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# Lectin Binding Affinities of Egg Coats and Sperm of the Freshwater Zebra Mussel, Dreissena polymorpha

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# Lectin Binding Affinities of Egg Coats and Sperm of the Freshwater Zebra Mussel, *Dreissena polymorpha*

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by
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Abstract

Carbohydrates have been shown to be an important component of cell-cell interactions such as sperm-egg association and binding. To investigate this process in the zebra mussel, *Dreissena polymorpha*, carbohydrate moieties associated with sperm and surface coats of eggs were probed with the FITC-conjugated lectins Concanavalin A (Con A), Wheat germ agglutinin (WGA), *Lens Culinaris* (LCA), and *Bandeirea simplicifolia* (BS-II). When treated at 30μg/ml, egg coats stained positive for glucose (Con A), mannose (Con A and LCA), and N-acetylneuraminic acid (WGA), but not N-acetyl-D-glucosamine (NAG)(BS-II). WGA stained the entire sperm, while Con A, LCA, and BS-II staining localized to the acrosome. For live eggs stained at 3 minutes post-insemination, discrete fluorescence was observed at sperm-egg binding sites in the presence of BS-II. Eggs preincubated for 20 minutes in 5mM glucose, mannose, glucose + mannose, or NAG were inseminated and assayed for sperm binding. Binding was significantly reduced by NAG compared to other sugars and controls. Incubation of eggs with sodium periodate does not interfere with fertilization, indicating that there are no surface carbohydrates which are essential to fertilization. These data suggest NAG plays a significant role in sperm-egg binding in *D. polymorpha*.

## Introduction

Surface interactions of gametes result in sperm association and binding, the first step in the fertilization process. The ova of many animals have an extracellular surface investment coat composed of glycoproteins. These glycoproteins can be peripherally embedded in the plasma membrane or "free" molecules deposited on the cell surface by the egg or follicle cells. For example, in mammalian species the investments include the cumulus oophorus, the corona radiata, and the zona pellucida, from the outermost to innermost layer (Talbot 1985). In the sea urchin, the egg investments are composed of a jelly coat and a vitelline layer (Kinsey *et al.* 1980). In each of these groups, the egg coat is known to be important in the initial association of the sperm and egg, as well as an initiator of the sperm acrosome reaction. In sea urchins, the jelly coat is responsible for initiating the acrosome reaction, causing a release of hydrolytic enzymes and concurrently preparing it to fuse with the egg. Sperm binding then occurs at the vitelline layer. Eggs devoid of a jelly coat but with an intact vitelline layer are not capable of binding sperm unless the sperm have been previously acrosome reacted (Kinsey *et al.* 1980). In the mammalian system, the acrosome reaction takes place in association with the zona pellucida after primary binding (Cherr 1986). Sperm then penetrate the zona pellucida and subsequently bind and fuse with the egg plasma membrane. Nucleus incorporation commences along with a host of events in the egg cytoplasm (Snell and White 1996).

Carbohydrates are known to be involved in other types of cell-cell associations, such as in mate recognition of rotifers, algae, and ciliates (Snell and Morris 1993). Carbohydrates have also been proposed as gamete recognition molecules in a variety of invertebrates and vertebrates and has been found on the sperm and/or the egg, depending on the system. In mammals, sea urchins, bivalve molluscs and marine shrimp the carbohydrate involved in sperm-egg association is

a terminal component of a glycoprotein on the egg surface (Snell and White, 1996; Vacquier *et al.*, 1995; Focarelli and Rosati, 1995; and Clark *et al.* 1994). This carbohydrate associates with a protein on the sperm surface, which leads to sperm binding and onset of the fertilization process (Ahuja, 1985; Snell and White 1996). Two terminal carbohydrates on a glycoprotein which appear to be critical in mammalian fertilization are galactose in β-linkage and N-acetylglucosamine. In the ascidian, *Ciona intestinalis,* the sperm and egg both appear to have a glycoprotein important to gamete interactions. In this paper we will show that the freshwater zebra mussel has a sperm-associated carbohydrate moiety that appears to contribute to sperm-egg interactions. Furthermore, there are no hexose carbohydrate components on the egg which are uniquely cued for fertilization.

Phillips et al. (1985) suggest that "the variety in gamete structure and function may have evolved as a mechanism for reproductive isolation." Thus, the use of different carbohydrates and proteins could assure species specific sperm-egg binding. In related species, often the sperm will associate with the egg, but binding sufficient for sperm entry will not occur. This also introduces the idea of surface components of sperm and eggs as vehicles of evolution and for speciation.

Lectins were used in this study to identify carbohydrate components of sperm and egg surfaces. Lectins are proteins which are produced in plants, and in a few animals which bind to specific carbohydrate linkages. The lectins we used were chosen because they had been shown to be involved in gamete recognition in other organisms. Conjugated to a fluorescent tag, these molecules can be used to observe carbohydrate involvement and interactions in cell processes. Because they are often non-toxic, lectins can be used on live cells as well as fixed samples. Lectin activity can be blocked by introduction of saturating amounts of the sugar or sugars for which it binds specifically. In this way, apparent binding can be evaluated for veracity.

