

# THE EFFECT OF COLD SHOCK AND FREEZE-THAWING ON RELEASE OF PHOSPHOLIPIDS BY RAM, BULL, AND BOAR SPERMATOOZA

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## Abstract

The effects of cold shock and freeze-thawing on the release of total phospholipid phosphorus and of specific phospholipids from ram, bull, and boar spermatozoa are examined. Species differences are apparent, both in the absolute amount of total phospholipids released and in the conditions required to effect a loss of individual phospholipids. The phosphoglycerides most affected by the temperature treatment are choline plasmalogen, phosphatidyl choline, and phosphatidyl ethanolamine. The loss of phospholipids is most specific in boar spermatozoa. Bull spermatozoa suffer a greater overall and more general breakdown of phospholipids, indicating disruption of the cell structure. This is in contrast to the more specific phospholipid losses from ram and boar spermatozoa, which may indicate a more localized region of damage and release of material, possibly acrosomal. The loss of phospholipid may be correlated with the species differences in the fertility of the frozen spermatozoa.

## I. INTRODUCTION

Much of the work on cold shock of ram, bull, and boar spermatozoa has been concerned with the effect on metabolism and ion balance. The importance of electrolyte concentration to spermatozoa was noted by Kampschmidt *et al.* (1953) and it was found that a sudden lowering of temperature to 0°C resulted in an influx of sodium and calcium and loss of potassium ions (Blackshaw and Salisbury 1957; Quinn and White 1966; Hood *et al.* 1970). According to Blackshaw and Salisbury (1957) changes in ionic concentrations in the seminal plasma following shock suggest breakdown in the selective permeability of the cell membrane, followed by passive transfer of the ions. Hood *et al.* (1970) have postulated a breakdown in the mechanisms providing energy for the maintenance of the cell's internal environment, possibly involving damage to the membranes. Cold shock has been shown to cause an irreversible decrease in motility, metabolism and level of ATP, a release of cytochrome *c* and other intracellular protein constituents, and the release of lipoprotein (Mann and Lutwak-Mann 1955; Blackshaw and Salisbury 1957; Walton 1957; Quinn *et al.* 1968*b*). White *et al.* (1954) showed that freezing resulted in a greater disruption of motility and metabolism.

Adding egg yolk or lecithin to the medium decreased damage to spermatozoan glycolysis caused by cold shock in the bull (Blackshaw 1954*a*; Blackshaw and Salisbury 1957) and reduced the accumulation of calcium in ram and bull spermatozoa

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(Quinn and White 1966). Miller and Mayer (1960) found that a lipoprotein extract was not as effective as egg yolk in preventing cold shock. Egg yolk was also superior to lecithin in protecting ram spermatozoa from cold shock, although the spermatozoa did not maintain full metabolic activity during subsequent incubation at 37°C (Blackshaw 1958). This was in contrast to the maintenance of metabolism under similar conditions observed by Blackshaw and Salisbury (1957) in the bull.

The presence of plasmalogen in mammalian spermatozoa has been noted by a number of workers (Lovern *et al.* 1957; Gray 1960; Hartree and Mann 1961; Masaki and Hartree 1962; Poulos *et al.* 1972), and detailed analyses of sperm phospholipid components have been reported for the ram (Quinn and White 1967; Scott *et al.* 1967; Scott and Dawson 1968; Poulos *et al.* 1972), bull (Pursel and Graham 1967; Poulos *et al.* 1973), and boar (Grogan *et al.* 1966; Johnson *et al.* 1967, 1969; Poulos *et al.* 1972).

The effect of cold shock and freeze-thawing on the phospholipids of spermatozoa has, however, received little attention. Phospholipids and lipoproteins were lost from erythrocytes under a variety of conditions including thermal shock (Lovelock 1954, 1955). There are also reports of loss of lipid from frozen bull spermatozoa (Pickett and Komarek 1964), and a 10% loss of plasmalogen from cold-shocked ram spermatozoa (Hartree and Mann 1959).

The intrinsic importance of phospholipids in membrane structure and in the expression of enzyme activity (Ansell and Hawthorne 1964) indicates that they play a significant role in cellular reactions to environmental change. Gross morphological changes, alteration in membrane permeability, and loss of cellular material are all features of the reaction of spermatozoa to sudden decreases in temperature and may reflect changes in the composition and binding of lipids to the membrane matrix. This has prompted an investigation of the effect of cold shock and freeze-thawing on the individual phospholipids of spermatozoa, using ram, bull, and boar spermatozoa for the purpose of comparison. A preliminary report of the results of this study has been presented (Darin *et al.* 1973).

