]), and medakas (*Oryzias latipes* [70]), as have the ovarian follicles of zebrafish (*D. rerio* [68]). However, few studies have focused on crustacean species. Experiments on chilling of *Stenopus hispidus*, *Trachypenaeus byrdi*, and *Penaeus stylirostris* embryos have revealed that embryos in the early developmental stage are more sensitive to chilling than are those in the late stage [3,33]). Weineck et al., 2018 [79] studied the effects of chilling on the adult shrimp *Procambarus clarkia* and *Litopenaeus vannamei*, discovering that their embryos were more sensitive than those of the crab *Callinectes sapidus* to chilling.

Glycans are structurally diverse molecules that evolve rapidly [72]. They are as common in nature as nucleic acids, proteins, lipids, and metabolites [38] and are essential to the existence of all living organisms [71]. Glycans are synthesized in the Golgi and endoplasmic reticulum (ER) [12] and feature prominently in the extracellular matrix and secreted soluble molecules [73]. Glycan structures form linearly as

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branches of carbohydrate units such as monosaccharides, disaccharides, and oligosaccharides [14]. The most common glycan structures in eukaryotic cells are N-linked glycans, which are associated with cell surfaces [46,58,80]. N-linked glycans comprise mannose, galactose, lactose, fucose, rhamnose, N-acetyl glucosamine, and N-acetyl galactosamine [46]. Glycan is vital to cell proliferation and protein trafficking in addition to cell recognition, signaling, defense, and immune functions [1,10,42,53,74,77,78]. Glycan structures also bind to molecules such as proteins and lipids. The current study focused on glycans that bind to the protein lectin [27], analyzing lectin expression to investigate how glycans respond to low temperatures in aquatic species.

Aquatic animals have complex glycans [1]. The major classes of glycan that have been identified in aquatic animals are L-rhamnose, D-galactose, α-galactoside, D-galacturonic acid, and fucose. The following aquatic organisms accumulate these glycans in their eggs and embryos: In their study, Huang et al. (2018) [22] discovered that mannose has the ability to impede the growth of microorganisms and expedite the elimination of bacteria in giant prawns (*Macrobrachium rosenbergii*). In addition, mannose and L-rhamnose contribute to the immune system in turbot (*Scophthalmus maximus* L. [15]), chum salmon (*Oncorhynchus keta* [44]), shishamo smelt (*Osmerus lanceolatus* [21]), and steelhead trout (*Oncorhynchus mykiss* [63]). D-galactose, α-galactoside, and D-galacturonic acid play a vital role in the antifungal and antibacterial activities observed in sea urchins (*Anthocidaris crassispina* [47]), catfish (*Silurus asotus* [60,61]), and sea hare slugs (*Aplysia kurodai* [63]). Moreover, research has demonstrated that D-galactose and D-galacturonic acid have the ability to inhibit the growth of tumors in sea hare embryos (*A. kurodai* [63]). Fucose, a significant glycan, had a crucial role in the process of agglutinating erythrocytes in the egg of sea bass (*Dicentrarchus labrax* L. [49]).

Glycans are sensitive and easily affected by chemical and environmental factors such as temperature and pH [6,26,62]. For example, Wang et al., 2018 [76] studied the effects of low-temperature stress on *Jatropha curcas* seedlings and demonstrated that at 12 °C, *J. curcas* exhibits resists chilling stress by reducing cellular osmotic potential through starch hydrolysis and accumulation of large quantities of soluble sugars (sucrose, fructose, glucose, maltose, and raffinose). A study on spinach (*Spinacia oleracea)* demonstrated that cold stress at 4 °C–7 °C markedly increased the presence of soluble sugars, free amino acids, and vitamin C in the spinach [82]. However, studies on the effects of chilling on lectin in aquatic cryopreservation are lacking.

One of the most widely studied aquatic species is *S. hispidus,* commonly known as banded coral shrimp, cleaner shrimp, or boxer shrimp because of its color and hardness [5]. These shrimp occur in the Indo-Pacific region, the Red Sea, and the Western Atlantic Ocean [20] and inhabit various shallow reef habitats [19]. Adults are typically found in mating pairs in coral reefs [9,23]. In addition, they are often kept in tanks because they are visually attractive and perform beneficial cleaning and scavenging in aquariums [75]. Cleaner shrimp can reduce the prevalence of parasites in their hosts, which are most frequently corals and anemones [39]. Moreover, these shrimps are common in the marine aquarium trade and the ornamental market because of their beautiful, bright coloration [16]. They are widely collected despite concerns that reductions in their numbers could harm reef ecosystems [75]. Reducing reef fishing is an essential strategy for preserving this species; however, alternative preservation strategies must also be explored. Since 1995, researchers have developed technologies for cultivating decapod species, including *S. hispidus*. However, few attempts at cultivating *S. hispidus* have been successful [13,35,47].

Embryogenesis is essential to the development of cell structures and is vital to cell functions [51]. Cryopreservation of embryos or larvae is an efficient method that could reduce the need for wild collection [33]. The current study screened the glycan profiles of *S. hispidus* and investigated the effects of chilling and cryoprotective agents (CPAs) on glycans in *S. hispidus* embryos to aid in developing techniques for embryo cryopreservation.

2. Materials and methods

2.1. Shrimp embryo collection

A total of 9 pairs were kept in 9 tanks with a flow-through seawater system. The water temperature, salinity, nitrate concentration, and pH were maintained at 26 °C ± 1.8 °C, 35.2 % ± 1.6 %, 5 ± 1.8 mg/L, and 8.0 ± 0.3, respectively. The tanks were maintained in a cycle of 10 h of light/14 h of darkness. The *S. hispidus* were fed frozen Antarctic krill twice a day, in the morning and evening at a rate of 5–10 % body weight. Insemination began naturally in the tank; embryos were spawned 15–25 min after fertilization, which lasted approximately 10 min before the female deposited a blue-green egg mass on the swimmerets. The embryo clusters were then removed from the female abdomen, collected in a 1-L beaker with seawater, and transferred to the laboratory for observation of embryonic development. Initially, clusters of embryos were separated into individual embryos using an enzymatic technique, as described by Lin and Tsai (2012) [30]. The embryonic cluster was exposed to a solution of trypsin at a concentration of 0.4 mg/mL for a duration of 10 min. This process took place in a plastic culture dish (Alpha Plus, Taiwan) at room temperature. The embryos were isolated using continuous pipetting until the embryo cluster was fully dispersed into individual embryos, and further rinsed three times with filtered seawater (FSW). Subsequently, the embryos were placed in a plastic culture dish and allowed to develop until the stage where the heartbeat is identified, which occurred after 240 h of spawning at room temperature. The stages of embryonic development was based on the classification proposed by Tsai and Lin in 2009 [67]. The embryos were collected in separate culture dishes with FSW for subsequent experiments.

2.2. Chilling experiments

A pool of 50 μL embryos in the heartbeat stage was incubated in a 1.5-mL Eppendorf tube with FSW, 1 M methanol (Sigma–Aldrich, St. Louis, MO, USA), 0.5 M ethylene glycol (EG, JT Baker, Phillipsburg, NJ, USA), and 0.5 M dimethyl sulfoxide (DMSO; Sigma–Aldrich) and then placed in a controlled-rate freezer (CLUBIO cb-1502, Medclub, Taoyuan, Taiwan) at 5 °C for 8 or 16 h. All CPAs were prepared at room temperature with no equilibration time. At the end of the estimated time, the embryos with CPAs were washed 3 times with FSW. The control samples were incubated at room temperature (26 °C) for 0, 8, or 16 h. At the end of the incubation period, the samples were stored in a −20 °C freezer with sample labels for further lectin array experiments.

