IDENTIFICATION AND DISTRIBUTION OF CARBOHYDRATES ON THE SURFACE OF GAMETES IN THE ZEBRA MUSSEL, *DREISSENA POLYMORPHA*

By

LINDSEY C. FALLIS

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Texas Christian University
Fort Worth Texas

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The invasive zebra mussel, *Dreissena polymorpha*, is a freshwater bivalve native to the Aral Sea in Europe. It was accidentally introduced into the Great Lakes region of the United States in 1986 through ballast waters (Herbert et al., 1989; Mackie et al., 1989). Since then, it has spread throughout the eastern half of the United States. Zebra mussels are now found as south as Louisiana and as west as Kansas. It has been predicted that the zebra mussel will eventually colonize the majority of permanent, inland freshwaters of the United States and southern Canada (Strayer, 1991; McMahon, 1992; Claudi and Mackie, 1993).

Natural History of the Zebra Mussel, *Dreissena polymorpha*

The affects of the zebra mussel can be devastating both ecologically and economically. Unlike other freshwater bivalves, zebra mussels attach to hard substrates via byssal threads. This attachment to hard substrates, also called biofouling, is a widespread phenomenon in marine environments as exhibited by barnacles and numerous bivalves including oysters and marine mussels. However, biofouling is relatively rare in freshwater habitats with the exception of the dressenid bivalves, including the zebra and quagga mussel. Zebra mussels are exceptional biofoulers that can grow to densities up to 700,000/m^2 (USGS, 2005). There are three main types of substrates that can be colonized by zebra mussels biological, geological, and anthropogenic.

Zebra mussels frequently attach to the hard outer shells of other aquatic animals, especially other freshwater bivalves. In many regions throughout the Great Lakes, native clam populations have decreased by up to 90% (Scholoesser et al., 1998). Zebra mussels either physically smother the bivalve they attach to or deprive them of nutrients. Zebra

mussels have also been shown to have a dramatic effect on phytoplankton abundance, water transparency, nutrient concentrations, and change benthic animal communities thus, dramatically modifying the food web in many freshwater lakes (Strayer and Malcolm, 2006). Zebra mussels also attach to man-made objects in the water column. This includes boat hulls, pilings, navigation buoys and, most commonly, water intake pipes to factories. The attachment of zebra mussels frequently causes the flow of water to be blocked. While the exact financial impact is debated, estimates range from millions to a billion dollars in cost to remove zebra mussels over the last decade.

Several aspects of the biology of zebra mussels contribute to their success as an invasive species. For example, their ability to attach to man-made structures, especially boats, allows for greater dispersal. This ability for dramatic and rapid dispersal is clearly demonstrated by the recent spread of the closely related quagga mussel (*Dreissena bugensis*) from the Great Lakes to Lake Mead in Nevada (Stokstad, 2007). Furthermore, the reproductive biology of zebra mussels promotes the broad spread of larvae.

Bivalve Fertilization

In most freshwater bivalves, fertilization of the female gamete occurs within the mantle cavity of the female. The developing embryos are maintained in a brood chamber and are not released into the water column until a later developmental stage (McMahon, 1991). *Dreissena polymorpha* is an exception among freshwater bivalves in that individuals broadcast spawn their gametes. Eggs and sperm are released directly into the external media where fertilization and larval development occurs. This reproductive strategy is more similar to marine bivalves that also exhibit broadcast spawning and external fertilization. The reproductive similarities between marine bivalves and zebra

mussels strengthens the theory that zebra mussels might be a secondary invasion into freshwater making them more closely related to marine bivalves then other freshwater bivalves (Ackerman et al., 1994).

Bivalves as a Study Organism

One scientific advantage to broadcast spawning is the increased ease of studying fertilization. This is clearly illustrated in the century-old use of sea urchins as the earliest model for fertilization. Similarly, many marine bivalves broadcast spawn and have been used as fertilization models. Studies have included the oyster *Crassostrea* spp. (Kyozuka and Osanai, 1985), the surf clam *Spisula soildissima* (Longo, 1973, 1976; Hylander and Summers, 1977), and the blue mussel *Mytilus edulis* (Longo and Anderson, 1969). Recently, *Dreissena polymorpha* has also been recognized as an ideal model for fertilization studies (Ram et al., 1993; Fong et al., 1995; Luetjens et al., 1998; Misamore et al., 1996).

Several other features of zebra mussels make them well suited for fertilization studies. Not only are zebra mussels broadcast spawners, but they release a relatively large number of gametes at one time and spawning is easily induced with external application of serotonin (Ram et al., 1993). Zebra mussel eggs have a transparent cytoplasm so that intracellular fertilization events can be viewed easily, and further more, fertilization events proceed relatively quickly (60-70 min post insemination) (Misamore and Lynn, 2000).

Zebra Mussel Fertilization

The fertilization events of D. polymorpha are like that of most invertebrate species. Sperm bind to the surface of the egg and undergo an acrosome reaction (AR).

During this reaction, the acrosomal vesicle fuses with the sperm plasma membrane, releases the contents of the acrosome, and exposes the previously hidden, inner acrosomal membrane to the egg surface. Sperm bind perpendicular to the egg surface and enter into the egg cortex through an insemination cone in a microfilament-dependent process (Misamore and Lynn, 2000). The sperm head and its associated mitochondria rotate 180°, and migrate laterally along the egg cortex. After the sperm nucleus enters the egg, a majority of the attached sperm axoneme is incorporated deeper into the egg cytoplasm in a microtubule-dependent process (Misamore et al., 1996; Misamore and Lynn, 2000).

After axoneme incorporation, the sperm becomes quiescent/nonmotile, the nucleus detaches from the mitochondria and axoneme, and the male chromatin begins to decondense. A pronuclear envelope forms around the decondensing sperm chromatin and the newly formed sperm pronucleus begins migrating toward the female chromatin. While the sperm chromatin begins its decondensation, the female chromatin completes meiosis, releases two polar bodies, and forms a female pronucleus. Pronuclear migration and first cleavage occurs within 90 minutes post insemination (PI) depending on temperature (Misamore et al., 1996; Luetjens and Dorresteijn, 1998).

Since gametes are released into the surrounding media, it is important for binding specificity to be great among broadcast spawned gametes. It has been suggested that the many different structures and functions of gametes could be used as a strategy for reproductive isolation (Phillips et al., 1985). Prominent among these mechanisms is the growing importance of carbohydrates in sperm-egg adhesion.