Carbohydrate studies were conducted on gametes of *Dreissena polymorpha*, a species exotic to the United States. Introduced in Michigan in 1986, zebra mussels now occupy areas of the Mississippi River to New Orleans, LA, illustrating the fecundity of this species (Dietz, personal communication, 1996). In addition to their high reproductive capacity, *D. polymorpha* is an excellent experimental animal for fertilization studies. As a broadcast spawning animal, gametes are released into the external environment where eggs and sperm are obtained with facility. The eggs are 50-60μm in diameter and have a relatively clear cytoplasm in which internal events can easily be observed with a light microscope. Sperm are flagellated, with a conical head of 1.4 μm in length and 0.7 μm in width. The animals are induced to spawn with 5-hydroxy-tryptamine and produce large numbers of gametes (Ram *et al.* 1993).

This study was conducted to evaluate the carbohydrate components of the zebra mussel egg and sperm membranes. FITC-conjugated lectins were used with fluorescence microscopy to identify the presence or absence of given carbohydrates. Specifically we are addressing the role of carbohydrates in zebra mussel sperm-egg binding.

**Materials and Methods**

Animals were obtained from Portage Lake, MI and the Mississippi River near Baton Rouge, LA in the summers of 1995 and 1996. Zebra mussels were maintained, unfed, in a Living Stream at 8-11° C using artificial pondwater (0.05 mM KCl, 0.7 mM $MgSO_4 \cdot 7H_2O$, 0.8 mM $NaHCO_3$, 0.6 mM $CaCl_2 \cdot 2H_2O$). The evening prior to spawning, animals were isolated into individual cups and allowed to warm to room temperature overnight. This was done in order to reduce gamete cross-contamination which could result from a natural spawn in response to the temperature increase. In the morning, animals were rinsed and subsequently treated in test tubes for twelve minutes with a $10^{-4}$ M solution of serotonin (5-

hydroxy-tryptamine) in pondwater (PW) to induce spawning (Ram *et al.* 1993). Animals were rinsed twice with PW and left in their respective test tubes with a small amount of fresh PW. The males would spawn 15-20 min after serotonin exposure and the females would begin to spawn 60-90 min post-serotonin. At the first sign of egg release, female mussels were isolated in crystallizing dishes in order to provide the females with more space, which has been observed to promote egg release (Nichols, personal communication).

For a fertilization series, 100µl of sperm were added to 10mL of eggs and gently mixed. At time points of 0, 5, 30, and 60 min, a 1-mL sample was taken from the beaker bottom where eggs had settled. For binding studies, 200µl of sperm were diluted in 10ml of pondwater and then 0.5mL of this solution was added to 0.5ml of eggs in a 1.5 ml cryovial. At three min post-fertilization, eggs were fixed by adding to the cryovial a volume of fixative (1.6% paraformaldehyde, 10% methanol, and 0.1% glutaraldehyde) equal to the volume of PW. After one hour, the sample was rinsed twice with a TAPS buffer (5.5 mM TAPS, 0.8 mM NaCl, 0.145 mM KCl, 0.8 mM $Na_2SO_4$, 0.887 mM $MgSO_4 \cdot 7H_2O$, 1.32 mM $NaHCO_3$, 1.19 mM $CaCl_2 \cdot H_2O$, .25% chloramphenicol, and 0.01% sodium azide) and stored at 4° to be used later for labeling. The chloramphenicol and sodium azide were used to prevent bacterial contamination.

Before labeling, the TAPS buffer was exchanged three times with phosphate buffered saline (Humanson 1967) containing 0.05 % BSA (bovine serum albumin) in order to reduce nonspecific binding. Four FITC-conjugated lectins were used for sugar specific labeling of egg coats and sperm; Con A, derived from Concanavalin A; LCA (*Lens Culinaris*); WGA (Wheat germ agglutinin, *Triticum vulgaris*); and BS-II (*Bandeirea simplicifolia*-II). Con A binds specifically to α-D-glucose and α-D-mannose, LCA binds to α-D-mannose, WGA to N-acetylneuraminic acid and β-N-acetyl-D-glucosamine, and BS-II to α- and β-N-acetyl-D-glucosamine. The labelled

lectins were prepared in stocks of 1mg/mL of deionized water and 30µl of this solution was added to 1ml of fixed eggs and allowed to incubate for 20 minutes. The samples were then rinsed three times with phosphate buffered saline (PBS) and observed using a Nikon Optiphot equipped with epifluorescence. Appropriate sugars were used as control blocks for the stain (Table 1). A 200-mM sugar solution was prepared in PW and 1ml was incubated with 30µl of the stain for 20 min before being incubated with the eggs. Eggs were rinsed in PBS and imaged as before.

Experiments were also performed with live eggs and sperm to assess the influence of the lectin on sperm-egg binding. The experimental treatments were comprised of the following: 1) incubation of live eggs with 30 µl lectin for 20 minutes prior to insemination with subsequent rinse in PBS; 2) introduction of sperm and 30 µl lectin to the egg simultaneously; and 3) control fertilizations in PW. For each condition, samples were fixed three min post-insemination as above. Sperm binding was evaluated with a Nikon Diaphot equipped with differential interference contrast (Nomarski) and epifluorescence. Sperm binding was assayed at a focal plane of the egg giving the largest diameter in sharp focus with a 40x or 60x DIC objective. Assuming random distribution of sperm on the egg surface, based on observations of Misamore et al., 1996, only sperm bound in this plane were counted. Counts were extrapolated to the whole egg on the basis of the calculated surface area of both the optical section and whole egg. (Surface area of sphere $=4\pi r^2$, thus S.A of egg = 11310 $\mu m^2$. Area of 10 µm optical [DIC] section = Area of a rectangle with a width of 10µm and a height of $\pi d$, i. e., $10(60)\pi$= 1885 $\mu m^2$. Thus, 11310/1885 = 6. Therefore, for every count of sperm around the periphery of the egg, the actual presumed number of bound sperm was six times greater.) All of the above staining procedures were performed with each of the four lectins.

