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Removal of surface glycoproteins and transfer among *Brachionus* species

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Abstract

Glycoproteins on the body surface of females of the rotifer *Brachionus plicatilis* are a key signal in their mate recognition system. When *B. plicatilis* Russian strain females were exposed to 50 mM EDTA or EGTA, several surface glycoproteins were removed. Females exposed to EDTA died, but remained intact and were used in mating bioassays with conspecifics males. Live control females elicited a male mating response in 21% of encounters, freeze-killed control females elicited responses in 23%, but EDTA extracted females elicited a mating response in only 5% of encounters. At least some of the EDTA-extractable proteins on the surface of females appear to be critical to male mate recognition. EDTA treated females could be exposed to proteins extracted from other females and some proteins re-attached to their body surface, restoring their attractiveness to males. SDS-PAGE of these proteins revealed 15–17 prominent bands, most ranging in molecular mass from 66 to 12 kD. The EDTA-extractable proteins were separated using ion exchange chromatography and each fraction was tested for its ability to restore female attractiveness. When proteins in fraction 22 were bound to females, they restored 80% of the females' ability to elicit male mating responses. Exposing EDTA treated females to bovine serum albumin or casein had no effect on their attractiveness to males. EDTA treated females from different *Brachionus* clades and species were exposed to proteins from fraction 22. Female attractiveness could be restored in most clades of *B. plicatilis*, but no transfer of mating attractiveness was observed to *B. rotundiformis* or *B. ibericus* females. Conspecific males treated with EDTA and exposed to proteins in fraction 22 could not be feminized and made attractive to other males. A sexual dimorphism in surface proteins therefore exists between *B. plicatilis* females and males. Successful transfer of glycoproteins critical in mate recognition is dependent on signal glycoprotein structure and the structure of the other proteins present on the surface of females.

Introduction

The taxon *Brachionus plicatilis* is actually a complex of several cryptic species (Gómez & Snell, 1996; Serra et al., 1997; Serra et al., 1998). Prior to 1995 only one of these was named (Segers, 1995), but now three are recognized by systematists (Ciros-Perez et al., 2001). Phylogenetic analysis of COI and ITS gene sequences suggests that there could be at least 11 more as yet unnamed species (Gómez et al., 2002; Derry et al., 2003; Suatoni, 2003). Many of these species are sympatric (Gómez & Serra, 1995; Gómez & Carvalho, 2000; Ortells et al., 2000), yet there is no evidence of hybridization or introgression between them (Ortells et al., 2000). These observations suggest that reproductive barriers among *B. plicatilis* species are well developed and effective. It also raises questions about the nature of the reproductive barriers, how they arose, and how they are maintained.

A key element in the sexual reproductive system of *B. plicatilis* is mate recognition by males of conspecific females. This is accomplished by contact chemoreception of a glycoprotein signal on the body surface of females (Snell & Hawkinson, 1983; Snell, 1989; Snell et al., 1995). There has been a long term effort to isolate and characterize the glycoprotein signal and its receptor, and to clone the underlying genes (Snell, 1998). In this paper, we describe how surface glycoproteins can be stripped from females by treatment with the chelator EDTA and re-attached to similarly treated females of the same or different species. This removal and re-attachment bioassay was developed for testing ion exchange chromatography and HPLC fractions for male mating activity. However, the technique also has allowed us to probe the phylogenetic distances over which transfer of these signal glycoproteins can occur and to develop hypotheses about the limitations on this transfer. We further have tested whether females of one species could be made attractive to males of a different species and whether males could be feminized by changing their surface glycoprotein profile. Such experiments are analogous to the strikingly successful epicuticular hydrocarbon transfer experiments in *Drosophila* (Coyne & Charlesworth, 1997; Blows & Allan, 1998; Etges & Ahrens, 2001). This approach has demonstrated that epicuticular hydrocarbons are essential elements of the *Drosophila* mate recognition system, that they are used as contact sex pheromones with information encoded in hydrocarbon composition and structure, and that there is epicuticular sexual dimorphism between males and females. We report here results of the application of a similar technique in rotifers.

Methods

The rotifers used in these experiments are part of the *B. plicatilis* species complex described by Gómez et al. (2002). The strains designated RUS, GP, AUS, CH, and L1 were originally collected from the Azov Sea (Russia), Gaynor Pond (Colorado, USA) Obere Halbjockchlacke (Austria), Tianjin (China), and Torreblanca (Spain), respectively, and maintained in the lab for many years as resting eggs. All are currently classified as members of the *B. plicatilis* morphospecies, but some clades are likely to be independent species. Strains LFL and IR2 were originally collected from Little Fish Lake, Nevada, and Indian Rocks Beach (Florida, USA, GPS 27.77° N, 82.68° W) [TS1] and are currently classified in the *B. ibericus* morphospecies. The ITS1 sequence of IR2 showed 100% similarity with the California1 populations of the ‚Almenara’ clade (Gómez et al., 2002, Genebank accession AF387222). The HAW strain is currently classified as *B. rotundiformis* morphospecies and was obtained from the Oceanic Institute in Hawaii, but its original collection site is unknown.