2.3. Protein extraction

Radioimmunoprecipitation assay buffer (RIPA buffer; Thermo Fisher Scientific) and 4 % protease inhibitor (PI; Thermo Fisher Scientific) were prepared in 550 μL and 22 μL solutions, respectively, in a 0.5-mL microcentrifuge tube (Scientific Specialties, Lodi, CA, USA) with 150 g of glass beads (425–600 μm, Sigma–Aldrich). A total of 50 μL of previously prepared samples was then added. The samples were simultaneously disrupted and homogenized with high-speed shaking for 5 min (TissueLyser LT, Qiagen, Venlo, the Netherlands). The supernatant solution was removed, and the sample was centrifuged at 21500 g at 4 °C for 5 min. Next, 550 μL of 20 % trichloroacetic acid (Sigma–Aldrich) was added and incubated overnight at 4 °C. The sample was rinsed in a 100-μL combination of 99.5 % acetone (Merck, Darmstadt, Germany) and 0.1 % dithiothreitol (GE Healthcare Life Sciences, München, Germany), and then centrifuged at 21500 g at 4 °C for 5 min. This procedure was performed for 3 replicates to ensure the sample was completely devoid of contaminants. Finally, the samples were centrifuged again using 21500 g at 4 °C for 5 min with 1 mL of 99.5 % acetone and were dried using a vacuum dryer (Napco 5831, National Appliance Company, Portland, OR, USA) for 30 min.

2.4. Protein concentration detection

Qubit Protein Assay Kits (500 assays; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used in accordance with the manufacturer's instructions. The dried sample was vortexed (Genie 2, Scientific Industries, Bohemia, NY, USA) and dissolved in 100 μL of 1 × sample buffer with 4 μL of PI (Thermo Fisher Scientific) to detect protein concentrations. After, 10 μL of the previously centrifuged and diluted sample was mixed with dissolved water in a 1:19 ratio. Subsequently, the 10-μL samples were added to 190 μL of working solution. The working solution was a mixture of Qubit (Thermo Fisher Scientific) and fluorescence (Thermo Fisher Scientific) at 199:1 ratio. The fluorescence intensity (FI) was measured using a Qubit 4 fluorometer (Thermo Fisher Scientific), and protein concentrations were extrapolated from the standard fluorescence curve.

2.5. Lectin array

S. hispidus embryos at the heartbeat stage at different incubation times under temperatures of 26 °C or 5 °C and subjected to different CPA treatments were assessed in terms of protein concentration and glycan profiles. A lectin array was obtained using a RayBiotech Lectin Array 70 (RayBiotech, Peachtree Corners, GA, USA), fluorescence, and GenePix microarray systems (Molecular devices, San Jese, CA, USA) according to the manufacturers' instructions. The Cy3 equivalent dye detection method was used to group carbohydrate glycans into the following 9 groups: 1) Gal (D-Galactose), 2) Gal-GalNAc (D-Galactose-N-Acetylglactosamine), 3) Gal-GlcNAc (D-Galactose-N-Acetylglucosamine), 4) GalNAc (N-Acetylglactosamine), 5) GlcNAc (N-Acetylglucosamine), 6) Fucose (L-Fucose), 7) Mannose, 8) LacNAc (Lactose), and 9) other glycans. In this process, the sample was first dialyzed with a 10 × dialysis buffer (a combination of a phosphate-buffered saline buffer [Sigma–Aldrich] and distilled water) at a ratio of 1:9. The sample was dialyzed using an orbital shaker (Major Science, Taoyuan, Taiwan) at 4 °C for 3 h. A 10 × dialysis buffer was changed after 3 h, with 3 replicates used during the procedure. Second, the sample was labeled using 3.3 μL of a 1 × labeling reagent solution. The mixture was incubated at room temperature for 30 min, with the tube tapped gently every 5 min to promote thorough mixing and ensure the chemicals underwent the optimal chemical reaction. Next, 3 μL of stop solution was added to the sample and immediately repeatedly dialyzed in a 10 × dialysis buffer. Third, the previously prepared lectin array was assembled using a glass slide, a silicone gasket, an incubation chamber, and 2 snap-on slides. The glass slide was maintained at room temperature for 30 min with a plastic cover used to prevent contamination from dirt. After 30 min, the glass slide was removed from the plastic cover, and the mixture was allowed to air dry at room temperature for 2 h. The sample was blocked and incubated, and 100 μL of the sample (consisting of 20 μL of the previously prepared dialyzed sample and 80 μL of a 10 × sample dilution) was transferred into each well of the glass slide and incubated at room temperature for 30 min for blocking. The glass slide was maintained overnight at 4 °C and covered with a glass slide cover film. The sample was decanted, and 150 μL of 1 × wash buffer I was added to each well. The glass slide was then placed in an orbital shaker for 5 min with 5 replicates for washing; the slide was washed twice for 15 min with a 1 × wash buffer, with the buffer carefully removed after each wash. The sample was then washed with 150 μL of 1 × wash buffer II and subjected to gentle shaking for 5 min. After, the sample was incubated with Cy3 equivalent dye-streptavidin, and 80 μL of prepared Cy3 (1400 μL of sample diluent added to Cy3) was loaded into each well of the glass slide, which was placed on a solid box to avoid exposure to light and was allowed to shake for 1 h. The sample was decanted from the wells and washed in 150 μL of 1 × wash buffer I for 5 min with gentle shaking, with the washing procedure repeated for the 5 replicates. Finally, the glass slide was disassembled from the incubation chamber by carefully pulling the snap-on sides from the bottom to the upper side without touching the surface of the glass slide. The glass slide was transferred into a container with 1 × wash buffer I or II to avoid exposure to light and was gently shaken for 15 and 5 min. The glass slide was allowed to dry completely, after which the data were analyzed using a microarray laser scanner (GenePix, PerkinElmer), as suggested by RayBiotech.

2.6. Statistical analysis

Statistical analyses were conducted using Microsoft Excel and SPSS version 17.0 (SPSS, Chicago, IL, USA). The normality of the data was assessed using the one-sample Kolmogorov–Smirnov test. A one-way analysis of variance was conducted to assess significant differences in the mean values for the samples subjected to different experimental treatments. If the differences did not meet the homogeneity requirements of Levene's test, Welch's test was performed, followed by the Games–Howell post hoc test.

3. Results

3.1. Glycan profiles at room temperature at various times

The FI for the heartbeat stage of *S. hispidus* at 0, 8, and 16 h of incubation at room temperature is presented in Table 1. The table reveals that mannose was the most abundant glycan, with 14 of the 15 lectins with a carbohydrate specificity to mannose. The second most abundant glycan FIs were glycans from the "other" group, with expressions of malectin and PSL1a being noted. Additionally, only 12 lectins were expressed from the groups of galactose, GlcNAc, GalNAc, fucose, and Gal-GlcNAc; these comprised discoidin II, UDA, STL, VVA, PA-IIL, discoidin I, CGL2, GS-I RS-Fuc, PHAP-P, ECA, and Lotus. Gal-GalNAc and LacNAc did not appear in the heartbeat stage of *S. hispidus*.

3.2. Effect of chilling on glycans at various times

Fig. 1 presents a comparison of the glycan profiles during chilling (5 °C) with those at room temperature (26 °C) at the exposure times of 0, 8, and 16 h. The results reveal that the glycan FI did not differ significantly ($P > 0.05$) between 5 °C and 26 °C at different exposure times for mannose (Fig. 1a), galactose (Fig. 1b), GlcNAc (Fig. 1c), or GalNAc (Fig. 1d). By contrast, the glycan FIs in fucose (Fig. 1e) and Gal-GlcNAc (Fig. 1f) at 5 °C differed significantly between exposure times ($P < 0.05$). Moreover, no significant differences in glycan FI were identified for different exposure times at 5 °C and 26 °C for the glycans in the "other" group (Fig. 1g).

Fig. 2 presents the FI results for individual lectin expression of the glycans under 5 °C and 26 °C at 0, 8, and 16 h. GNA (Fig. 2a), GRFT (Fig. 2b), and HHA (Fig. 2c) exhibited no significant differences in FI at different exposure times at 5 °C and 26 °C. However, at 5 °C, the FI of LcH (Fig. 2d) significantly decreased at 8 h then recovered at 16 h ($P < 0.05$); the FI differed significantly at 16 h under 5 °C and 26 °C ($P < 0.05$). The results were similar for lentil (Fig. 2e); under 5 °C and 26 °C, the FI decreased at 8 h and then increased at 16 h. However, no significant differences in the lectin FIs were observed at any temperature or time for NPA (Fig. 2f), orysata (Fig. 2g), PALa (Fig. 2h), or PSA (Fig. 2i). The FI of ASA (Fig. 2j) at 5 °C significantly differed at 0 and 16 h ($P < 0.05$), but no significant difference between exposure times was noted at 26 °C. This finding was similar to that regarding the FI for banlec (Fig. 2k) at both temperatures and for BC2L-A at 26 °C (Fig. 2l). However, the FI of BC2L-A at 5 °C significantly decreased at 8 h ($P < 0.05$). Calsepa (Fig. 2m), Con A (Fig. 2n), CGL2 (Fig. 2o), and discoidin II (Fig. 2p) exhibited no significant differences in FI at any exposure time at either temperature. Exposure time significantly affected the FI of STL (Fig. 2q) at both temperatures ($P < 0.05$). UDA (Fig. 2r), PA-IIL (Fig. 2s), and RS-Fuc (Fig. 2t) exhibited no differences in FI at any time or temperature. The FI in discoidin I (Fig. 2u) gradually decreased at the temperature of 26 °C. In addition, no differences in FI were observed in

Table 1
Glycan profiles of *S. hispidus* at the heartbeat stage at room temperature. Different colors correspond to varying levels of fluorescence intensity.