Carbohydrates as Cell Recognition Molecules

Carbohydrates as cell recognition molecules have been observed in even the simplest organisms, bacteria and viruses, as well as very complex, higher order organisms such as mammals. In the bacteria *Escherichia coli*, surface carbohydrates, α-D-mannose, bind to receptors on human oral epithelial cells, initiating infection. Viruses such as the influenza viruses have a surface "attachment protein" on the viral envelope, which recognizes and binds to a "virus receptor" on a target-cell surface (Brandley and Schnaar, 1986). Higher organisms such as mammals use carbohydrates in many different biological processes. Some of these events include cell migration, organ formation, immune defense, microbial infection, and embyogenesis, as well as fertilization (Sharon and Lis, 1989).

Carbohydrates are critically important in fertilization (Mengerink and Vacquier, 2001). Species and gamete specificity could be accomplished through the role of surface carbohydrates and proteins. Vertebrates, such as the anuran, *Xenopus laevis*, rely on glycosylated proteins on the egg to be present in order for sperm binding to take place (Olson and Chandler, 1999). In the mammalian system, three main glycoproteins have been identified in the mouse. These proteins have been designated the names ZP1, ZP2, and ZP3. It is believed that ZP3 mediates the initial binding of sperm and the acrosome reaction, while ZP2 is involved in adhesion to the sperm's inner acrosomal membrane (Dell et al., 1999).

Invertebrates seem to also rely on this mechanism for successful sperm-egg binding. The sea urchin, for example, relies on protein-carbohydrate interactions in the egg jelly and vitelline layers. Sperm first come in contact with the jelly layer where a

receptor on the sperm binds with a fucose sulfate polymer on the egg jelly and triggers the acrosome reaction in the sperm (Mengerink and Vacquier, 2001). Plants also use carbohydrates as a means of finding and binding gametes together. For example, the red algae, *Aglaothamnion oosumiense*, use at least two sets of carbohydrate-receptor systems in gamete recognition. These carbohydrate moieties are found on the male gamete, while the complimentary receptors found on the female gamete (Kim and Kim, 1999).

In the freshwater bivalve, *Unio elongatulus*, the glycoprotein, Gp273, has been isolated as the ligand molecule for sperm-egg interaction during fertilization. This glycoprotein has functional carbohydrate epitopes in common with the human zona pellucida glycoprotein, ZP3, and has been found to induce the acrosome reaction of human sperm (Patrizi et al., 2001; Focarelli et al., 2001).

Lectins

One way of studying surface carbohydrates is through the role of lectins. Lectins can be either a protein or a glycoprotein that have the ability to bind to a carbohydrate with great specificity and selectivity. Lectins have two or more carbohydrate binding sites and are not enzymes or antibodies. Lectins have also been found naturally on cell surfaces to mediate cell-cell interactions by combining with complementary carbohydrates on apposing cells. For example, the selectin family of adhesion molecules is characterized by a lectin-like mechanism that binds to carbohydrates on the surface of neighboring cells facilitating cell-cell adhesion. Lectins are thought to play a key role in various biological functions including normal and pathological processes (Sharon and Lis, 1989).

Objective

Most other studies on zebra mussels have focused on the physical mechanisms of fertilization (Misamore et al., 1996) while the role of carbohydrates has yet to be determined. The objective of this study is to identify the surface carbohydrates on *D. polymorpha* gametes and to analyze the possible role of these carbohydrates during fertilization.

MATERIALS AND METHODS

Animal Collecting and Handling

Zebra Mussels (*Dreissena polymorpha*) were collected from Ann Arbor, Michigan. Animals were kept in aquaria filled with artificial pond water (PW) (0.1 mM KCl, 0.7 mM $MgSO_4$, 0.8 mM $NaHCO_3$, 0.6 mM $CaCl_2$). Animals were kept at 9°C and unfed until needed for use. Twenty-four hours prior to use, the animals were isolated in 120-ml specimen cups containing cold PW and allowed to acclimate to room temperature over night (~21°C). Isolation ensured that no cross mixing of gametes occurred prior to induction of spawning and premature release of gametes due to increases in temperature. All procedures were carried out at room temperature (20-22°C).

Prior to spawning, individual zebra mussels were rinsed with deionized (DI) water and transferred to 25-ml flat-bottom tubes. Spawning was induced by submerging the animals in 0.2 mM 5-hydroxytryptamine (serotonin) for 12 minutes (Ram et al., 1993). Animals were then rinsed twice with DI water and covered with PW. Males typically began spawning 5-10 min post serotonin treatment. After the release of gametes, males were removed from the flat-bottom tubes, in order to minimize damage to sperm caused

by continued filtering of the water by the male. Females generally began to spawn 0.5-1.0 hr post serotonin treatment. When females started to spawn, they were transferred to 70 x 50 mm crystallizing dishes filled with PW. A second batch of animals was spawned and sperm from the second spawning was used in fertilization experiments to ensure viable sperm. Spawned eggs were used within 2 hrs and sperm from the second spawn were used within 30 min.

Gamete Handling

For fertilization experiments, freshly spawned eggs and sperm were added to a 10-mL beaker. Final concentrations of eggs were approximately 30,000 - 40,000 eggs per female and sperm concentrations were roughly 10^3 - 10^6 sperm per male. Microscopic observations were achieved by placing egg/sperm mixture on a slide and four silicone grease spots were used to support a cover slip to prevent the eggs from being smashed. Fertilization events and timing were determined by visual observation and digital photography. Fixed time points were also used to characterize the timing of events.

To fix samples, egg/sperm mixtures were added to a paraformaldehyde fixative (4% Paraformaldehyde, 5.0 mM TAPS, 0.2 mM KCL, 2 mM NaCl, 1.8m M Na_2SO_4, 1.25 mM $MgSO_4$, 2.0 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 20% MeOH) in a 1:1 ratio. Time points were taken at 1, 2, 5, 7, 10, 15, 20, 25, 30 min intervals. Samples were fixed for 2-24 hrs and washed twice with mussel buffer (5mM TAPS, 0.8mM NaCl, 0.145mM KCl, 1.8mM Na_2SO_4, 0.887mM $MgSO_4 \cdot 7H_2O$, 1.32mM $NaHCO_3$, 1.9mM $CaCl_2 \cdot 7H_2O$, pH=7.6) and kept at 4°C until later use.

Lectin Labeling and DNA Staining

Lectins used in this study were as follows: wheat germ agglutinin (WGA), concanavalin A (ConA), *Lens culinaris* (LcH), and *Griffonia simplicifolia* (GSII) (Table 1). Lectins were prepared from stocks of 1mg/ml in DI water. For labeling individual gametes, eggs and sperm were mixed with 30 μg/ml FITC (fluorescein isothiocyanate) conjugated lectin (EY Labs, San Mateo, CA) for 10 min. To determine the localization of the lectin during fertilization, eggs and sperm were mixed and labeled with 30 μg/ml of FITC conjugated lectin 1 min post insemination (PI). Lectin labeled samples were allowed to set for a minimum of 30 seconds before live observations or for subsequent fixation in the paraformaldehyde fixative.