## II. MATERIALS AND METHODS

### (a) Collection of Semen

Ram semen was obtained by electrical ejaculation (Blackshaw 1954b) and was maintained at 20°C during transfer to the laboratory. Boar semen was collected using a dummy sow and samples of the sperm-rich fraction from three boars were pooled. Ram and boar semen was processed within 30 min of collection. Bull semen was collected by means of an artificial vagina at the Artificial Insemination Centre at Berry, N.S.W. The samples were placed in an insulated container inside a thermos flask at 8°C, to ensure slow cooling of the spermatozoa, which were received within 3 hr of collection. Only spermatozoa displaying good initial motility at the time of processing were used.

### (b) Treatment of Semen and Extraction

Pooled samples of ram and bull semen were diluted with three volumes of calcium-free Krebs-Ringer phosphate solution (pH 7.2-7.4) (Umbreit *et al.* 1959) at 30°C and centrifuged at 500 *g* for 20 min at room temperature (20-25°C). Owing to the large volume, boar semen was not diluted prior to centrifugation. The spermatozoa were gently resuspended in 30 ml of the Krebs-Ringer solution containing 30 mg glucose, and the suspension equilibrated at 30°C for 20 min. The final spermatozoal concentrations per millilitre were  $4.5-8.0 \times 10^8$  (ram),  $2.7-9.3 \times 10^8$  (boar), and  $1.7-3.9 \times 10^8$  (bull) as determined by taking an average of three haemocytometer counts. An aliquot

of the sperm suspension was extracted prior to treatment and a control aliquot kept at 30°C throughout the temperature treatment. A third aliquot was cold-shocked in a pre-cooled conical flask and maintained at 0°C for 10 min with constant agitation. A fourth aliquot was frozen in ethanol-dry ice for 10 min.

Each aliquot was then kept at 30°C for 30 min and the motility of the spermatozoa scored (Emmens 1947) to check the immediate effect of treatment. After centrifuging (500 *g*) for 20 min at room temperature (20–25°C), the supernatants were passed through a 2- $\mu$ m Nucleopore filter to remove any contaminating whole spermatozoa, then extracted by the method of Bligh and Dyer (1959). The lower phase resulting from the extraction procedure was evaporated to dryness in a rotary evaporator at 40°C and the residue treated by the method of Ansell and Hawthorne (1964) to remove any proteolipids. The final residue was taken up in chloroform-methanol (4 : 1 v/v) and stored under nitrogen at –10°C.

Phosphorus assays were carried out as described by Owens and Hughes (1970) and  $\alpha,\beta$ -unsaturated ether analyses by the method of Gottfried and Rapport (1962), using cetyl vinyl ether as a standard. Two-dimensional thin-layer chromatographic analyses of the phospholipid mixtures were performed as described by Poulos and White (1973). The yield of phosphorus recovered from the plate was generally in excess of 90% and less than 2% of this was located at the origin. The amount of plasmalogen estimated by this method agreed well with the amount detected by the  $\alpha,\beta$ -unsaturated ether analysis.

#### (c) Proof of Identity of Phospholipids

The proof of identity of the phospholipids was based on a comparison of their mobilities on thin-layer chromatography with phospholipid standards in three separate solvent systems. In addition, lipids isolated by preparative thin-layer chromatography were hydrolysed according to the procedure of Dawson (1960) and the liberated water-soluble phosphate esters were identified by paper chromatography in water-saturated phenol-acetic acid-water (50 : 5 : 6 v/v).

#### (d) Statistical Analysis

Data for percentage composition were subjected to arcsin transformation before analysis of variance (Snedecor 1959). The interaction mean square has been used to calculate the standard error of the differences between means as presented in Table 2, and this has been used in *t*-tests comparing each treatment with the control.

### III. RESULTS

#### (a) Total Phospholipid Released

The amount of phospholipid phosphorus extracted from untreated spermatozoa, and the quantity released by the sperm into the supernatant under control conditions, after cold shock and freeze-thawing is shown in Table 1.

TABLE 1

PHOSPHOLIPID PHOSPHORUS RELEASED FROM SPERMATOZOA AFTER TEMPERATURE STRESS

Data are expressed as ng-atoms of phospholipid phosphorus per 10<sup>9</sup> spermatozoa  $\pm$  standard error.