Glycan	Lectin	0h	8h	16h	Glycan	Lectin	0h	8h	16h
Mannose	GNA	268848	235315	259128	GalNAc	CNL	0	0	0
	GRFT	316363	406856	355249		DBA	0	0	0
	HHA	158974	114087	159720		Discoidin I	18253	8962	2380
	LcH	4824	0	3524		LBA	0	0	0
	Lentil	42451	23333	63604		PTL	0	0	0
	NPA	149121	113236	149716		SAMB	0	0	0
	Orysata	183648	182144	232609		SBA	0	0	0
	PALa	27159	5977	34684		SJA	0	0	0
	PSA	37751	16297	57630		SNA-I	0	0	0
	VFA	0	0	0		SNA-II	0	0	0
	ASA	287339	254382	289200		VVA	13822	12815	19564
	BanLec	322930	323946	381016		WFA	0	0	0
	BC2L-A	247738	189604	233120		**Total sum**	32075	21777	21944
	Calsepa	0	19867	3261	Gal-GlcNAc	PHA-E	0	0	0
	Con A	211066	175751	239846		PHA-L	0	0	0
	Total sum	2258212	2060792	2462306		PHA-P	4598	3326	2251
Galactose	CGL2	2496	7328	10177		MAA	0	0	0
	Discoidin II	61333	83151	117051		ECA	0	0	3986
	EEL	0	0	0		MOA	0	0	0
	Gal1	0	0	0		Gal7-S	0	0	0
	GS-I	0	0	11647		**Total sum**	4598	3326	6237
	PA-IL	0	0	0	Gal-GalNAc	ACL	0	0	0
	Total sum	63829	90479	138875		BPA	0	0	0
GlcNAc	DSA	0	0	0		Jacalin	0	0	0
	F17AG	0	0	0		MPL	0	0	0
	GS-II	0	0	0		PNA	0	0	0
	LEA	0	0	0		PPL	0	0	0
	STL	38038	18250	47845		**Total sum**	0	0	0
	UDA	52631	42818	39691	LacNAc	Gal1-S	0	0	0
	UEA-II	0	0	0		Gal2	0	0	0
	WGA	0	0	0		Gal3	0	0	0
	Total sum	90669	61068	87536		Gal3C-S	0	0	0
Fucose	Lotus	0	740	0		Gal9	0	0	0
	PA-IIL	11984	11378	14560		LSL-N	0	0	0
	RS-Fuc	5373	4393	1759		**Total sum**	0	0	0
	UEA-I	0	0	0	Other	Malectin	141798	122396	140569
	AAA	0	0	0		PSL1a	22474	14097	32972
	AAL	0	0	0		ACG	0	0	0
	BC2LCN	0	0	0		**Total sum**	164272	136493	173541
	Total sum	17356	16511	16318	0	<9,999	10,000-99,999	>100,000	

VVA (Fig. 2v) at any temperature or time. The results also demonstrate that the FI of PHA-P (Fig. 2w) at 5 °C significantly decreased at 8 h ($P < 0.05$) but recovered at 16 h. Finally, the FI of malectin (Fig. 2x) and PSL1a (Fig. 2y) exhibited no significant differences under any temperature or time ($P > 0.05$).

3.3. Effects of CPAs on glycans at various times

Fig. 3 depicts the effects of CPAs on glycans at 8 and 16 h of chilling exposure. No significant differences were observed for the glycan FIs at 8 and 16 h when 1 M methanol, 0.5 M EG, and 0.5 M DMSO were used (Fig. 3a and b; $P > 0.05$). Similarly, no differences in glycan FI were identified at 8 or 16 h of chilling using these CPA treatments (Fig. 3a and b).

Fig. 4 depicts the lectin FI expressed in the embryos of *S. hispidus* after exposure to various CPAs at 5 °C at 8 and 16 h. Several CPAs significantly affected the expression of 9 of the 70 lectins ($P < 0.05$), namely, NPA, orysata, PALa, ASA, discoidin II, discoidin I, UDA, PA-IIL, and PHA-P (Fig. 4a and b). When comparing lectin expression between the CPA-treated group and the non-treated group, it was found that six specific lectins, namely orysata, ASA, discoidin II, UDA, PA-IIL, and PHA-P, were significantly different ($P < 0.05$) (Fig. 4a and b). The use of methanol and EG had a bigger effect on lectin expression than the use of DMSO, based on the overall intensity after 8 h and 16 h of chilling (Fig. 4a and b). Moreover, the CPAs triggered expression of PHAP-E, DBA, and MOA, which was noted only after the samples were treated with 1 M methanol, 0.5 M EG, or 0.5 M DMSO, respectively (Fig. 4a and b). In addition, the expression of HHA, ASA, PHA-P, malectin, and UDA was significantly affected by the use of 0.5 M EG and 1 M methanol at 8 and 16 h, resulting in an 80 % and 20 % change, respectively ($P < 0.05$;