Rock shrimp egg envelopes (*Sicyonia ingentis*) were used as positive controls for lectin staining. Envelopes were provided by Dr. Pat Glas (EPA Lab, Gulf Breeze,

Florida). These envelopes were chosen as representatives of biological material with known lectin binding characteristics for the lectins selected (Pillai and Clark 1990). A volume of 200 µl of shrimp egg envelopes were added to 800 µl of PBS, and centrifuged to a pellet. The egg envelopes were stained according to the procedure above and evaluated for agreement with the findings of Pillai and Clark. This confirmed that the lectins used were specific for the appropriate carbohydrates.

Fresh sperm spawned into PW were used for labelling. Sperm were centrifuged in microfuge tubes for two min at 4000 rpm. Thirty µL of lectin were introduced into 1mL of sperm, incubated 25 min and washed three times with PBS. Sperm labelling was evaluated on a Nikon Optiphot equipped with epifluorescence.

Ability of sugars to block sperm binding was investigated with glucose, mannose, glucose + mannose, or NAG. The first trial was conducted with a 10mM sugar solution in pondwater and a second with a 5mM sugar solution. The concentration of sugar in the second trial was lower to reduce potential osmotic stress on the eggs. One ml of the sugar solution was added to 50µl eggs and allowed to incubate for ten min before the addition of sperm. Sperm were diluted 400µl in 10ml PW and then 0.5 ml were added to 0.5ml eggs in the sugar solution. Eggs were fixed in 1.6% paraformaldehyde after three min and sperm binding was assessed on an equatorial plane using an inverted Nikon DIC microscope. Numbers of sperm bound on the equatorial plane were adjusted to be representative of the numbers of sperm bound to the entire surface area of the egg. All data were analyzed using an ANOVA test.

## Results

*Lectin Binding Characteristics of Sperm and Egg*

Upon staining with Con A, the egg coat fluoresced brightly when compared to the egg cytoplasm (Fig 1A and B). Staining was restricted to the egg coat as

evidenced by optical sectioning in the confocal microscope (Fig 2). When the lectin was preincubated with glucose, mannose, or glucose + mannose, the staining was diminished. Fluorescence was significantly reduced when glucose was used as a block, increasingly diminished with mannose, and maximally reduced with glucose + mannose. The intensity of labelling was evaluated by eye in the microscope and in photographs (Table 1). Eggs treated with LCA or WGA also fluoresced brightly in the egg coat. However, no fluorescence was observed with BS-II (Table 1). Thus, N-acetyl-D-glucosamine (NAG) is not present on the egg coat. However, glucoside, mannoside, and N-acetylneuraminic acid (sialic acid) linkages are suggested by positive staining with ConA, LCA, and WGA respectively. WGA stains for N-acetylneuraminic acid as well as NAG. The fluorescence observed with WGA was most likely related to the presence of sialic acid, since BS-II, which only stains for NAG, did not label the coat.

Sperm were also probed with the lectins and the acrosome area of the sperm specifically fluoresced with Con A, BS-II, and LCA (Fig 3). WGA, however, fluoresced along the entire surface of the sperm membrane. This suggests that a sperm has N-acetylneuraminic acid residues along the entire membrane and shows no localized prominence. Live sperm stained with WGA became immotile after exposure to fluorescence. Because the other three lectins are localized to the acrosome the carbohydrate they tag may be involved in sperm-egg binding, but neither mannose nor glucose is a likely candidate as a binding sugar because these sugars are also present on the surface of the egg.

*Sperm-Egg Binding*

The number of sperm bound to eggs was affected by the presence of each lectin during fertilization. A difference in levels of sperm binding was observed in some of the eggs treated with lectins when compared to the control group. This difference was dependent on both the lectin used and the treatment performed. An

ANOVA comparing sperm binding between the control, simultaneously treated and preincubated treated eggs was performed. Appropriate multiple comparisons were then performed between groups. For LCA, the simultaneous sperm-egg treatment bound a significantly lower number of sperm than either the control or the preincubated eggs in every trial. The results for the preincubated eggs compared to the control are variable for each trial. On average, the eggs which were preincubated with LCA bound fewer sperm than the control. Trials with BS-II showed that the difference between the binding with the simultaneous and preincubated eggs was not significant. In fact, in two out of three trials, the BS-II did not significantly differ between the control and either treatment. Eggs preincubated with Con A had a significant increase in binding in each of the three trials when compared to both conditions of simultaneous introduction and the pondwater control. This may be due to the dual binding sites of Con A. However, when the lectin was introduced concurrently, there was no significant difference in sperm binding from the control. For the WGA trial, the results were particularly interesting. When the egg was preincubated with WGA, there was no sperm binding in any of the three trials (n=90 eggs / trial). Sperm were present in the areas between the eggs, however. When the stain was introduced simultaneously with the sperm, there was an extremely low level of binding (94 bound sperm for 270 eggs), although more than for the preincubation trial. This may be linked to the observation that the lectin labels the entire surface of the sperm, which may indirectly affect sperm binding.