To visualize genetic material, samples were added to bisbinzimide (Hoechst 33342, Sigma Chemicals, St. Lewis, MO) or DAPI (Invitrogen/Molecular Probes, Carlsbad, CA) immediately before viewing. Samples were stained with 1μg/ml final concentration of Hoechst 33342 or DAPI and allowed to set for a minimum of 5 min.

Induction of the Acrosome Reaction

To induce the acrosome reaction, thereby exposing the inner acrosomal components for lectin labeling, sperm were incubated in with 4μg/ml ionomycin Calcium, an ionophore, (Sigma Laboratories) for 5 min. Ionomycin opens Ca+ channels and is a tool for inducing the AR in other animals, including human sperm (Thomas and Meizel, 1988).

Quantification of Sperm Binding and Incorporation

To determine number of sperm bound, a focal plane at the equator of the egg was established using a 40x objective and the number of sperm bound to the egg surface in

Table 1. List of lectins and associated carbohydrates.

Lectin Name	**Lectin Abbreviation**	**Target Carbohydrate**	**Carbohydrate Abbreviation**
Triticum vulgare (Wheat Germ Agglutinin)	WGA	β-N-acetylglucosamine(1,4)>> N-acetylneuraminic acid >>N-acetylgalactosamine	GlcNacβ(1,4) Neu5Ac GalNAc
Canavalia ensiformis (Concanavalin A)	ConA	α-methyl-mannopyranside >α-D-Mannose > α-D-glucose > α-N-acetyl-D-glucosamine	
Lens culinaris	LcH	D-Mannose and D-Glucose	
Griffonia simpliciforlia	GSII	N-acetyl-D-glucosamine (A terminal only)	GlcNac

this focal plane was counted. This technique ensured that only sperm bound to the egg were counted and not sperm in close proximity to the egg surface at more polar fields of focus. A total of 40 eggs from three independent fertilizations (different males and females) were scored.

To quantify fertilization, the number of sperm nuclei inside each egg was counted. Fertilized sperm were distinguished from bound sperm using two criteria. First, the position of the sperm relative to the egg (on the surface versus inside the egg cytoplasm) was used as an initial determining factor. Secondarily, for sperm in more polar planes of focus, where distinguishing exact position of the sperm relative to the egg surface is more difficult, the state of the sperm chromatin was used. The chromatin of bound sperm remains tightly packed with distinct lateral margins. The chromatin of fertilizing sperm has begun to decondense and their later margins become less defined (Misamore et al., 1996, 2006).

Microscopy

Light and fluorescent microscopy was performed on a Zeiss Axiovert 200 and a Nikon Optiphot equipped with phase-contrast and epifluorescence. Digital micrographs were captured using a Zeiss AxioCam MRm and Axiovision software. Adobe Photoshop was used for final image processing.

Scanning electron microscopy was performed using a JOEL model JSM-6100 scanning electron microscope. The samples for electron microscopy were fixed with the same 4% Paraformaldehyde as before and washed with 0.03M sodium cocadylate buffer three times. Samples were additionally fixed with a 1:1 solution of 2% osmium tetroxide and 0.05M sodium cacodylate buffer for 1 hour. Samples were washed 3 more times

with 0.03M sodium cacodylate buffer. Each sample was then taken through an ethanol dehydration series consisting of washings with 30%, 50%, 95%, and 100% ethanol for 10 minutes each. Samples were then critical point dried and sputter coated with gold.

Statistical Analysis

For each of three trials, forty eggs were counted and scored for bound sperm, incorporated sperm, and/or GSII patches. Statistical analysis was performed using Microsoft Excel.

RESULTS

Lectin Labeling of Gametes

For both egg and sperm, a small group of commonly used FITC conjugated lectins was tested for affinity to certain areas of the gametes (Table 2). When stained with WGA, the lectin uniformly labeled the entire egg surface (Figure 1A). Similarly, ConA (Figure 1B) and LcH (Figure 1C) also uniformly labeled the entire egg surface. Conversely, GSII showed no detectable labeling of the egg surface (Figure 1D).

WGA labeled the entire surface of the sperm (Figure 2A). There was uniform distribution of the lectin along the length of the flagella, midpiece, and sperm head. There was a slightly greater concentration of label in the sperm acrosomal region. Unlike WGA, the other three lectins (ConA, LcH, GSII), only labeled the sperm acrosomal region (Figure 2B-D). They did not label any other portion of the sperm.

The labeling of the inner acrosomal membrane appears to be dependent on the state of the sperm acrosome. In sperm that had not undergone the acrosome reaction (unreacted) labeled with WGA, the outer surface of the intact sperm acrosome was

Table 2. Summary of lectin labeling of gametes

Lectin	Eggs	Sperm
WGA	Entire surface	Entire surface
Con A	Entire surface	Activated acrosome
LcH	Entire surface	Activated acrosome
GSII	No label	Activated acrosome