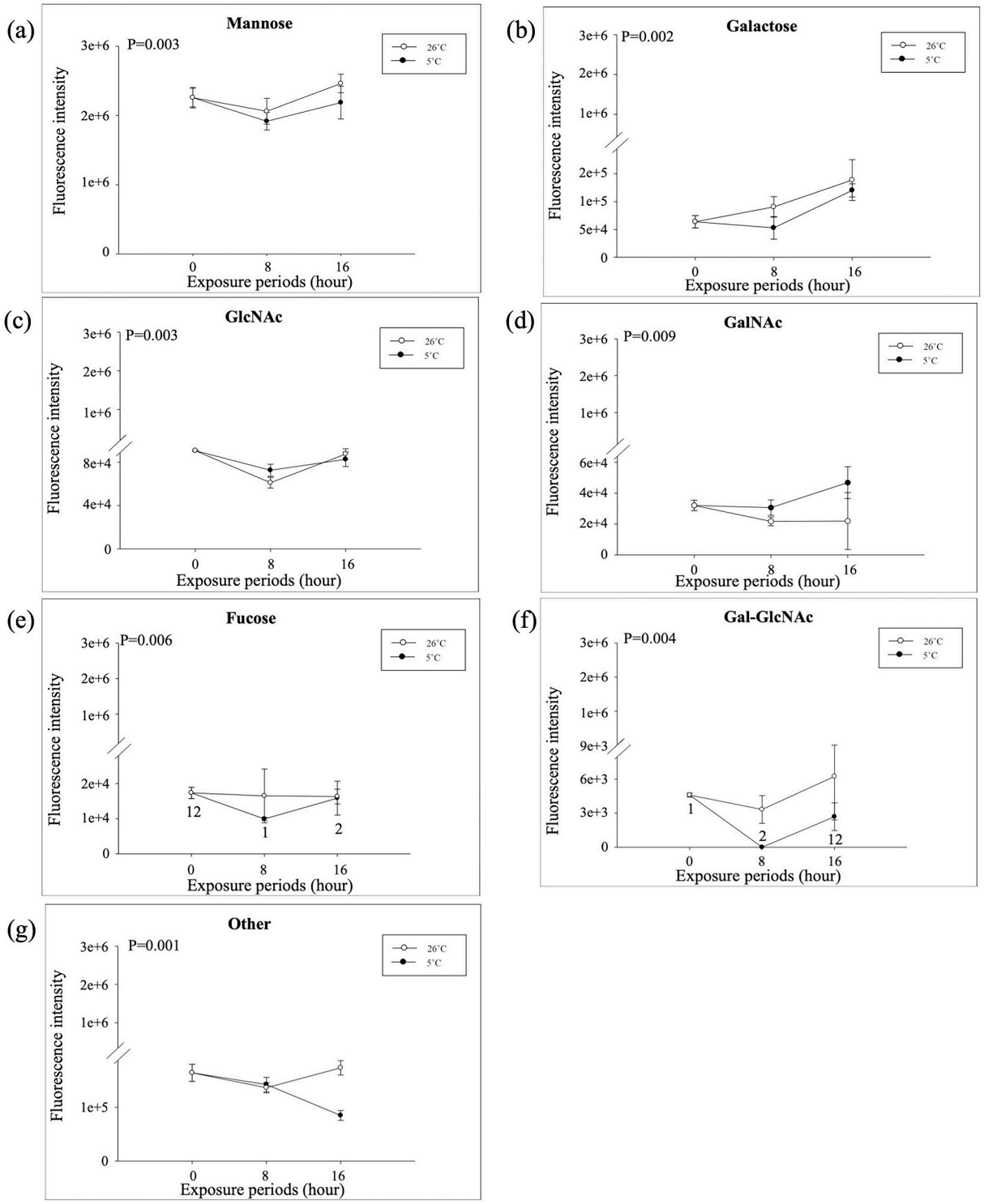

Fig. 1. Effect of 5 °C exposure at 0, 8 and 16 h compared with the control sample at 26 °C in seven glycans. (a) mannose, (b) galactose, (c) GlcNAc, (d) GalNAc, (e) fucose, (f) Gal-GlcNAc and (g) other. The bar represents the standard deviation. The different numbers indicate significant differences (Welch's $P < 0.05$) for the 5 °C sample.

Fig. 4a and b).

4. Discussion

Mannose is the most abundant glycan in *S. hispidus* embryos. Similarly, glycan profiling indicates that Symbiodiniaceae such as *B. minutum* [65], *C. goreaui*, and *F. kawagutii* [66] exhibit the highest FI for mannose. Mannose is believed to be the sugar that attaches most readily to N-linked glycans [57].

According to Ref. [52], malectin has a carbohydrate specificity to other glycans; this finding is consistent with the results of the present study, in which malectin was the second most abundant glycan. Malectin is also frequently expressed in the ER during cell processes such as protein synthesis, protein quality control, lipid synthesis, cell signaling,

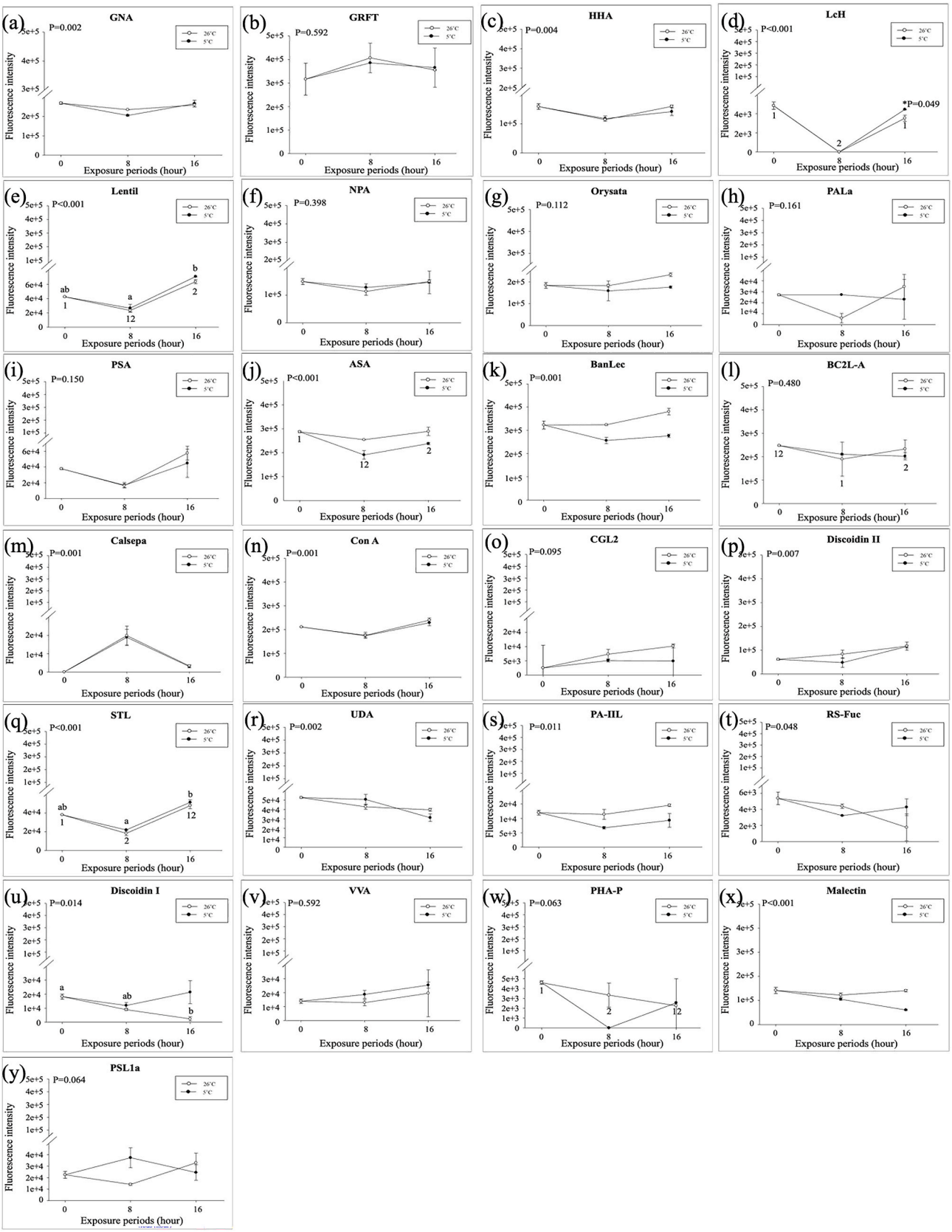

Fig. 2. Effects of 5 °C exposure at 0, 8, and 16 h compared with those of 26 °C exposure at the same times on 25 lectins: (a) GNA, (b) GRFT, (c) HHA, (d) LcH, (e) lentil, (f) NPA, (g) orysata, (h) PALa, (i) PSA, (j) ASA, (k) BanLec, (l) BC2L-A, (m) calsepa, (n) Con A, (o) CGL2, (p) discoidin II, (q) STL, (r) UDA, (s) PA-IIL, (t) RS-Fuc, (u) discoidin I, (v) VVA, (w) PHA-P, (x) malectin, and (y) PSL1a. Bars represent standard deviations. Letters and numbers indicate significant differences (Welch's $P < 0.05$) at 26 °C and 5 °C, respectively. Asterisks indicate significant differences between 5 °C and 26 °C (Welch's $P < 0.05$) at the same exposure times.