*Lectin Binding in Inseminated Eggs*

During these experiments, we also looked at the eggs which were stained with the lectins simultaneous with sperm introduction. The Con A, LCA, and WGA fluoresced in the entire egg coats, as before for uninseminated eggs. In contrast, the eggs stained with BS-II fluoresced only where the sperm had bound to the egg (Fig 4A and B). This suggested that NAG, the sugar stained by the lectin BS-

II, may be involved in the binding or recognition of sperm and egg. In addition, the label was left on the surface of the egg even after the sperm head has entered the egg. (Figure 4C and D). An aperture where the nucleus passed through the egg coat and membrane was visible with the fluorescent patch left on the egg surface.

*Attempts to block sperm binding with sugar preincubation*

To observe the effectiveness of sugars in blocking sperm binding, the sperm and eggs were preincubated with sugars. Theoretically, the sugar should occupy the binding sites on the egg coat which the sugar on the acrosome of the sperm would normally bind to. Because sperm bind randomly across the entire egg surface (Misamore *et al.* 1996), the number of binding sites is probably large and if the osmolarity of the pondwater increases significantly, the sperm and egg interactions may be indirectly affected. In the control eggs, 22.3 sperm bound to the egg (Figure 5). When preincubated with glucose the number of sperm bound was 10.8 and with glucose + mannose it was 10.4. Mannose alone was 9.7 (Fig 5). Each of these was significantly different from number of sperm bound to the control egg, and possibly due to the osmolarity effects and not the action of the sugar. Each was statistically insignificant from one another. However, only 7.8 sperm bound per NAG treated egg, a number which was significantly different from the controls and sugar pretreatments except mannose.

**Discussion and Conclusions**

We have shown that N-acetylglucosamine may play a role in zebra mussel fertilization. This finding avers the current literature, which states that NAG is a likely candidate for modulation of sperm-egg interactions in other species. This carbohydrate is located on the surface of the sperm, probably attached to the plasma membrane through a glycoprotein.

This conclusion is supported by several observations. 1) The NAG-specific lectin, BS-II, labels the sperm only in the acrosomal region. It does not label any of the egg surface. This data concurs with the most simplistic sperm-egg binding scenario. 2) In eggs where the sperm and egg are bound, the NAG is present only in the area of sperm binding, in an area greater than the tip of the sperm surface, which corresponds to the interior of the acrosome. 3) Disrupting the carbohydrate structure of the egg does not adversely affect sperm binding. Treatment with sodium periodate, a chemical which cleaves hexose sugars at the site of two adjacent hydroxyl groups, producing aldehydes, does not significantly alter sperm binding properties (Misamore, personal communication). 4) Presence of 5 mM NAG in the egg solution for 20 min prior fertilization significantly inhibits sperm binding. Incubation with any sugar before fertilization decreases the level of sperm binding, probably due to osmotic factors. Although the concentration of sugar is outside of the normal range for this animal, sperm binding occurs. However, preincubation with NAG reduces binding even further than the other three carbohydrates combinations tested. This indicates that the sugar is occupying binding sites which prevent sperm from binding. The inconclusive results of the lectin experiment are likely to have been caused by the small sample size of 90 for each trial. An extended sample size would be required to further test for significance. Additionally, steric hindrance provided by the large lectin (1130 kD) may have also been a factor in the inconclusive results of that experiment by causing indirect effects.

The role of NAG in sperm-egg binding has further implications because it shows striking similarities in the process of fertilization between molluscs and mammals. In the mouse the glycoprotein is on the egg surface rather than the sperm surface as it is in the zebra mussel. The oligosacchiride is believed to be an O-linked terminal galactose in β-linkage or N-acetylglucosamine in β-linkage, or both. Three proteins are currently regarded as candidates as adhesion proteins on

the sperm (Snell and White 1996). In the sea urchin the 30.5 kDa protein, "bindin," has been shown to be the sperm molecule important to fertilization and it interacts with an O-linked glycoprotein receptor on the surface of the egg (Kinsey *et al.* 1980, Vacquier *et al.* 1995). Similarly, in shrimp a protein is found on the sperm while the critical carbohydrate moiety is located on the egg vitelline envelope (Clark *et al.* 1994). The only example of a glycoprotein associated with sperm is in *Ciona intestinalis*, an ascidian species (Casazza *et al.* 1987). The egg of this species also possesses a glycoprotein which is essential to fertilization. The sperm-egg binding specifics of the zebra mussel and mouse possesses a common putative glycoprotein, N-acetyl-glucosamine. The sperm of both the zebra mussel and ascidian carry a glycoprotein involved in sperm-egg binding. The molecules above may not be the only molecules involved in fertilization. As the process of fertilization is an essential one for the continuance of a species, it is likely that there would be a redundancy in function of several molecules.

There are many opportunities for future studies in this system. Further sugar block trials should be run in order to understand more precisely the profile of inhibition of binding. Also, we would like to locate the precise area of the sperm acrosome which is the source of N-acetylglucosamine. Using other time points, the morphology of the carbohydrate patch could be observed and investigation could be continued with transmission electron microscopy and gold-labelled lectins. Enzymes could also be used to alter the egg surface of the gametes. Trypsin could be used to destroy the proteins of the egg plasma membrane and sperm binding could be tested. Enzymes can also be used to determine the types of carbohydrate linkage which would provide more information about the relationship of fertilization tactics between zebra mussels and other animals. The long range goal of this research is to ascertain the molecules of the sperm and egg which are critical to

fertilization. From this information, the molecular interactions responsible for binding and recognition could be derived.