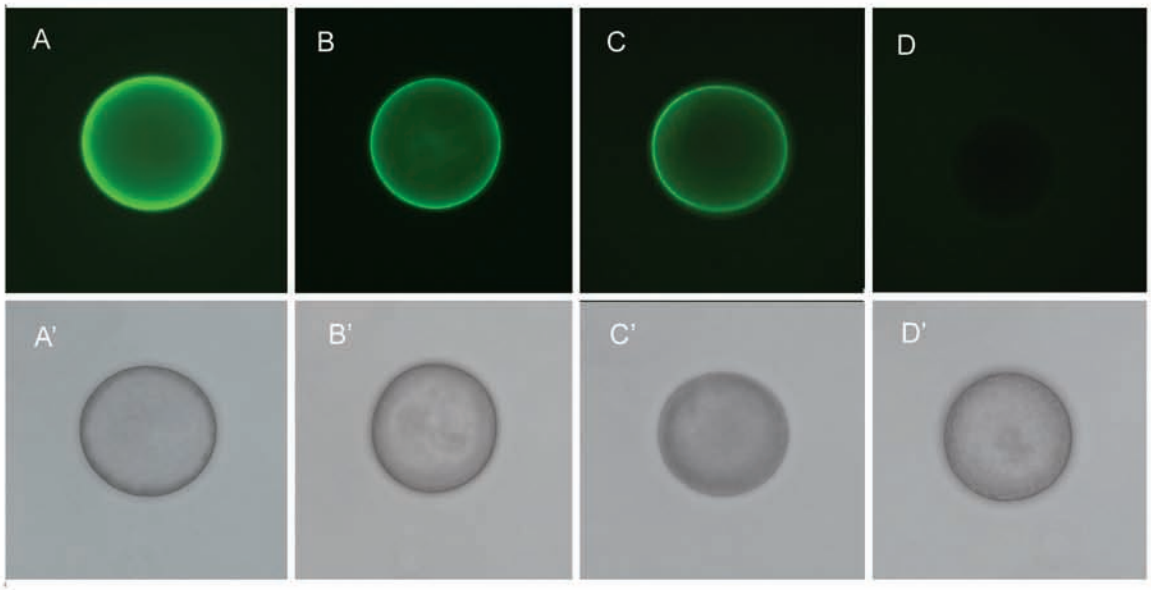

Figure 1. Fluorescent and phase micrographs of zebra mussel eggs prelabeled with the lectins Wheat Germ Agglutinin (WGA), Concanavalin A (ConA), *Lens culinaris* (LcH), and *Giffonia simplicifolia* (GSII). Fluorescent micrographs (A-D) with corresponding phase micrographs (A'-D') of eggs labeled with (A) WGA, (B) ConA, (C) LcH, (D) GSII. Lectin bound to the entire surface of the egg for all lectins (A-C, A'-C') except GSII (D, D').

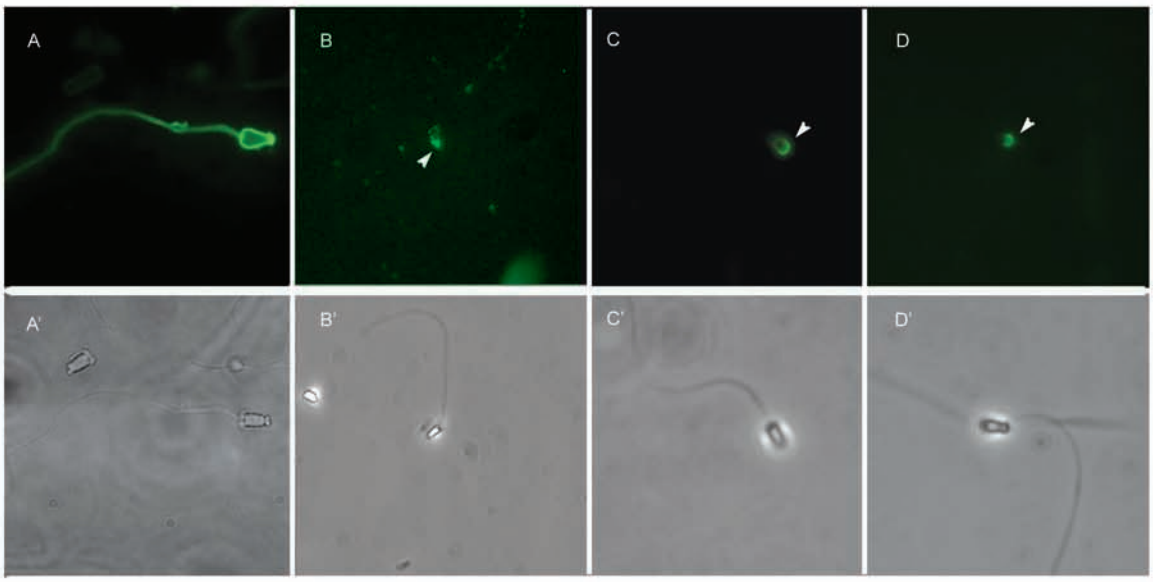

Figure 2. Fluorescent and phase micrographs of zebra mussel sperm prelabeled with the lectins Wheat Germ Agglutinin (WGA), Concanavalin A (ConA), *Lens culinaris* (LcH), and *Giffonia simplicifolia* (GSII). Fluorescent micrographs (A-D) with corresponding phase micrographs (A'-D') of sperm labeled with (A) WGA, (B) ConA, (C) LcH, (D) GSII. Lectin bound to the entire surface of sperm labeled with WGA (A, A'). ConA, LcH, and GSII labeled the inner acrosomal region (arrowhead) of acrosome reacted sperm (B-D, B'-D').

clearly visible and no intense labeling was observed on the inner acrosomal region. In sperm induced to undergo the acrosome reaction by treatment with ionomycin, WGA intensely labeled the inner acrosomal region (Figure 3B). GSII was unable to label unreacted sperm (Figure 3C), but intensely labeled the inner acrosomal region and acrosomal filament in acrosome reacted sperm (Figure 3D). Similar results were observed with ConA and LcH.

Additionally, a slight difference in acrosomal labeling was observed between spontaneously reacted acrosomes (Figure 2D) relative to ionomycin induced acrosome reacted sperm (Figure 3D). There was stronger labeling in the ionomycin induced acrosomes especially on the acrosomal filament. To verify that ionomycin was increasing the number or AR sperm and to determine the ability of ionomycin to induce the acrosome reaction, 100 sperm treated with ionomycin and not treated with ionomycin were labeled with WGA and scored for the presence of an intact acrosome. In the ionomycin treated sample, 90% of the sperm were acrosome reacted. In the untreated sample, only 38% of the sperm had undergone spontaneous acrosome reactions.

In summary, WGA labeled the entire surface of both eggs and sperm. Con A and LcH labeled eggs and only the inner acrosomal region of reacted sperm. GSII was the only lectin that specifically labeled just the inner acrosomal region of reacted sperm. Further characterization of this lectin was performed as described in the methods.

Distribution of GSII Labeling For Fertilizing Sperm

To determine the fate of the GSII-labeled inner acrosomal region of the sperm during fertilization, we mixed eggs and sperm together and added FITC conjugated GSII lectin 1 min PI. Immediately after sperm binding to the egg surface, the GSII labeling