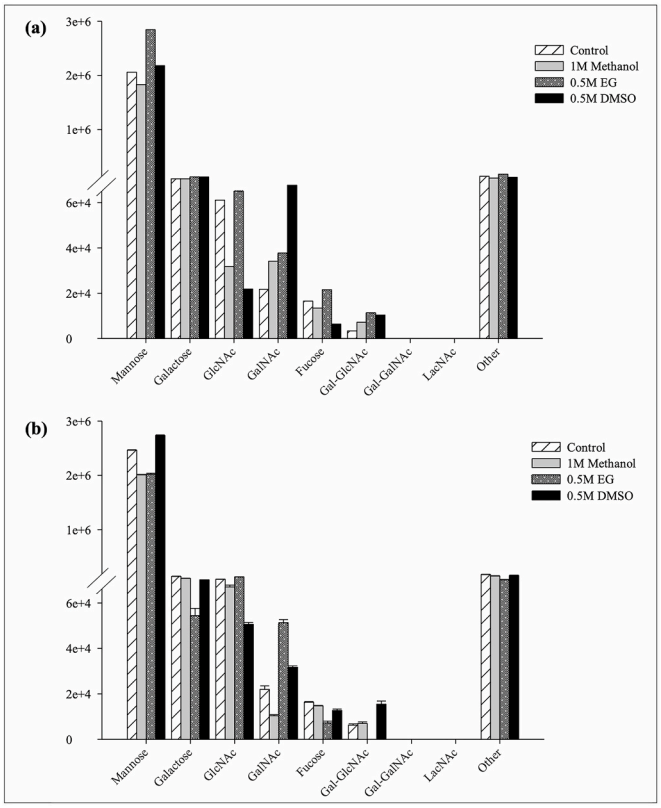

Fig. 3. Glycan profile at 5 °C revealing no significant differences (Welch's $P > 0.05$) in the same glycans under treatment with various CPAs at (a) 8 and (b) 16 h of exposure. Bars represent standard deviations.

and immune functions [42,53,77,78]. A study by Kelly et al., 2000 [25] indicated that malectin plays a crucial role in the embryonic development of *Xenopus laevis*. Malectin in the ER may be essential to embryogenesis, particularly in the cell growth and differentiation processes, such as that of organogenesis in the well-developed structures of late-stage *S. hispidus* embryos. One study reported that malectin is involved in the immune systems of scallops, particularly *Chlamys farreri* [77,78]; the yellow croaker (*Pseudosciaena crocea*; [42]) and the big-belly seahorse (*Hippocampus abdominalis*; [54]). The eggs and embryos of marine invertebrates are typically carried and protected by a parent but lose their protection when they are hatched [8]. For example, embryos of *S. hispidus* are deposited under the female abdomen [67], and once hatched, the newborn larvae must defend themselves against diseases and predators. Therefore, late-stage embryos may develop an early immune system that enables them to survive in the absence of their parents [64].

Maltose is the first disaccharide that binds to malectin [53]. Maltose is not involved in embryogenesis but breaks down into glucose [53] a monosaccharide similar to mannose in *S. hispidus*. Glucose and mannose are essential in providing energy to organisms, especially during the energy-intensive cell creation processes of embryonic development [43]. In the present study, the most abundant glycans were observed after 16 h of chilling the embryos at the heartbeat stage (when late-stage embryos transition to the pre-hatch stage [67]).

Monosaccharides are signaling molecules that control developmental processes and enhance the ability of cells to adapt to cold environments [56,82]. The present study revealed that chilling affects glycan expression, increasing FI at the late stages of the chilling process, although some glycans did not significantly differ at different stages of chilling. For the 9 glycan groups of this study, chilling only significantly affected the FIs of fucose and Gla-GlcNAc. However, when the lectin FI was individually analyzed, significant changes were observed in LeH, lectil, ASA, BC2L-A, STL, and PHA-P. Because mannose, galactose, fucose, GlcNAc, and GalNAc are monosaccharides [17], they may participate in osmotic regulation of cells and serve as signaling molecules that assist *S. hispidus* embryos in adapting to cold environments. The results of the present study are consistent with those of [76], who observed that the maltose, glucose, and fructose content in *J. curcas* seedlings changed during the early phase of chilling and that only glucose and fructose increased during the late stages of chilling. They concluded that chilling stress promotes soluble sugar accumulation in cells. Chilling also affects glycans because embryos adapt to chilling by using monosaccharides. The results of the present study are thus consistent with those of [37], who reported that chilling at 4 °C reduced the expression of lecRLK1. In addition, [6] revealed that temperature affected the stability of the lectins SELfd and ebulin f, which bind to D-galactose. The FI decreased as the temperature increased up to 90 °C, leading to protein denaturation. However, temperatures of 4 °C have been demonstrated to not affect the content of D-galactose and galactose [6,62]. Glycans thus enhance the ability of embryonic cells to adapt to the chilling process, as the present study demonstrated.

Although CPAs did not significantly affect glycan content in the present study, the results of the present analysis of the expression of individual lectins revealed that CPAs trigger expression of NPA, orysata, PALa, ASA, discoidin II, discoidin I, UDA, PA-IIL, and PHA-P. Methanol, EG, and DMSO are the most common CPAs and have low molecular weights, enabling them to easily penetrate cell membranes [55,81]. These CPAs reduce ice crystal formation and prevent most cryogenic injuries [32,40,48]. Moreover, CPAs are crucial to minimizing cell damage during exposure to low temperatures and to preserving cell structure; cell membrane properties; and cell functions, such as lipid storage and creation, protein expression, and the creation of mitochondrial deoxyribonucleic acid and adenosine triphosphate [7,29,34,69]. The present study demonstrated that CPAs trigger glycan expression, particularly EG and methanol. Based on the total intensity after 8 h and 16 h of chilling, the addition of EG and methanol had a more

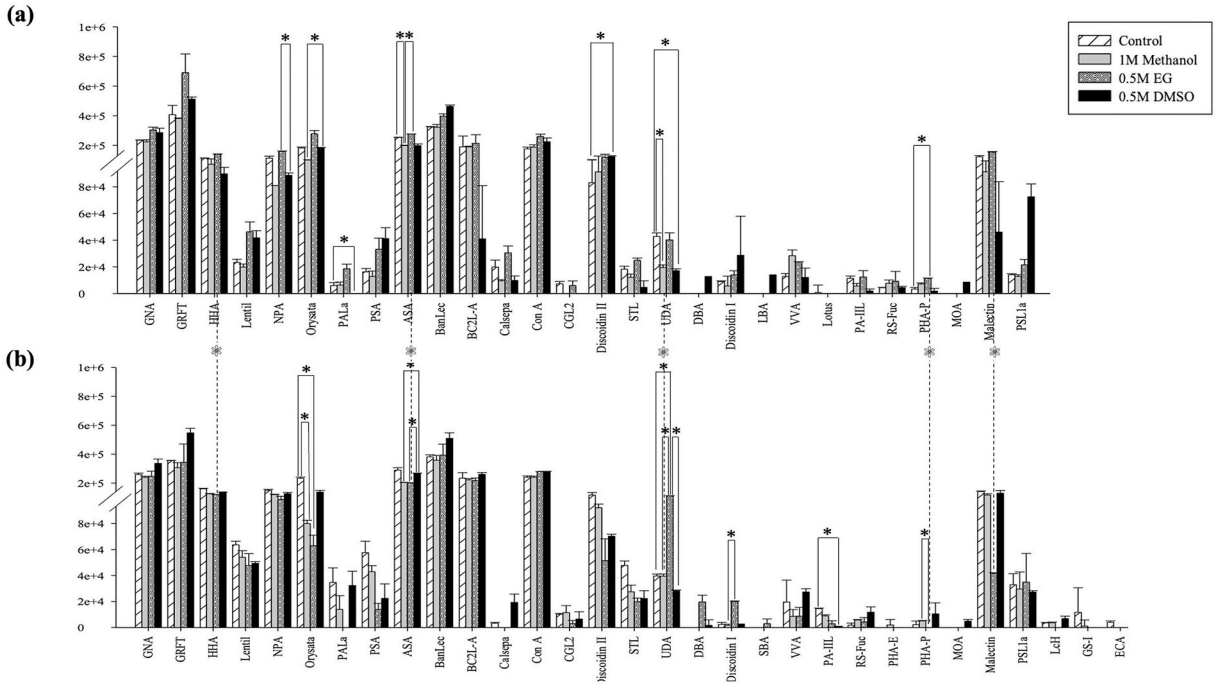

Fig. 4. Changes in lectin expression under treatment with various CPAs at (a) 8 and (b) 16 h of exposure at 5 °C. Bars represent standard deviations. Asterisks indicate significant differences (Welch's $P < 0.05$) between CPAs in the same lectins and between exposure times for treatment with the same CPAs.

significant impact on lectin expression compared to that of DMSO. However, EG and methanol was reported to be the least toxic CPA and to have been successfully used for embryo and larval cryopreservation of prawns (*Penaeus indicus* [45]), *S. hispidus* [67]), sea urchins (*Strongylocentrotus intermedius* and *Paracentrotus lividus* [4,48] and fruit flies (*Drosophila melanogaster* [59]). Numerous vertebrate and invertebrate species have successfully cryopreserved gametes and embryos using DMSO as an appropriate CPA [67].