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## Figure Legends

Figure 1 This figure shows a phase contrast micrograph (A) and an epifluorescent micrograph (B) of an egg stained with a 30 μg/ml final concentration of Con A. The staining regions are localized to the membrane of the egg.

Figure 2 Figure 2 shows a series of fixed eggs which were stained with Con A under various conditions and visualized using confocal laser scanning microscopy. A) Digital pseudocolor image of an untreated egg stained with Con A. Note that the periphery of the egg is brightly fluorescing. B), C), and D) Each image shows an egg which was stained for 20 minutes using a 30 μg.ml final concentration of lectin after a 20 minute preincubation with the appropriate blocking sugar. B) preincubated with glucose, C) preincubated with mannose and D) preincubated with both glucose and mannose. The amount of peripheral staining decreases with each subsequent condition.

Figure 3 Phase contrast (A-D) and corresponding fluorescent (E-H) micrographs of sperm stained with the following FITC-conjugated lectins: BS-II (A,E), Con A (B,F), LCA (C,G), WGA (D,H). All lectins bind to the sperm acrosome (arrow). However, when incubated in WGA (D,H), the lectin was bound around the periphery of the entire sperm.

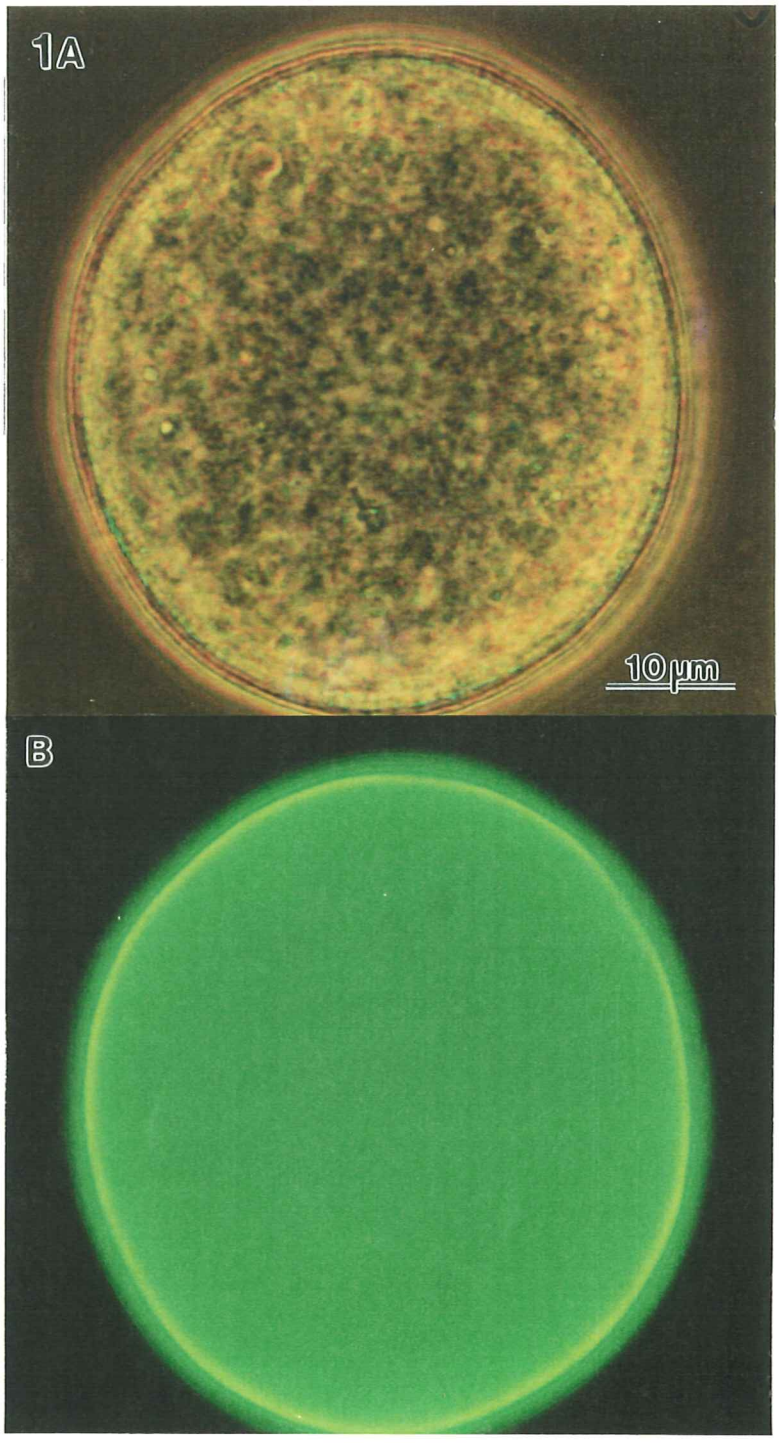
Figure 4 Phase and corresponding fluorescent micrographs of Dreissena polymorpha eggs stained with a FITC-conjugated lectin (BS-II) which is specific for N-acetyl-D-glucosamine residues. Bound sperm fluoresce brightly at the point of attachment to the egg (large arrow). In phase micrograph (C), one sperm can be seen bound to the egg surface while another can be observed in the cytoplasm of the egg