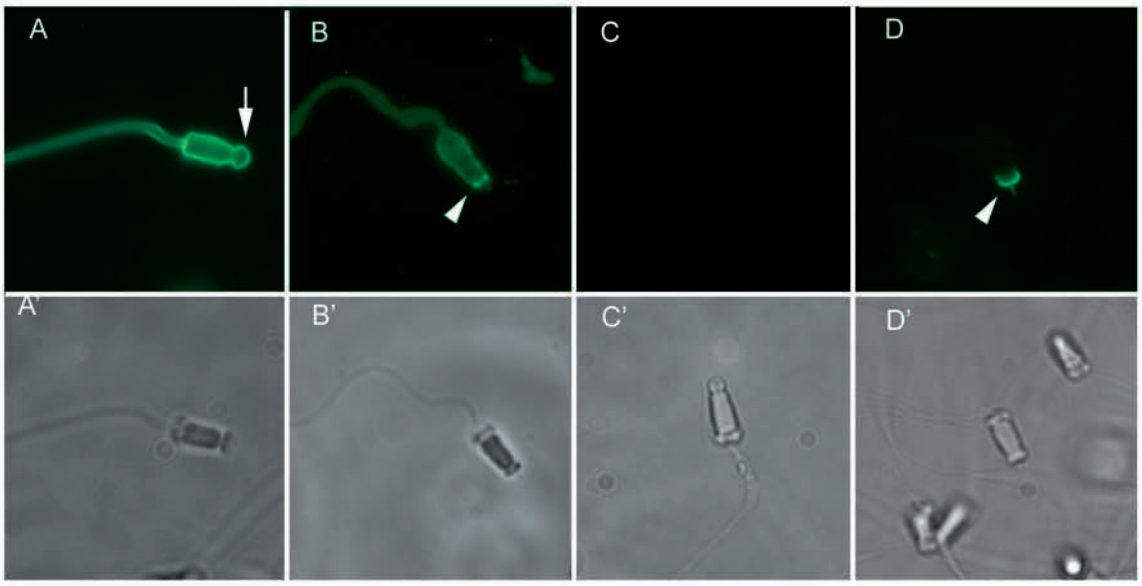

Figure 3. Fluorescent (A-D) and corresponding brightfield (A'-D') micrographs of acrosome reacted and unreacted zebra mussel sperm. Unreacted sperm labeled with WGA (A,A') showed uniform labeling over the entire sperm surface and an intact acrosome (arrow). Acrosome reacted sperm labeled with WGA (B, B') showed uniform labeling over the entire sperm surface with intense labeling in the acrosomal region (arrowhead). Unreacted sperm labeled with GSII (C,C') showed no labeling. Acrosome reacted sperm labeled with GSII (D,D') showed intense labeling of the inner acrosomal region including the acrosomal filament (arrowhead).

was detected between the sperm head and the egg surface (Figure 4 A'). This labeling was localized to a distinct region at the tip of the sperm where it contacts the egg surface (Figure 4A'). Under scanning electron microscopy, this region displayed a ring-like structure associated with the sperm acrosome bound to the egg surface (Figure 5C).

After the sperm was incorporated into the egg cytoplasm (Figure 4B''), the GSII labeling remained on the surface of the egg (Figure 4B', Figure 5A). With the entire sperm inside the egg, the GSII-label looked like a ring or "doughnut" on the surface of the egg when viewed perpendicularly (Figure 5B). We termed this characteristic labeling a "GSII patch." This patch remained on the egg surface throughout the early fertilization events and eventually degraded at approximately 30-40 minutes PI or later. At this point in the fertilization process, the female and male pronuclei are moving toward each other and are close to fusing. Even in the case of polyspermic eggs, all fertilizing sperm left distinct GSII patches on the surface of the egg.

Distribution of GSII of Non-Fertilizing Sperm

For the many sperm that bind to the egg, but do not fertilize it, the sperm eventually detach from the egg surface. To determine the timing of detachment, visual observation of detaching sperm were made. Detachment of non-fertilizing sperm from the egg surface occurred at approximately 10-15 minutes PI. This timing of sperm detachment roughly corresponds to the release of the first polar body and the initial sperm decondensation of fertilizing sperm.

When studying the localization of the GSII patch of non-fertilizing sperm, we observed that the sperm detached from the egg surface and took their associated GSII patch (Figure 6). Thus, when sperm are released off the egg surface, the lectin GSII is

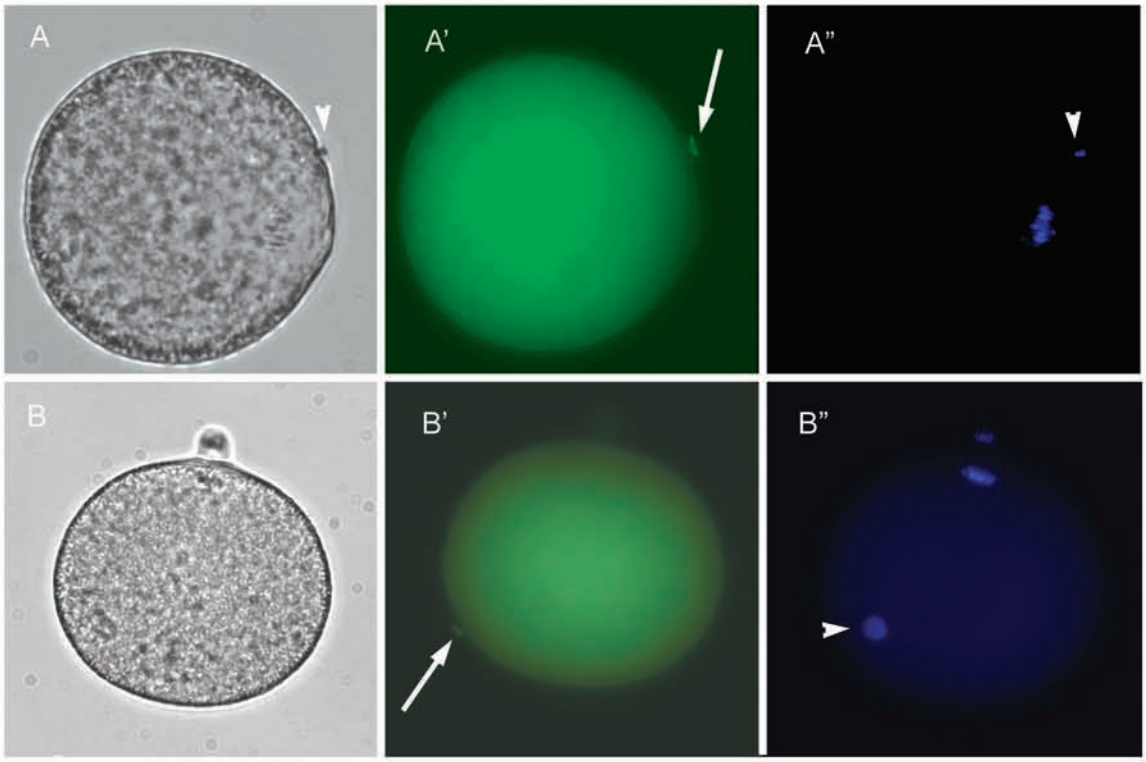

Figure 4. Fluorescent and phase micrographs of eggs and sperm labeled with GSII lectin (green) or DNA-specific stains DAPI or Hoechst 33342 (blue). (A-A") Corresponding images of a bound sperm on the egg surface at 5 min PI. A distinct GSII patch (arrow in A') was found between the egg and the bound sperm (arrowhead). (B-B') Corresponding images of an egg at 15 min PI with fertilizing sperm (arrowhead) undergoing nuclear decondensation. A distinct GSII patch (arrow) remained on the egg surface after the sperm nucleus entered the egg cytoplasm.