Glycan expression is affected by chemicals such as lecRLK, which binds nonenzymatically to specific carbohydrates. LecRLK expression may be reduced by abscisic acid and methyl jasmonate treatments [37]. In addition, glycan may be affected by environmental factors such as pH and salinity. Acidic environments have been reported to alter the structure of D-galactose [6,26]. Expression of lecRLK and O-linked glycans such as Sp7 and Sp245 may be reduced by salinity stress [2,37]. Further studies are required to investigate the effects of environmental and biochemical factors on glycans in marine organisms.

In conclusion, this study is the first to investigate the effects of chilling and CPAs on glycans in *S. hispidus* embryos. Chilling and CPAs triggered glycan expression. The use of DMSO presented less effect on glycan content when compared to methanol and EG. Selecting the suitable CPA for *S. hispidus* embryo cryopreservation necessitates further research to develop an optimal freezing protocol. The results of this study can serve as a reference for developing cryopreservation techniques for the embryos of aquatic organisms. Additionally, this study advances understanding of the roles of saccharides in embryo formation, which may assist research in developmental biology, embryogenesis, aquaculture, and biotechnology.

Ethical approval

Not applicable.

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Availability of data and materials

The data and materials are available on request.

CRediT authorship contribution statement

Kanokpron Loeslakwiboon: Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Hsing-Hui Li:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Sujune Tsai:** Conceptualization, Data curation, Resources, Software, Supervision, Validation, Writing – review & editing. **Zhi-Hong Wen:** Project administration, Resources, Supervision, Validation. **Chiahsin Lin:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

I declare that the authors have no competing interests as defined by the journal, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

References

[1] M.K. Ahmmed, S. Bhowmik, S.G. Giteru, M.N.H. Zilani, P. Adadi, S.S. Islam, O. N. Kanwugu, M. Haq, F. Ahmmed, C.C.W. Ng, Y.S. Chan, M. Asadujjaman, G.H. H. Chan, R. Naude, A.E.D.A. Bekhit, T.B. Ng, J.H. Wong, An update of lectins from marine organisms: characterization, extraction methodology, and potential biofunctional applications, Mar. Drugs 20 (2022), https://doi.org/10.3390/md20070430.

[2] S.A. Alenkina, M.A. Kupryashina, Influence of *Azospirillum* lectins on the antioxidant system response in wheat seedling roots during abiotic stress, Soil Res. 60 (2022) 197–209, https://doi.org/10.1071/SR21092.

[3] J. Alfaro, J. Komen, E.A. Huisman, Cooling, cryoprotectant and hypersaline sensitivity of penaeid shrimp embryos and nauplius larvae, Aquaculture 195 (2001) 353–366, https://doi.org/10.1016/S0044-8486(00)00557-3.

[4] E. Asahina, T. Takahashi, Freezing tolerance in embryos and spermatozoa of the sea urchin, Cryobiology 15 (1978) 122–127, https://doi.org/10.1016/00112240(78)90016-0.

[5] R. Calado, L. Lin, A.L. Rhyne, R. Araújo, L. Narciso, Marine ornamental decapods: popular, pricey and poorly studied, Crustacean Biol. 23 (2003) 963–973.

[6] C. Carrillo, D. Cordoba-Diaz, M. Cordoba-Diaz, T. Girbés, P. Jiménez, Effects of temperature, pH and sugar binding on the structures of lectins ebulin f and SELfd, Food Chem. 220 (2017) 324–330, https://doi.org/10.1016/j.foodchem.2016.10.000.

[7] S. Chen, J. Ren, R. Chen, Cryopreservation and desiccation preservation of cells, Comprehensive Biotechnol. 5 (2019) 157–166, https://doi.org/10.1016/B978-0-444-64046-8.00451-1.

[8] F.B. Christiansen, T.M. Fenchel, Evolution of marine invertebrate reproductive patterns, Theor. Popul. Biol. 16 (1979) 267–282, https://doi.org/10.1016/0040-5809(79)90017-0.

[9] P.L. Colin, Caribbean Reef Invertebrates and Plants, THF Publications, Inc., Ltd, Neptune City, 1978, p. 512.

[10] K.D. Cox, D.R. Layne, R. Scorza, G. Schnabel, Gastrodia anti-fungal protein from the orchid *Gastrodia elata* confers disease resistance to root pathogens in transgenic tobacco, Planta 224 (2006) 1373–1383, https://doi.org/10.1007/s00425-006-0322-0.

[11] A. Dinnyés, B. Urbányi, B. Baranyai, I. Magyary, Chilling sensitivity of carp (*Cyprinus carpio*) embryos at different developmental stages in the presence or absence of cryoprotectants: work in progress, Theriogenology 50 (1998) 1–13, https://doi.org/10.1016/s0093-691x(98)00108-3.

[12] S. Emr, B.S. Glick, A.D. Linstedt, J. Lippincott-Schwartz, A. Luini, V. Malhotra, B. J. Marsh, A. Nakano, S.R. Pfeffer, C. Rabouille, J.E. Rothman, G. Warren, F. T. Wieland, Journeys through the Golgi-taking stock in a new era, J. Cell Biol. 187 (2009) 449–453, https://doi.org/10.1083/jcb.200909011.

[13] D.J. Fletcher, I. Kötter, M. Wunsch, I. Yasir, Preliminary observations on the reproductive biology of ornamental cleaner prawns *Stenopus hispidus*, Lysmata amboinensis and Lysmata debelius, International Zoo Yearbook 34 (1995) 73–77, https://doi.org/10.1111/j.1748-1090.1995.tb00661.x.

[14] H.J. Gabius, The sugar code: why glycans are so important, Biosystems 164 (2018) 102–111, https://doi.org/10.1016/j.biosystems.2017.07.003.

[15] C. Gao, B. Su, D. Zhang, N. Yang, L. Song, Q. Fu, S. Zhou, F. Tan, C. Li, l-rhamnose-binding lectins (RBLs) in turbot (*Scophthalmus maximus L.*): characterization and expression profiling in mucosal tissues, Fish Shellfish Immunol. 80 (2018) 264–273, https://doi.org/10.1016/j.fsi.2018.06.008.

[16] R.A. Gregati, V. Fransozo, L.S. López-Greco, M.L. Negreiros-Fransozo, Reproductive cycle and ovarian development of the marine ornamental shrimp *Stenopus hispidus* in captivity, Aquaculture 306 (2010) 185–190, https://doi.org/10.1016/j.aquaculture.2010.05.009.

[17] M.E. Griffin, L.C. Hsieh-Wilson, Tools for mammalian glycoscience research, Cell 185 (2022) 2657–2677, https://doi.org/10.1016/j.cell.2022.06.016.

[18] J.C. Gwo, K. Strawn, C.R. Arnold, Changes in mechanical tolerance and chilling sensitivity of red drum (*Sciaenopus ocellatus*) embryos during development, Theriogenology 43 (1995) 1155–1161, https://doi.org/10.1016/0093-691X(95)00087-O.

[19] L.B. Holthuis, The stenopodidae, nephropsidae, scyllaridae and palinuridae, the Decapoda macrura of the snellius expedition 1, Temminckia 7 (1946) 1–178.

[20] L.B. Holthuis, The recent genera of the caridean and stenopodidean shrimps *(Crustacea, Decapoda)* with an appendix on the order Amphionidacea, Nation. Natuurhistorisch Museum, Leiden, The Netherlands (1993) 328.

[21] M. Hosono, S. Sugawara, A. Matsuda, T. Tatsuta, Y. Koide, I. Hasan, Y. Ozeki, K. Nitta, Binding profiles and cytokine-inducing effects of fish rhamnose-binding lectins on Burkitt's lymphoma Raji cells, Fish Physiol. Biochem. 40 (2014) 1559–1572, https://doi.org/10.1007/s10695-014-9948-1.

[22] X. Huang, K. Han, T. Li, W. Wang, Q. Ren, Novel L-type lectin from freshwater prawn, *Macrobrachium rosenbergii* participates in antibacterial and antiviral immune responses, Fish Shellfish Immunol. 77 (2018) 304–331, https://doi.org/10.1016/j.fsi.2018.03.061.

[23] Jr.V.R. Johnson, Individual recognition in the banded shrimp *Stenopus hispidus* (Olivier), Anim. Behav. 25 (1977) 418–428, https://doi.org/10.1016/0003-3472(77)90017-3.