The number of replicates of each species is shown in parentheses

Material	Ram (4)	Bull (6)	Boar (4)
Control supernatant	110.0 $\pm$ 12.8	166.7 $\pm$ 25.5	33.7 $\pm$ 7.0
Cold-shocked supernatant	256.1 $\pm$ 4.1	484.2 $\pm$ 19.4	121.8 $\pm$ 13.9
Frozen-thawed supernatant	496.7 $\pm$ 65.2	663.2 $\pm$ 72.7	214.5 $\pm$ 10.5
Untreated spermatozoa	2127.3 $\pm$ 93.0	2074.0 $\pm$ 166.4	2852.4 $\pm$ 304.0

Approximately 2  $\mu$ g-atoms of phospholipid phosphorus were extracted from 10<sup>9</sup> untreated ram or bull spermatozoa. The amount was greater for boar spermatozoa,

TABLE 2  
COMPOSITION OF PHOSPHOLIPID RELEASED FROM RAM, BULL, AND BOAR SPERMATOCYTES AFTER TEMPERATURE TREATMENT

Each lipid value is expressed as a percentage of the total phospholipid released. s.e., standard error. Values which are significantly different from the control are indicated by asterisks

Component	Ram			Bull			Boar		
	Control	Cold-shocked	Frozen-thawed s.e.	Control	Cold-shocked	Frozen-thawed s.e.	Control	Cold-shocked	Frozen-thawed s.e.
Spingomyelin	13.3	14.1	1.3	11.6	9.8***	10.3**	32.4	23.5***	23.4***
Phosphatidylserine	2.7	3.5	0.2***	0.6	0.8	0.8	6.8	2.9**	4.4**
Choline plasmalogen	32.7	40.2***	40.7***	46.2	46.0	42.6**	4.8	8.0***	9.4***
Phosphatidylcholine	15.1	17.7***	18.7***	18.5	17.8	19.0	11.9	30.1***	32.8***
Ethanolamine plasmalogen	4.4	4.2	3.6***	5.6	5.4	6.1	21.1	11.9***	13.6***
Phosphatidylethanolamine	1.9	1.5	5.4***	1.8	1.6	2.8**	5.9	11.5***	13.2***
Cardiolipin	8.0	1.0	†	2.3	1.9**	2.2	1.9	0.6	2.1
Other lipids†	21.9	17.8	17.4	13.4	16.7	16.2	15.2	11.5	1.1

\*\*\* $P < 0.01$ .      \*\*\* $P < 0.001$ .

† This includes material remaining at the origin in both the first and second dimension of the thin-layer chromatographic analysis, any phosphatidic acid, proteolipid, or phosphatidyl inositol present, and trace amounts ( $< 1\%$ ) of a number of unidentified phospholipids.

‡ Insufficient for statistical analysis.

but in spite of this less phospholipid phosphorus was released into the supernatant under control and treatment conditions than was the case with bull or ram spermatozoa. In each instance, a significant release of phospholipid occurred after cold shock with a further amount released after freezing and thawing; the latter varied from 7.5% (boar) to 32% (bull) of the total phospholipid content. However, in the boar, cold shock caused a greater relative increase in the phospholipid phosphorus release over the control. In spite of differences in the amount of phospholipid released, no differential effect on motility was observed. Samples kept at 30°C maintained maximal motility of 4, while the cold-shocked and frozen-thawed samples exhibited motilities of 1.0–1.5 and 0 respectively. Varying the duration of cold shock of ram spermatozoa from 5 to 120 min had no appreciable effect on phospholipid release.

The released material measured under these conditions was unlikely to be due to the extraction of unsedimented whole or fragmented spermatozoa, as the supernatants were subsequently passed through filters with pore sizes ranging from 0.2 to 2.0  $\mu\text{m}$  with little effect on the data shown in Table 1.

The bulk of the released material appeared to be particulate in nature and was not substantially due to contaminating seminal plasma or release of soluble lipoprotein. This was shown by high-speed centrifugation (100,000  $g$  for 75 min at 2°C) of the filtrate containing the material released from ram spermatozoa after cold shock; the sediment contained 93% of the phospholipid phosphorus. Samples of ram spermatozoa maintained at 30°C for 140 min released 20% more phospholipid than non-incubated samples. However, as 85% of this material was liberated during manipulation and prior to incubation, extending the incubation time beyond the 30 min of these experiments should not have influenced the validity of the cold shock data.