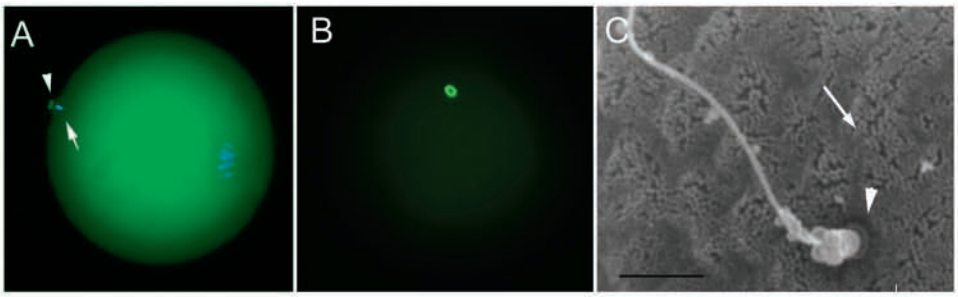

Figure 5. (A) Fluorescent micrograph of an egg 5 min PI dual labeled with GSII (green) and Hoechst 33342 (blue). The fertilizing sperm has entered the egg cytoplasm while the GSII patch (arrowhead) remains on the egg surface. (B) A confocal micrograph showing the distinct, doughnut-shaped GSII patch on the surface of an egg. (C) A scanning electron micrograph (3000X) of sperm attached to the egg surface. Notice the microvilli on the egg surface (arrow).

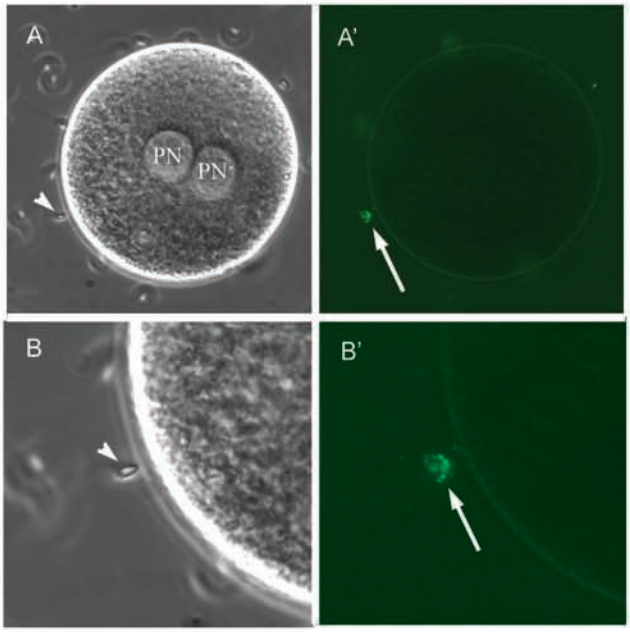

Figure 6. Corresponding phase (A, B) and fluorescent (A', B') micrographs of a sperm detached from the surface of an egg. (A, A') The fertilized egg shows fusing pronuclei (PN) with a sperm detached from the surface of the egg (arrowhead). The GSII patch remains associated with the detached sperm (arrowhead). (B, B') A higher magnification of the egg in A, A' showing a space separating the detached sperm (arrowhead) from the egg surface. The GSII patch remained with the detached sperm and not the egg surface (arrow).

released with the detaching sperm and does not remain on the egg surface as found with fertilizing sperm.

To quantify the relationship between fertilizing sperm, non-fertilizing sperm, and the location of their GSII patch, samples of fertilized eggs were fixed at various time points. There was a significant decrease in the number of bound sperm over time ($P<0.01$) (Figure 7A). The most dramatic decrease in numbers of bound sperm occurred between 10 and 15 min PI. Patch numbers also significantly decreased through time, having a very similar trend to that of bound sperm with a significant drop between the 10 and 15 min time points (Figure 7B). Incorporated sperm averages increased through time PI and then leveled off close to the expected amount of 1 sperm per egg (Figure 7C).

DISCUSSION

Carbohydrates are believed to be an integral part of many fertilization molecules (Mengerink and Vacquier, 2001). Considerable effort over the past decades have focused on specific proteins involved in sperm-egg binding such as bindin in sea urchins, (Vacquier and Moy, 1977), fertilin and izumo in mammals (Wassarman et al., 2004), Spe9 in *C. elegans* (Singson et al., 1998) and fus1 in *Chlamydomonas* (Misamore et al., 2003). More recently, efforts have focused on the role of carbohydrates in fertilization. While the extremely complex nature of glycobiology creates numerous obstacles, recent studies have illustrated the essential role of carbohydrates in fertilization (Mengerink and Vacquier, 2001; Wassarman, 2004; Dell, 1999). In mammals, modification of the carbohydrates located on ZP proteins has been shown to greatly alter sperm binding. Rankin, et al., (1998) showed that mouse sperm were able to bind to human ZP3 proteins