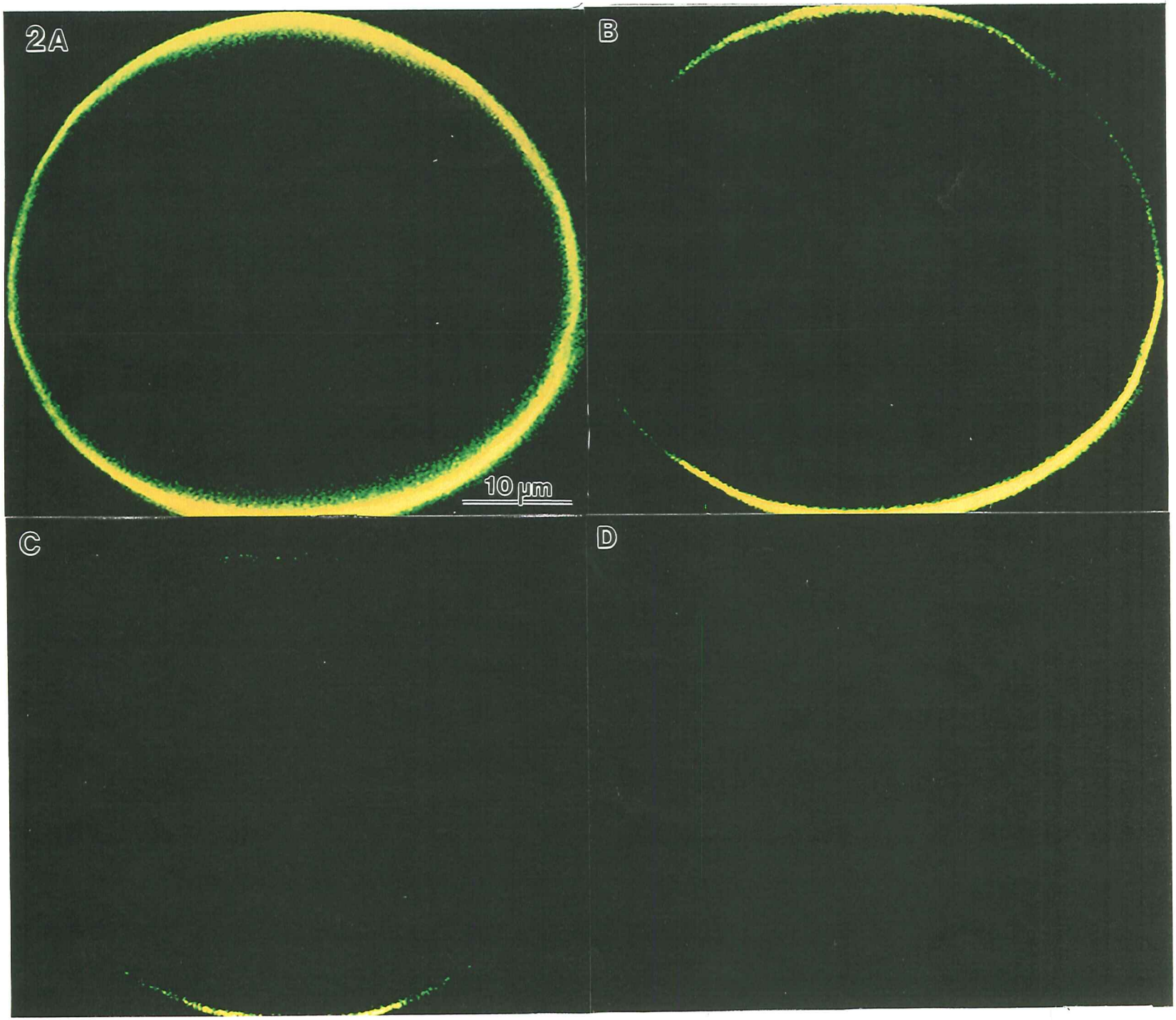
(arrow). The fluorescent image of this egg (D) shows the binding of BS-II to the site of sperm-egg attachment (small arrowhead), as well as the site of former attachment (large arrowhead) of the cytoplasmic sperm which has already begun to rotate in the cytoplasm.