#### (b) Individual Phospholipids Released

The percentage amount of each component in the total phospholipid released into the supernatant is shown in Table 2. The variability in readings for the phospholipids comprising a low (<5) percentage of the total is due to the fact that these involved optical density readings under 0.05, with a high experimental error.

"Sphingomyelin" actually refers to the sphingomyelin area on the chromatogram. One of these spots ran like a standard sphingomyelin marker while the other component migrated with a slightly lower  $R_F$  in all solvent systems. Although this unidentified spot was of little quantitative importance in the extract from untreated ram and bull spermatozoa (2.3 and 2.6% respectively), it formed 9.9% of the untreated boar sperm extract and 31.6% of the material released from boar spermatozoa under control conditions, although this latter value decreased to 20.2% after cold shock. As there is evidence that sphingomyelin may run as two components (Rouser *et al.* 1963), the two values were combined and shown in Table 2 as "sphingomyelin".

#### *Ram*

There was a significant increase in the amounts of choline plasmalogen and phosphatidyl choline released after cold shock, and a slight further increase in phosphatidyl choline after freezing and thawing. The latter treatment also resulted in an increase in the percentage content of phosphatidyl ethanolamine. The decrease in

TABLE 3

EXTENT OF PHOSPHOLIPID LOSS FROM RAM, BULL, AND BOAR SPERMATOZOA AFTER COLD SHOCK AND FREEZE-THAWING AND THE COMPOSITION OF THE RELEASED MATERIAL AFTER SUBTRACTING CONTROL VALUES

Values for phospholipids liberated from the spermatozoa are expressed as: A, a percentage of the total phospholipid released after each treatment; or B, a percentage of the average absolute amount of that phospholipid present in an extract from untreated spermatozoa. Standard errors are shown after mean values for untreated spermatozoa

Phospholipid	Ram						Bull						Boar							
	Untreated sperm		Cold-shocked		Frozen-thawed		Untreated sperm		Cold-shocked		Frozen-thawed		Untreated sperm		Cold-shocked		Frozen-thawed			
			A	B	A	B			A	B	A	B			A	B	A	B	A	B
Sphingomyelin	11.4±0.6		14.7	8.9	14.2	22.6	10.5±1.3		8.9	12.9	9.9	22.5	12.6±1.4		20.1	4.9	21.7	10.9		
Phosphatidylserine	1.0±0.1		4.1	0.3	0.0	0.0	1.8±1.0		0.9	7.7	0.9	11.5	2.4±0.3		1.4	1.8	3.9	10.4		
Choline plasmalogen	40.8±1.1		45.9	7.7	43.0	19.1	44.8±2.2		45.9	15.9	41.4	22.1	9.8±0.8		9.2	2.9	10.3	6.7		
Phosphatidylcholine	17.3±1.7		19.6	7.8	19.7	20.7	15.5±1.0		17.3	17.2	19.2	29.6	37.4±1.5		37.1	3.1	36.7	6.2		
Ethanolamine plasmalogen	5.9±0.3		4.1	4.8	3.4	10.4	5.5±0.4		5.4	14.8	6.3	27.3	10.8±0.9		8.4	2.4	12.2	7.2		
Phosphatidylethanolamine	5.6±0.5		1.2	1.4	6.4	20.7	3.5±0.5		1.5	6.2	3.1	20.6	13.5±0.5		10.6	3.1	14.6	6.8		
Cardiolipin	5.6±0.5		0.0	0.0	0.0	0.0	3.4±0.9		1.7	7.7	2.2	15.3	3.0±0.7		0.1	0.1	2.2	4.6		
Other lipids	12.4		15.0																10.5	

percentages of phosphatidyl serine and ethanolamine plasmalogen after freezing were possibly due to the dilution of these components by other phospholipids lost from the spermatozoa. A decrease in cardiolipin content after cold shock was also apparent.

### *Bull*

The composition of phospholipids released after cold shock was not appreciably different from material released under control conditions, although decreases were observed in spingomyelin and cardiolipin. Freeze-thawing resulted in an increase in phosphatidyl ethanolamine and a decrease in the choline plasmalogen contribution to the released material.

### *Boar*

When boar spermatozoa were cold-shocked there were significant increases in the release of choline plasmalogen, phosphatidyl choline, and phosphatidyl ethanolamine, with some further increases observed after freeze-thawing. No other phospholipid was specifically affected by the more severe treatment. Decreases were observed in the relative amounts of sphingomyelin, phosphatidyl serine, and ethanolamine plasmalogen released after cold shock.