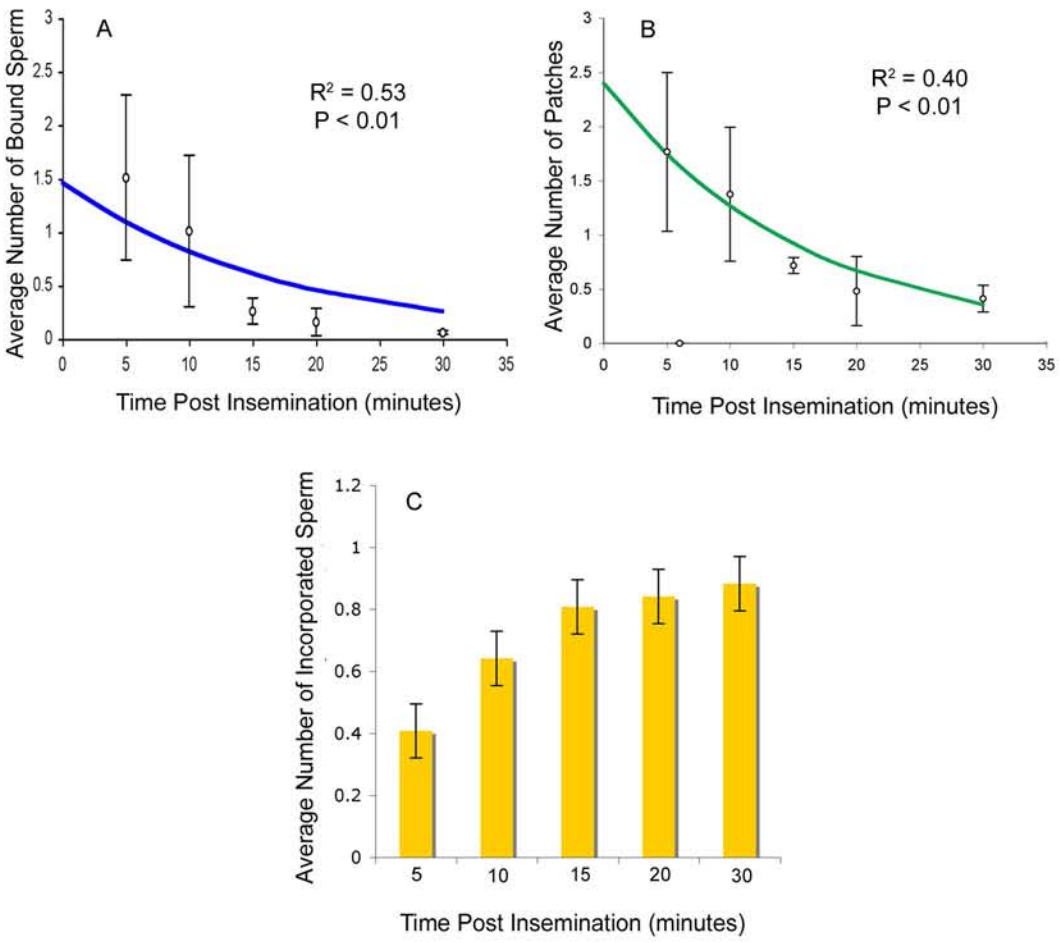

Figure 7. To determine the timing of sperm detachment, inseminated eggs were labeled with GSII and fixed at various time points PI. Sperm binding was assayed by counting the number of bound sperm and GSII patches in the equatorial focal plane. There was a significant decrease in the number of bound sperm and GSII patches over time ($p<0.01$). The numbers of bound sperm (A) decreased over time PI with the most dramatic decrease occurring between 10 and 15 min PI. A similar trend was observed in the numbers of GSII patches (B). The lines represent log-transformed regressions illustrating the downward trends. The number of incorporated sperm (C) increased over time PI and leveled off after 15 min PI. Bars - standard error.

expressed on mouse eggs showing that the mouse glycosylation on a human protein was sufficient to retain mouse species specificity.

In the present study we have begun to characterize the carbohydrates associated with the gametes of zebra mussels. We found similar carbohydrate moieties on both eggs and sperm while at least one unique moiety was associated only with sperm. Moreover, we were able to induce the acrosome reaction in zebra mussels with ionomycin.

Induction of the Acrosome Reaction

Ionomycin and other ionophores have been used in other organisms to induce AR in sperm. Most of these organisms have been mammals, including the human (Thomas and Meizel, 1988). However, this is the first time ionomycin has been used to successfully induce the acrosome reaction in the zebra mussel. Unfortunately, acrosome reacted sperm lost their ability to undergo fertilization. These results contrast with the high levels of fertilization observed in untreated sperm samples that contain numerous spontaneously acrosome reacted sperm. Here are several suggestions on why this may be happening.

First, ionomycin induces the acrosome reaction by creating an influx of calcium. When the sperm acrosome is opened, enzymes are released, which are believed to help sperm get through the egg jelly and vitelline coat (Dan, 1967; Franklin, 1970; Levine et al., 1978). If sperm acrosomes react before insemination, the enzymes needed to fertilize may be unavailable by the time a sperm comes in contact with an egg. The fertilizations observed in untreated sperm may be due solely to the unreacted sperm in the sperm samples. Second, the ionomycin may be opening other calcium channels on the sperm

surface, other than the acrosome, that might inhibit the sperms ability to fertilize. For instants, there may be calcium channels on the sperm flagella that dictate sperm motility. Sperm flagellar calcium channels are shown to be important for fertilization in other species (Suarez and Dai, 1995).

Another potential source for the prevention of fertilization in ionomycin treated eggs is the opening of calcium channels on the egg. It is impossible to eliminate all of the ionomycin from the sperm sample without damaging the fragile sperm during multiple centrifugation washes. In many species including sea urchins and mammals, calcium channels initiate the block to polyspermy preventing supernumerary sperm binding (Baker and Whitaker, 1978; Sun, 2003). Zebra mussels may employ a similar method.

Lectin Labeling of Gametes

Zebra mussel eggs are surrounded by a relatively small, transparent, outer jelly layer (Misamore et al., 1996). This layer is not overtly distinct and its potential role in sperm chemotaxis or induction of the acrosome reaction is unclear. None of the four lectins labeled the zebra mussel jelly layer, suggesting that none of the carbohydrates associated with these lectins are found in the egg jelly. In contrast to these results the lectin WGA bound to the outer most layer of the egg jelly of Xenopus laevis (Mozingo, 1999).

There is a more distinct vitelline envelope immediately surrounding the egg surface. Embedded in the vitelline layer are numerous microvilli that uniformly surround the egg surface. Three of the four lectins tested labeled the surface of zebra mussel eggs. This suggests that the sugars associated with WGA, ConA, and LcH are located on the

egg surface (Table 1). With all the lectins, the distribution appeared uniform. This supports the theory that zebra mussel eggs exhibit little or no polarity across the egg surface prior to fertilization since sperm binding can occur uniformly across the surface of the egg (Misamore et al., 1996). No obvious punctate labeling was observed suggesting no particular lectin was associated with only the microvilli. Additionally, zebra mussels do not exhibit the prominent vitelline spikes found in the closely related marine bivalve *Mytilus* (Focarelli, 1991). The exact location of the carbohydrate moieties associated with the lectins to the vitelline coat and/or microvilli will require additional research and electron microscopy.

WGA labeled the entire surface of the sperm including the acrosome suggesting that one or all of its associated sugars are found along the entire surface of the sperm (Table 1). In sea urchins, WGA was shown to specifically bind to sperm and was able to block fertilization (Vacquier, 1986). Since WGA labels the entire surface of both egg and sperm and sperm are only able to bind to the egg via their acrosome, the role of WGA-associated sugars as a specific gamete adhesion molecule is unlikely. Furthermore, Misamore et al. (1996) found that eggs pretreated with 10ug/ml WGA were still able to undergo normal fertilization.

ConA and LcH labeled the inner acrosomal region of the sperm in *Dreissena polymorpha* (Figure 2B,C) suggesting a potential role in sperm-egg binding. However, similar carbohydrate moieties are also found on the egg surface (Figure 1B,C) making the specific role of these carbohydrates in either sperm binding to an egg receptor or egg carbohydrate binding to a sperm receptor more difficult to confirm. However, inhibition

studies with these lectins and their associated sugars are needed to make this assumption valid.