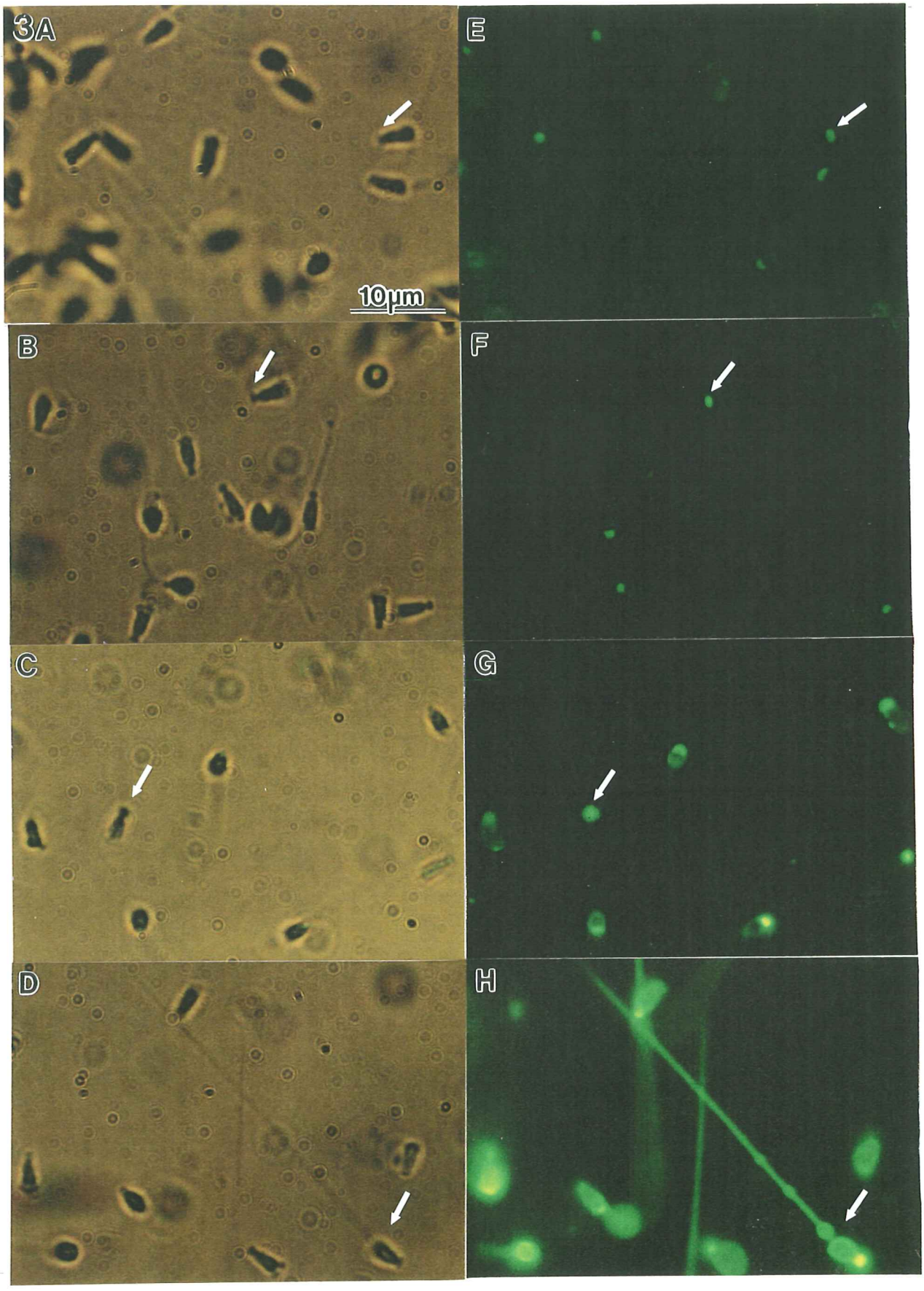
Figure 5 This graph shows the average number of sperm bound to an egg which was preincubated with a sugar for 20 minutes. The statistics show that the control eggs are significantly different than all of the sugar treated eggs. This is likely due to an osmolarity difference in the solutions. In addition, glucose and glucose + mannose were significantly different from the NAG, while the mannose alone was not. Overall, it appears that NAG may have somewhat more of an effect on sperm binding than the other sugar treatments.