[24] C.L. Juurakko, G.C. diCenzo, V.K. Walker, Cold acclimation and prospects for cold-resilient crops, Plant Stress 2 (2021), https://doi.org/10.1016/j.stress.2021.100028.

[25] O.G. Kelly, D.A. Melton, Development of the pancreas in *Xenopus laevis*, Dev. Dynam. 218 (2000) 615–627, https://doi.org/10.1002/1097-0177.

[26] F. Khan, A. Ahmad, M.I. Khan, Chemical, thermal and pH-induced equilibrium unfolding studies of *Fusarium solani* lectin, IUBMB Life 59 (2007) 34–43, https://doi.org/10.1080/15216540601178075.

[27] A. Kuno, A. Matsuda, Y. Ikehara, Differential glycan profiling by lectin microarray targeting tissue specimens, Methods Enzymol. 478 (2010) 165–179, https://doi.org/10.1016/S0076-6879(10)78007-1.

[28] H. Laczkó-Dobos, B. Szalontai, Lipids, proteins and their interplay in the dynamics of temperature-stressed membranes of a cyanobacterium, Synechocystis PCC 6803, Biochemistry 48 (2009) 10120–10128, https://doi.org/10.1021/bi9011034.

[29] J.E. Lannan, Experimental self-fertilization of the pacific oyster, *Crassostrea gigas*, utilizing cryopreserved sperm, Genetics 68 (1971) 599–601, https://doi.org/10.1093/genetic.

[30] C. Lin, S. Tsai, Development of an enzymatic method for individual separation of banded coral shrimp (*Stenopus hispidus*) embryos, Aquacult. Res. 43 (2012) 1509–1514, https://doi.org/10.1111/j.1365-2109.2011.02953.x.

[31] C. Lin, S. Tsai, The effect of cryopreservation on DNA damage, gene expression and protein abundance in vertebrate, Ital. J. Anim. Sci. 11 (2012) 119–122, https://doi.org/10.4081/ijas.2012.e21.

[32] C. Lin, S. Tsai, Fifteen years of coral cryopreservation, Platax 17 (2020) 53–75, https://doi.org/10.29926/platax.202012_17.0004.

[33] C. Lin, C.C. Han, S. Tsai, Effect of thermal injury on embryos of banded coral shrimp (*Stenopus hispidus*) under hypothermal conditions, Cryobiology 66 (2013) 3–7, https://doi.org/10.1016/j.cryobiol.2012.05.005.

[34] C. Lin, T. Zhang, F.W. Kuo, S. Tsai, Gorgonian coral (*Junceella juncea* and *Junceella fragilis*) oocyte chilling sensitivity in the context of adenosine triphosphate response (ATP), Cryo Lett. 32 (2011) 141–147.

[35] J. Lin, H. Shi, Effect of broodstock diet on reproductive performance of the golden banded coral shrimp *Stenopus scutellatus*, World Aquacult. Soci. 33 (2002) 383–386, https://doi.org/10.1111/j.1749-7345.2002.tb00515.x.

[36] K. Liu, T. Chou, H. Lin, Cryopreservation of goldfish embryos after subzero freezing, Aquatic Living Recour. 6 (1993) 145–153, https://doi.org/10.1051/alr:1993007.

[37] S. Liu, J. Wang, K. Chen, Z. Zhang, P. Zhang, The L-type lectin receptor-like kinase (PnLecRLK1) from the Antarctic moss *Pohlia nutans* enhances chilling-stress tolerance and abscisic acid sensitivity in Arabidopsis, Plant Growth Regul. 81 (2017) 409–418, https://doi.org/10.1007/s10725-016-0217-4.

[38] J.D. Marth, A unified vision of the building blocks of life, Nat. Cell Biol. 10 (2008) 1015–1016, https://doi.org/10.1038/ncb0908-1015.

[39] A. McCammon, Snapping Shrimp Protect Host Anemones from Predators. M.Sc. Thesis, Florida Atlantic University, 2010.

[40] C. Molinet, N. Barahona, P. Araya, C. Arriagada, C. Balboa, C. Bravo, E. Diaz, P. Díaz, C. Eisele, C. Montenegro, C. Olguín, A. Ruiz, P. Subiabre, Actualización de parámetros biológicos del erizo rojo Loxechinus albus en la X y XI regions, FIP. Puerto Montt: Universidad Austral de Chile, Puerto Montt (2014).

[41] G.J. Morris, Membrane model of chilling and cold shock, Cryobiology 71 (2015) 174, https://doi.org/10.1016/j.cryobiol.2015.05.041.

[42] Y. Mu, M. Li, F. Ding, Y. Ding, J. Ao, S. Hu, X. Chen, Characterization of the spleen transcriptome of the large yellow croaker (*Pseudosciaena crocea*) and analysis of the immune relevant genes and pathways involved in the antiviral response, PLoS One 9 (2014) e97471, https://doi.org/10.1371/journal.pone.0097471.

[43] B.V. Navarro, P. Elbl, A.P. De Souza, V. Jardim, L.F. De Oliveira, A.F. Macedo, A.L. W. Dos Santos, M.S. Buckeridge, E.I.S. Floh, Carbohydrate-mediated responses during zygotic and early somatic embryogenesis in the endangered conifer, *Araucaria angustifolia*, PLoS One 12 (2017) 1–20, https://doi.org/10.1371/journal.pone.0180051.

[44] R. Nemoto, S. Yamamoto, T. Ogawa, R. Naude, K. Muramoto, Effect of chum salmon egg lectin on tight junctions in Caco-2 cell monolayers, Molecules 20 (2015) 8094–8106, https://doi.org/10.3390/molecules.

[45] S.S. Newton, T. Subramoniam, Cryoprotectant toxicity in penaeid prawn embryos, Cryobiology 33 (1996) 172–177, https://doi.org/10.1006/cryo.1996.0017.

[46] C.L. Nilsson, Lectins: Analytical Tools from Nature, Analytical Technologies, 2007, https://doi.org/10.1016/B978-044453077-6/50002-8.

[47] M.R. Palmtag, G.J. Holt, Captive rearing of the shrimp (*Lysmata debelius*), Texas Sea Grant College Program Research Report (2001) 1–4.

[48] E. Paredes, Biobanking of a marine invertebrate model organism: the sea urchin, J. Mar. Sci. Eng. 4 (2016) 7, https://doi.org/10.3390/jmse4010007.

[49] M.G. Parisi, M. Cammarata, G. Benenati, G. Salerno, V. Mangano, A. Vizzini, N. Parrinello, A serum fucose-binding lectin (DlFBL) from adult *Dicentrarchus labrax* is expressed in larva and juvenile tissues and contained in eggs, Cell Tissue Res. 341 (2010) 279–288, https://doi.org/10.1007/s00441-010-1004-6.

[50] P.J. Quinn, Effects of Temperature on Cell Membranes, vol. 42, Symposia of the Society for Experimental Biology, 1988, pp. 237–258.

[51] H.J. Ray, L. Niswander, Mechanisms of tissue fusion during development, Development (Cambridge, England) 139 (2012) 1701–1711, https://doi.org/10.1242/dev.068338.

[52] RayBiotech, RayBiotech lectin array 70 kit. https://www.raybiotech.com/lectin-array-70, 2016.

[53] T. Schallus, C.K. Jaeckh, A.S. Palma-Fehér, Y.J. Liu, C. Simpson, M. Mackeen, G. Stier, T.J. Gibson, T. Feizi, T. Pieler, C. Muhle-Goll, Malectin: a novel carbohydrate-binding protein of the endoplasmic reticulum and a candidate player in the early steps of protein N-glycosylation, Mol. Biol. Cell 19 (2008) 3404–3414, https://doi.org/10.1091/mbc.e08-04-0354.

[54] S. Sellaththurai, K.A.S.N. Shanaka, D.S. Liyanage, H. Yang, T.T. Priyathilaka, J. Lee, J. Molecular, And functional insights into a novel teleost malectin from big-belly seahorse *Hippocampus abdominalis*, Fish Shellfish Immunol. 99 (2020) 483–494, https://doi.org/10.1016/j.fsi.2020.02.044.