GSII was the only lectin that specifically localized to only one gamete – the sperm (Figure 2D). Moreover, it appears to localize to the inner acrosomal region and the acrosomal filament that tightly bind to the egg surface during fertilization (Figure 2D, 3D, and 4C) (Misamore et al., 1996). This suggests GSII might play an important role in acrosomal binding to the egg surface and might serve as a useful tool for identifying its associated glycoprotein.

The acrosome of an unactivated zebra mussel sperm consists of a central actin-filled acrosomal filament and a complex electron-dense basal region. Upon undergoing the acrosome reaction, the inner basal region of the zebra mussel acrosome opens up to form a circular structure with the central acrosomal filament. Both the inner acrosomal membrane and the acrosomal filament bind to the egg surface during fertilization (Misamore et al., 1996). The activated inner acrosomal membrane forms a structure that corresponds nicely to the GSII patch reported here. Further analysis at the electron microscopy level is needed before exact correlation between the GSII patch and the inner acrosomal membrane can be made.

While the sperm nucleus, mitochondria, and flagella enter into the egg (Misamore et al., 2006), the GSII patch remains on the egg surface (Figure 4, 5) suggesting that the inner acrosomal membrane remains on the egg surface. This corresponds well with Misamore et al. (2006) that showed the WGA labeling is associated with the rest of the sperm plasma membrane that also remained on the egg surface following sperm incorporation.

The doughnut shape of the GSII patch suggests that fertilizing sperm pass through the central hole to enter into the egg cytoplasm. The center of the doughnut could be the remains of the plasma membrane found on the exterior of the remaining sperm body. In *Dreissena polymorpha*, sperm incorporation includes that of the sperm nuclear membrane and axoneme. The sperm plasma membrane and flagellar membrane is left associated with the exterior of the egg (Misamore et al., 2006). The unlabeled surface carbohydrates could be the center of the doughnut.

Sperm Detachment

After a time period of approximately 10-15 min PI, sperm detached from the outer surface of fertilized eggs. Based on live and epifluorescence observations, the GSII patch released from the egg surface with the detaching sperm (Figure 6). This suggests that the cleavage that is responsible for detaching bound sperm is not directly targeting the N-acetyl-D-glucosamine (GlcNac) moiety. The mechanism for sperm detachment may be targeting egg-specific proteins similar to the zona reaction in mammals where the egg receptor is modified (Hoodbhoy and Dean, 2004). Preliminary data suggests that a trypsin-like enzyme is used to release bound sperm as SBTI shows the release of bound sperm during zebra mussel fertilization (John Lynn, Louisiana State Univ., per. comm.). Similar results have been shown in sea urchins where a trypsin-like enzyme alters sperm binding sites (Longo, 1974). Whether this trypsin-like enzyme is targeting the GlcNac receptor on the egg surface or a completely different system, requires further investigation.

When sperm are followed through time post insemination, several trends are observabled (Figure 7). As expected, the amount of bound sperm per egg decreased

significantly over time post insemination (Figure 7A). Our data shows a general downward trend with a break at approximately 15 min PI. This break is consistent with the timing of sperm detachment from our visual observations. Similarly, the amount of incorporated sperm increased over time and then leveled off after 15 min PI (Figure 7C) suggesting no additional sperm incorporation can occur due to the detachment of non-fertilizing sperm.

The number of patches on the outer surface of the egg decreased significantly over time post insemination (Figure 7B). This data supports visual observations suggesting that patches are detached from the egg with detaching sperm. The number of patches was slightly higher than the amount of bound sperm. This is suggested by the presence of GSII patches left on the egg surface by fertilizing sperm.

Summary

In summary, a detailed description of the distribution of several carbohydrates on the surfaces of gametes was performed. Several of the lectins labeled both eggs and sperm while only GSII was specific to just one gamete. The GSII patch corresponds nicely with the inner acrosomal region of the sperm. The distribution of this patch during the fertilization process is described. Further work is needed to identify the specific glycoprotein associated with the GSII patch and its precise role in sperm-egg binding.

APPENDIX: GLOSSARY OF ABBREVIATIONS

AR – acrosome reaction

ConA – *Canavalia ensiformis*

DI – deionized

GlcNac – N-acetyl-D-glucosamine

GSII – *Griffonia simplicifolia*

LcH – *Lens culinaris*

PI – post insemination

PW – pond water

SBTI – soy bean trypsin inhibitor

WGA – wheat germ agglutinin

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VITA

Lindsey Fallis was born in Wellington, Kansas on March 10, 1983. She grew up in Harper, Kansas and graduated as valedictorian of her class from Chaparral High School in 2001. She received her Bachelor of Science degree in Biology from Texas Christian University in May 2005. Lindsey entered the biology graduate program in August 2005 at Texas Christian University, where she received her Masters of Science in Biology in August 2007. There she served as a teaching assistant for biology majors. Lindsey plans on continuing her education at Kansas State University where she has been accepted to a biology doctoral program in August 2007 under the direction of Dr. Ted Morgan.

Lindsey is a member of the American Society of Cell Biology (ASCB) and the National Geographic Society.

Lindsey is the daughter of Jim and Pam Fallis, sister of Lauren and Leanna Fallis.

ABSTRACT

IDENTIFICATION AND DISTRIBUTION OF CARBOHYDRATES ON THE SURFACE OF GAMETES IN THE ZEBRA MUSSEL, *DREISSENA POLYMORPHA*

By Lindsey Caroline Fallis, M.S., 2007

Department of Biology

Texas Christian University

Thesis Advisors: Dr. Michael Misamore, Assistant Professor of Biology
Dr. Giridhar Akkaraju, Assistant Professor of Biology
Dr. Ernest Couch, Associate Professor of Biology

The objective of this study is to identify the surface carbohydrates on *Dreissena polymorpha* egg and sperm and to analyze the role of carbohydrates during fertilization. The lectins WGA, ConA, LcH, and GSII were tested for affinity to both eggs and sperm. The surface of eggs labeled evenly with WGA, ConA, and LcH. GSII was the only lectin that did not label the egg surface. When sperm were treated with lectin WGA it labeled the entire surface, while ConA, LcH, and GSII only labeled the inner acrosomal region of acrosome reacted sperm. Therefore, GSII is the only lectin to label a carbohydrate moitey on the sperm and not on the surface of the egg suggesting N-acetyl-D-glucosamine may have a role in sperm-egg binding. When sperm pretreated with GSII was allowed to fertilize eggs a GSII "patch" is left on the surface. This patch corresponds to the area where the sperm binds to the egg surface. When a sperm does not fertilize the egg, it detaches from the surface with its corresponding GSII patch at approximately 15 min post insemination.