The results in Table 2 reflect the composition of the material released by the sperm after the temperature treatments and indicate any specific loss of individual phospholipids. However, the data for the cold-shocked and frozen-thawed spermatozoa include all phospholipid released and do not take into account material released under control conditions. Columns designated A in Table 3 show the composition of the sperm phospholipids after correcting for the release during the control incubation at 30°C. To arrive at these values the results in Table 2 were converted from a percentage to ng-atoms of phospholipid phosphorus released per  $10^9$  spermatozoa and the amounts released under control conditions were subtracted from the cold-shocked and frozen-thawed values. Columns B in Table 3 also show the net loss expressed as a percentage of the amount of the particular phospholipid present in untreated spermatozoa (see Table 1). It is evident from Table 3 that the extent of disruption of individual phospholipids varied with the species, and that the composition of the phospholipid material specifically released after the temperature treatment does not necessarily parallel the composition of the untreated spermatozoa.

Boar spermatozoa suffered the least overall loss of phospholipids. Bull spermatozoa lost a greater proportion of their phospholipid components after cold shock, in particular plasmalogens and phosphatidyl choline, although after freeze-thawing the release of the individual phospholipid components of ram spermatozoa closely paralleled that of the bull. Generally, a high proportion of choline rather than ethanolamine phosphoglycerides was released after treatment, but there did appear to be more general release of phospholipid in bull spermatozoa, judging from the amount of cardiolipin released.

## IV. DISCUSSION

The varying susceptibility of the spermatozoa of different species to cold shock has been usually attributed to intrinsic differences in the cell, rather than purely environmental factors (Lasley and Bogart 1944a, 1944b; Wales and White 1959;

White and Wales 1960). Quinn *et al.* (1968a), however, explained differences in the resistance to cold shock of ram spermatozoa collected by an artificial vagina and electrical ejaculation as being due to differences in the pH of the seminal plasma.

The release of membrane-bound phospholipid after cold shock and freezing agrees with theories (Mann and Lutwak-Mann 1955; Walton 1957; Wales and White 1959; White and Wales 1960) that the changes involve a loosening of the cell surface, causing release of proteins which would normally be bound with phospholipids in the membrane structure. It has been suggested that a sensitizing factor exists in the fluids of the seminal vesicles of bulls (Fulka *et al.* 1965) and possibly other accessory glands in different species (Morita and Chang 1970). This factor is presumably antigenic (Matousek 1968) and is absorbed onto bull spermatozoa (Fulka *et al.* 1965). It would be highly dependent for its effectiveness on the structure of the sperm membrane and therefore on the phospholipid conformation. From an examination of acrosomal alterations in boar spermatozoa after cold shock, Pursel *et al.* (1972) postulated that the morphological changes may affect the membrane-antigen relationship. Presumably this would be caused by the loss of phospholipid from the membrane, with either accompanying loss of protein or at least a profound effect on protein and membrane conformation and therefore on binding ability.

Other workers have examined the composition of spermatozoan phospholipids. The results presented here for the ram are in reasonable agreement with those of Scott *et al.* (1967) and Neill and Masters (1972) and those for boar spermatozoa agree with the data of Johnson *et al.* (1969). Differences between present findings and some of those mentioned in Section I are probably due to differences in the amount of total phospholipid extracted and the analytical techniques employed. Pickett and Komarek (1964) have found that bull spermatozoa released more total lipid after freezing than did ram spermatozoa. It is unlikely that the released material is due to the breakdown of cytoplasmic droplets because practically all the droplets would remain in suspension during the initial centrifugation and thus be removed in the supernatant (Harrison and White 1972). In any case, any residual droplets would contain only small amounts of phospholipid which is of similar composition to that of the untreated spermatozoa (Poulos and White, unpublished data) and therefore would not be responsible for the specific release observed.