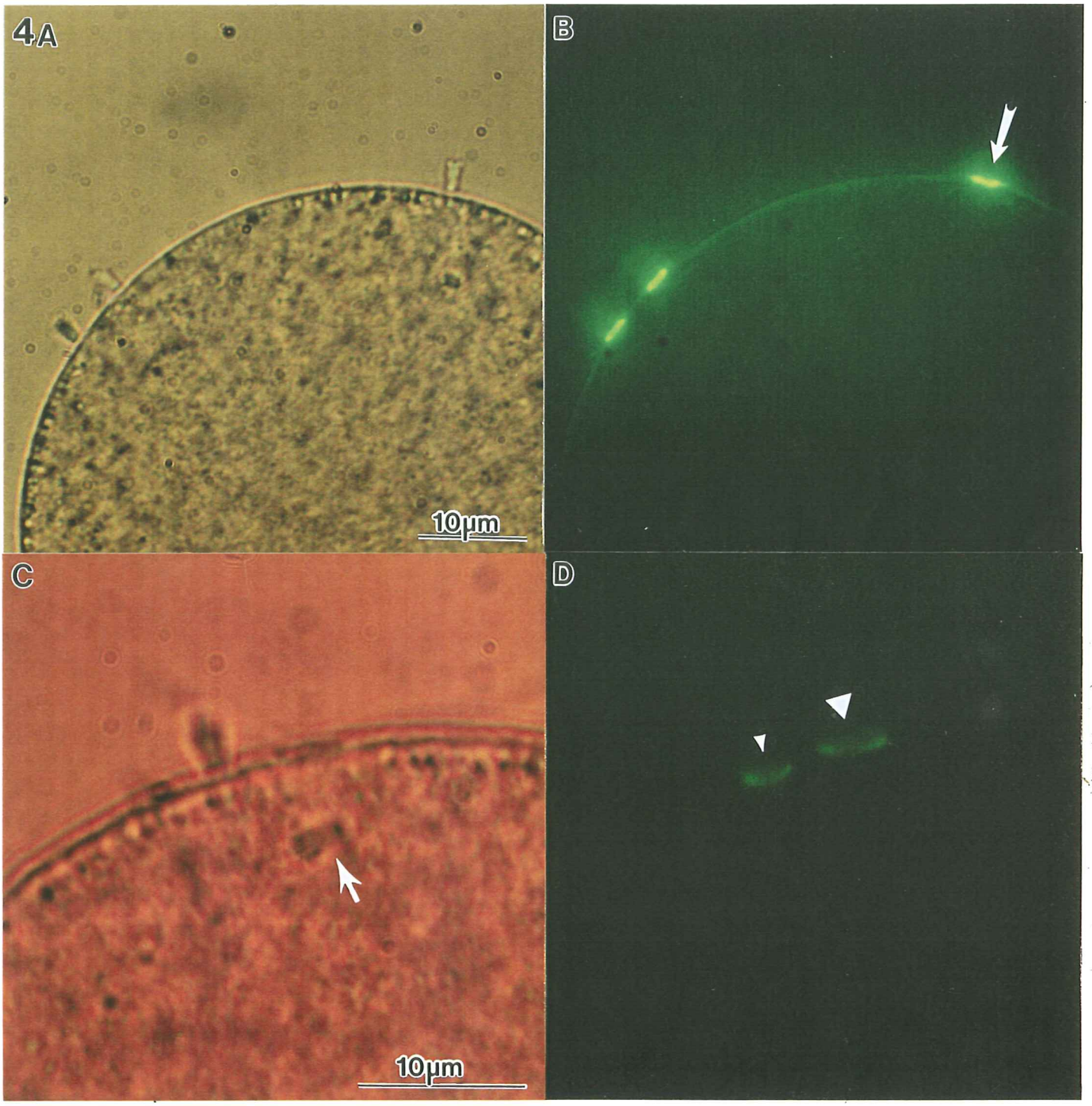
**Table 1**

| Relative Fluoresence Intensity | | |
|---|---|---|
| | Lectin | Lectin + Sugar |
| Con A | *** | glucose - |
| | | mannose -- |
| | | glucose & mannose --- |
| WGA | *** | N-acetylglucosamine ** |
| | | sialic acid -- |
| | | NAG & sialic acid ** |
| LCA | *** | mannose -- |
| BS-II | -- | NA |

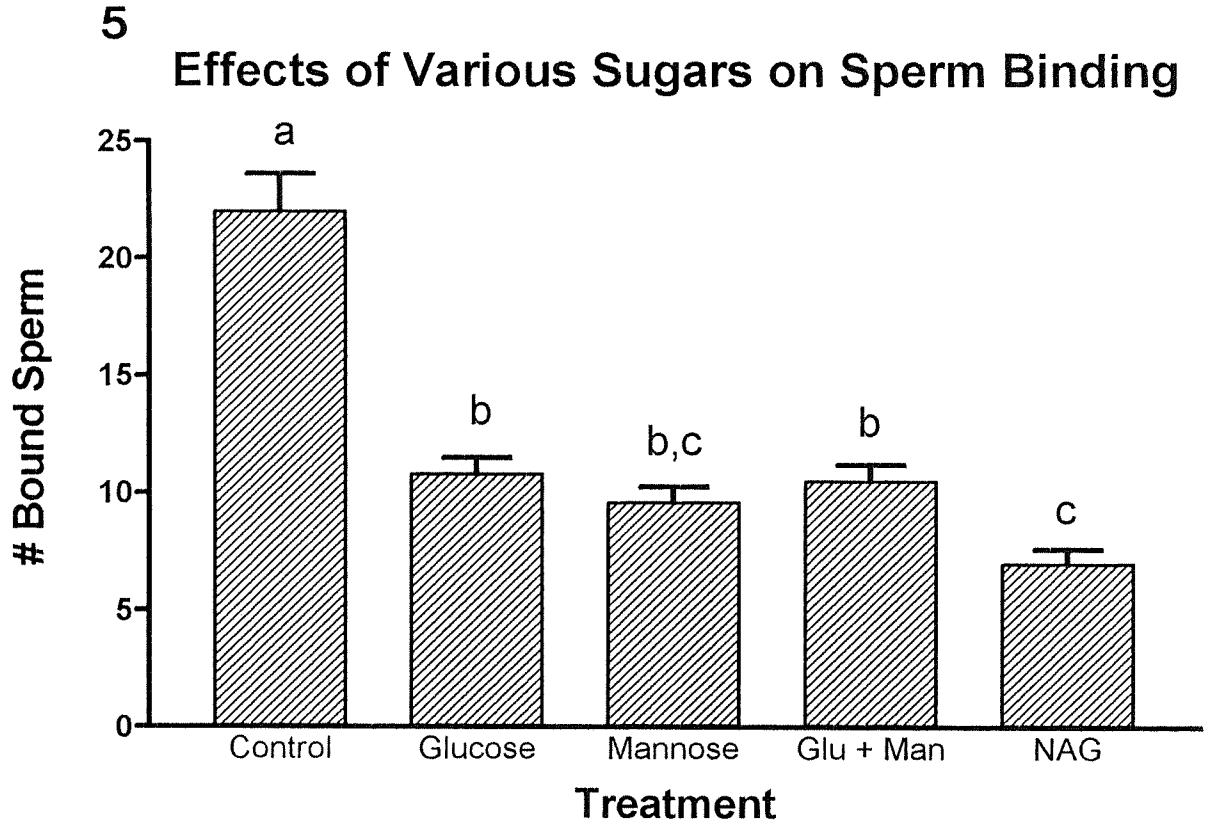

1A
10µm
B


2A
B
10 µm
C
D


3A
10µm
B
C
D
E
F
G
H


4A
10µm
B
C
10µm
D

5


Effects of Various Sugars on Sperm Binding
# Bound Sperm
25
20
15
10
5
0
a
b
b,c
b
c
Control
Glucose
Mannose
Glu + Man
NAG
Treatment