[55] F. Shaluei, M.R. Imanpoor, A. Shabani, M.H. Nasr-Esfahani, Effect of different concentrations of permeable and non-permeable cryoprotectants on the hatching rate of goldfish (*Carassius auratus*) embryos, Asian Pacific J. Reproduct. 2 (2013) 185–188, https://doi.org/10.1016/S2305-0500(13)60144.

[56] J. Sheen, L. Zhou, J. Jang, Sugars as signaling molecules, Curr. Opin. Plant Biol. 2 (1999) 410–419, https://doi.org/10.1016/s1369-5266(99)00014.

[57] G. Sorrenti, A. Bagnoli, V. Miraglia, F. Crocetta, V. Vitiello, F. Ristoratore, P. Cirino, G. Sansone, P. Sordino, Investigating sperm cryopreservation in a model tunicate, *Ciona intestinalis* sp, Cryobiology 68 (2014) 43–49, https://doi.org/10.1016/j.cryobiol.2013.11.005.

[58] P. Stanley, K.W. Moremen, N.E. Lewis, N. Taniguchi, M. Aebi A. Varki, R. D. Cummings, J.D. Esko, G.W. Hart, N-Glycans, in: Essentials of Glycobiology, fourth ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2022 (Chapter 9).

[59] P.L. Steponkus, S.P. Myers, D.V. Lynch, L. Gardner, V. Bronshteyn, S.P. Leibo, W. F. Rall, R.E. Pitt, T.T. Lin, R.J. MacIntyre, Cryopreservation of *Drosophila melanogaster* embryos, Nature 345 (1990) 170–172, https://doi.org/10.1038/345170a0.

[60] S. Sugawara, Application of lectin from catfish eggs to cancer therapy: a fundamental study, J. Pharm. Soc. Jpn. 138 (2018) 1451–1459, https://doi.org/10.1248/yakushi.18-00128.
[61] S. Sugawara, C. Im, T. Kawano, T. Tatsuta, Y. Koide, D. Yamamoto, Y. Ozeki, K. Nitta, M. Hosono, Catfish rhamnose-binding lectin induces $G_{0/1}$ cell cycle arrest in Burkitt's lymphoma cells via membrane surface Gb3, Glycoconj. J. 34 (2017) 127–138, https://doi.org/10.1007/s10719-016-9739-2.
[62] N.A.M. Sultan, M. Kavitha, M.J. Swamy, Purification and physicochemical characterization of two galactose-specific isolectins from the seeds of *Trichosanthes cordata*, IUBMB Life 61 (2009) 457–469, https://doi.org/10.1002/iub.174.
[63] H. Tateno, A. Saneyoshi, T. Ogawa, K. Muramoto K, H. Kamiya, M. Saneyoshi, Isolation and characterization of rhamnose-binding lectins from eggs of steelhead trout (*Oncorhynchus mykiss*) homologous to low density lipoprotein receptor superfamily, J. Biol. Chem. 273 (30) (1998) 19190–19197, https://doi.org/10.1074/jbc.273.30.19190.
[64] A.M. Thomas, V.H.W. Rudolf, Challenges of metamorphosis in invertebrate hosts: maintaining parasite resistance across life-history stages, Ecol. Entomol. 35 (2010) 200–205, https://doi.org/10.1111/j.1365-2311.2009.01169.x.
[65] T.R. Tivey, J.E. Parkinson, P.E. Mandelare, D.A. Adpressa, P. Wenjing, X. Dong, Y. Mechref, V.M. Weis, S. Loesgen, N-Linked surface glycan biosynthesis, composition, inhibition, and function in cnidarian-dinoflagellate symbiosis, Microb. Ecol. 80 (2019) 223–236.
[66] G. Tortorelli, C. Rautengarten, A. Bacic, G. Segal, B. Ebert, S.K. Davy, M.J.H. van Oppen, G.I. McFadden, Cell surface carbohydrates of symbiotic dinoflagellates and their role in the establishment of cnidarian–dinoflagellate symbiosis, ISME J. 16 (2022) 190–199, https://doi.org/10.1038/s41396-021-01059-w.
[67] S. Tsai, C. Lin, Effects of cryoprotectant on the embryos of banded coral shrimp (*Stenopus hispidus*); preliminary studies to establish freezing protocols, Cryo-Letters 30 (2009) 373–381.
[68] S. Tsai, D.M. Rawson, T. Zhang, T. Studies on chilling sensitivity of early stage zebrafish (*Danio rerio*) ovarian follicles, Cryobiology 58 (2009) 279–286, https://doi.org/10.1016/j.cryobiol.2009.02.002.
[69] S. Tsai, P. Thongpooe, F.W. Kuo, C. Lin, Impacts of low temperature preservation on mitochondrial DNA copy number in oocytes of the hard coral *Echinopora* sp, Mitochondrial DNA 27 (2015) 2512–2515, https://doi.org/10.3109/19401736.2015.1036254.
[70] D.M. Valdez, A. Miyamoto, T. Hara, K. Edashige, M. Kasai, Sensitivity to chilling of medaka (*Oryzias latipes*) embryos at various developmental stages, Theriogenology 64 (2005) 112–122, https://doi.org/10.1016/j.theriogenology.2004.11.006.
[71] A. Varki, Evolutionary forces shaping the Golgi glycosylation machinery: why cell surface glycans are universal to living cells, Cold Spring Harbor Perspect. Biol. 3 (2011) 1–14, https://doi.org/10.1101/cshperspect.a005462.
[72] A. Varki, Biological roles of glycans, Glycobiology 27 (2017) 3–49, https://doi.org/10.1093/glycob/cww086.
[73] A. Varki, N. Sharon, in: Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2009, pp. 1–22.
[74] I.M. Vasconcelos, J.T. Oliveira, Antinutritional properties of plant lectins, Toxicon: Offic. J. Int. Soci. Toxinol. 44 (2004) 385–403, https://doi.org/10.1016/j.toxicon.2004.05.005.
[75] C. Wabnitz, M. Taylor, E. Green, T. Razak, From Ocean to Aquarium, UNEP-WCMC, Cambridge, UK, 2003.
[76] H. Wang, M. Gong, H. Xin, L. Tang, D. Dai, Y. Gao, C. Liu, Effects of chilling stress on the accumulation of soluble sugars and their key enzymes in *Jatropha curcas* seedlings, Physiol. Mol. Biol. Plants 24 (2018) 857–865, https://doi.org/10.1007/s12298-018-0568-6.
[77] M. Wang, B. Wang, K. Jiang, X. Liu, L. Shi, A. Wang, Mitochondrial manganese superoxide dismutase involved in innate immunity is essential for the survival of *Chlamys farreri*, Fish Shellfish Immunol. 72 (2018) 282–290, https://doi.org/10.1016/j.fsi.2017.11.010.
[78] M. Wang, B. Wang, M. Liu, K. Jiang, L. Wang, The first identification of a malectin gene (CfMal) in scallop *Chlamys farreri*: sequence features and expression profiles, Invertebr. Surviv. J. 16 (2019) 25–33, https://doi.org/10.25431/1824-307X/isj.v0i0.25-33.
[79] K. Weineck, A.J. Ray, L.J. Fleckenstein, M. Medley, N. Dzubuk, E. Piana, R. L. Cooper, Physiological changes as a measure of crustacean welfare under different standardized stunning techniques: cooling and electroshock, Animals 8 (2018) 1–21, https://doi.org/10.3390/ani8090158.
[80] B.S. Weston, E.W. Charles, Chapter twenty-two high throughput RNAi screening for N-Glycosylation Dependent Loci in *Caenorhabditis elegans*, Methods Enzymol. 480 (2010) 477–493, https://doi.org/10.1016/S0076-6879(10)80021-7.
[81] D. Whaley, K. Damyar, R.P. Witek, A. Mendoza, M. Alexander, J.R.T. Lakey, Cryopreservation: an overview of principles and cell-specific considerations, Cell Transplant. 30 (2021) 1–12, https://doi.org/10.1177/0963689721999617.
[82] Y.E. Yoon, S. Kuppusamy, K.M. Cho, P.J. Kim, Y.B. Kwack, Y.B. Lee, Influence of cold stress on contents of soluble sugars, vitamin C and free amino acids including gamma-aminobutyric acid (GABA) in spinach (*Spinacia oleracea*), Food Chem. 215 (2017) 185–192, https://doi.org/10.1016/j.