Only ram and boar spermatozoa are specifically affected by cold shock, the choline phosphoglycerides being first affected, and to a greater extent than other phospholipids. The major components released from ram and boar spermatozoa after cold shock were choline plasmalogen and phosphatidyl choline respectively. Phosphatidyl ethanolamine was lost from boar spermatozoa on cold shock; however, the more severe treatment of freeze-thawing was required to release this phospholipid from ram and bull spermatozoa. In almost all instances the amount of the various phospholipids released after cold shock increased substantially after freeze-thawing (Table 3), suggesting more extensive membrane breakdown. Thus the percentage amount of some phospholipid components, and in particular phosphatidyl ethanolamine, was considerably higher in material released after freeze-thawing than after the milder cold shock treatment. Possibly due to their location within the cell, the more severe conditions were required to effect their release. The decreases in percentage amounts observed, particularly in sphingomyelin and phosphatidyl serine after



cold shock and freezing, was most likely due to dilution with other phospholipids released by the treatments. The virtual absence of cardiolipin in the material released from cold-shocked ram and boar spermatozoa is of particular importance. As an essential component of the inner mitochondrial membrane, its presence in the released material would indicate breakdown of the midpiece. It thus seems unlikely that this has occurred, at least in ram and boar spermatozoa, which agrees with earlier electron microscope evidence (Quinn *et al.* 1968b). The data shown in Table 3 confirm that boar spermatozoa suffered the least overall breakdown, whereas bull spermatozoa lost a large proportion of their plasmalogens and phosphatidyl choline after cold shock; after freezing the disruption in ram and bull spermatozoa was comparable.

Electron microscope studies have indicated that the acrosome is specifically affected by cold shock and freezing (Quinn *et al.* 1968b; Pursel *et al.* 1972; Jones and Martin 1973; Darin-Bennett and White, unpublished data). Quinn *et al.* (1968b) found that most of the acrosomal damage was confined to the anterior and terminal parts, with little apparent change occurring in the smooth portion. The nature of the acrosomal change appeared as a swelling and blebbing, the latter probably being associated with a loss of material. Vesiculation and even total loss of the boar acrosome after cold shock has been described by Boender (1968) and Pursel *et al.* (1972), and an irregular acrosomal outline loosely enveloping the anterior part of the sperm head has been reported in bull spermatozoa after thermal shock (Hancock 1952).

Perhaps the specific phospholipids released from ram and boar spermatozoa are acrosomal membrane components. These are detected in the supernatant, at least in the ram, in a particulate form and are presumably part of the vesiculation and "blebbing" observed by Healey (1969). Lovelock (1955) suggested that the cause of the mechanical fracture of membranes after cold shock was that lipids were rendered brittle at low temperatures under the stress of differential thermal contraction. He found that phosphatidyl choline was removed as a result of cold lysis of red blood cells and that sensitivity was not altered by removal of protein but by amounts of cholesterol and phosphatidyl choline present. Lovelock (1954) found that loss of lipids in an indiscriminating manner appeared to cause no great harm; in contrast, loss of phospholipid selectively rendered the cell "metastable" and subject to rapid lysis on cooling to less than 20°C, lysis and the loss of phospholipids being manifestations of the same general phenomenon.

Damage after freezing in protective diluents is greatest in boar, less in ram, and least in bull spermatozoa (Healey 1969; Watson and Martin 1972). The fertility of frozen spermatozoa follows a similar species pattern (Emmens and Martin 1961; Emmens and Robinson 1962; Lightfoot and Salamon 1970) and the results can be correlated with the pattern of phospholipid release.

It is suggested that cold shock and freeze-thawing may affect the acrosomal phospholipids specifically, or cause more general loss of phospholipids from the spermatozoon, the former being the more vital factor in reducing fertility. The release of non-specific phospholipid membrane components, particularly cardiolipin, from cold-shocked bull spermatozoa may indicate that a proportion of the spermatozoa of this species are broken down; the remaining whole spermatozoa may possess acrosomes which are virtually undamaged after cold shock and only slightly affected by freeze-thawing. On the other hand, ram and to a greater extent boar acrosomal

phospholipids may be specifically affected by these temperature treatments, leaving other structures relatively undamaged. The release of specific phospholipids could cause membrane changes and determine the relative species susceptibility, both structurally and functionally, to cold shock. The effect is heightened by freeze-thawing, a process which probably also damages cell structures. Protective diluents would therefore have a greater effect in preventing general disruption of bull spermatozoa rather than in acting on specific phospholipid loss from the spermatozoa of the other two species.

It is postulated that the presence of the normal phospholipid complement gives rise to a certain membrane conformation that allows binding of protective agents, e.g. egg yolk. Specific loss of phospholipids, brought about by cold shock or freeze-thawing, would alter the membrane conformation and therefore alter its ability to bind protective diluents. It is concluded that it is the loss of specific phospholipids, rather than the absolute amount of phospholipid lost, which affects the behaviour of the spermatozoa under conditions of cold shock and freezing.

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