Rotifers were hatched from resting eggs and cultured in 15 ppt artifical seawater (Instant Ocean) at 25 °C on a diet of *Tetraselmis suecica* in 5 l bags that were lightly aerated. Constant fluorescent illumination of approximately 2000 lux was provided. Males and females in log-phase populations were filtered from about 200 ml of culture using a 68 μm screen and re-suspended in clean seawater. Experimental animals were isolated under a stereomicroscope at 10× magnification using a narrow bore micropipet and separated into Petri dishes according to sex in 5 ml seawater. Only vigorous, fast swimming males (ages unknown) were isolated and mated with young (<24 h old), non-ovigerous females.

The positive control mating bioassay was performed by placing 7–10 males and 4–6 live females into about 50 μl of seawater on the inverted top of a 96-well plate, which provides a flat, clear viewing surface. Mating behavior was videotaped for 5 min under a stereomicroscope at 10× magnification using a CCD camera. The number of male–female encounters and the number of matings initiated (circlings) by males were recorded in three replicate trials for each treatment. A second positive control was conducted using females that were killed by freezing at –80 °C for 1 h. Male matings with control females were compared to matings with females exposed to a variety of treatments. Surface proteins were removed from females by exposing them to 100 mM EDTA prepared in 2 ppt seawater for 15 min. Approximately 50 females were pipetted into a minimum volume in a nine spot glass depression plate. We used glass so that the plates could be baked overnight at 100 °C between experiments. Addition of 1 ml of 100 mM EDTA to the rotifers in the well dilutes it to about 50 mM EDTA which is enough to cause most females to stop swimming and fall to the bottom after about 15 min. As much of the solution as possible was then removed, being careful not to remove rotifers, and replaced with fresh EDTA solution for another 15 min of incubation. Females were then washed by serial transfer through three rinses of 2 ml of clean seawater, being careful to transfer minimum volumes to each well. At this point, females were immobile and 4–6 were transferred for a final wash to a well containing 2 ml clean seawater. EDTA treated females have a strong tendency to pick up proteins, so glass micropipets must be baked overnight at 100 °C between experiments and changed between each treatment. The 4–6 females then were transferred in about 20 μl to a spot on the 96-well lid to begin the bioassay. Care was taken so that females were arrayed towards the middle of the spot and not trapped in the surface tension or along the edges. About 7–10 young, fast males were transferred in minimum volume to the spot and excess seawater removed so that the spot was flat. If EDTA had not been completely removed in the washing steps, male swimming markedly slowed, rendering the replicate unusable. Mating behavior was videotaped for 5 min and scored as described above.

Females treated with EDTA were exposed to ion exchange fractions (see below) containing proteins to test their ability to elicit male mating. In this experiment, 6–8 EDTA treated females were transferred in minimum volume to a well in a 96-well plate. About 1–2 μl of the test fraction was added, followed by 20 μl of seawater to mix thoroughly, and incubated for 5 min. Females then were transferred to a well containing 2 ml of seawater for washing. Finally, they were transferred in minimum volume to the lid of a 96-well plate, males added, and the bioassay was conducted as described above. Treatments with EGTA, bovine serum albumen, and casein followed similar protocols. The active ion exchange fraction number 22 was boiled for 10 min to test its thermal stability.

Approximately 20–30 g wet-weight RUS clade biomass was filtered from mass cultures and re-suspended in 5 l of clean seawater for 3–4 h with aeration. Seawater was replaced with clean seawater every hour so that the rotifer guts were cleared. Proteins for ion exchange chromatography were extracted from rotifer biomass using 2× volume of 100 mM EDTA in 2 ppt seawater containing a cocktail of protease inhibitors (Roche Complete Mini protease inhibitor cocktail, 1 tablet/7 ml). The biomass was shaken on a rotary shaker for 1 h to solubilize surface proteins. Rotifers were separated from soluble proteins by decanting off the liquid, then centrifuging at 20,000 × g for 30 min at 4 °C. Supernatant was collected and EDTA was removed by ultrafiltration using a 10,000 Dal molecular weight cut-off filter that retained the proteins of interest. Proteins were re-suspended from the membrane in a 20 mM Tris–HCl buffer, pH 8.0, containing 50 mM NaCl. This solution was applied to Q Sepharose (Amersham Pharmacia) high performance ion exchange resin packed in a 1 cm diameter glass column to a height of about 3 cm. Proteins were eluted with a linear gradient from 50 to 1000 mM NaCl over 40 min in 1 ml fractions collected each minute. Samples were stored at –80 °C until tested for mating activity.

EDTA-extractable proteins were separated and visualized by SDS-polyacrylamide gel electrophoresis performed according to the protocol described by Snell et al. (1995). EDTA or NaCl was removed from electrophoresis samples by centrifugation with 10 000 Da MWCO filters. Proteins were re-suspended in DI water, then electrophoresis sample solution was added in a ratio of 1 to 3 parts sample volume. Proteins were separated on 12% acrylamide gels and visualized with Sypro Orange protein gel stain (Molecular Probes) according to the manufacturer's protocol.

Results

When *B. plicatilis* Russian females were treated with either EDTA or EGTA they elicited about 4-fold fewer mating responses from conspecific males (Fig. 1). Males initiated mating (circled) freeze-killed females with the same propensity as live females. When EDTA treated females were exposed to proteins in ion exchange fraction 22 (see below), their ability to elicit male mating

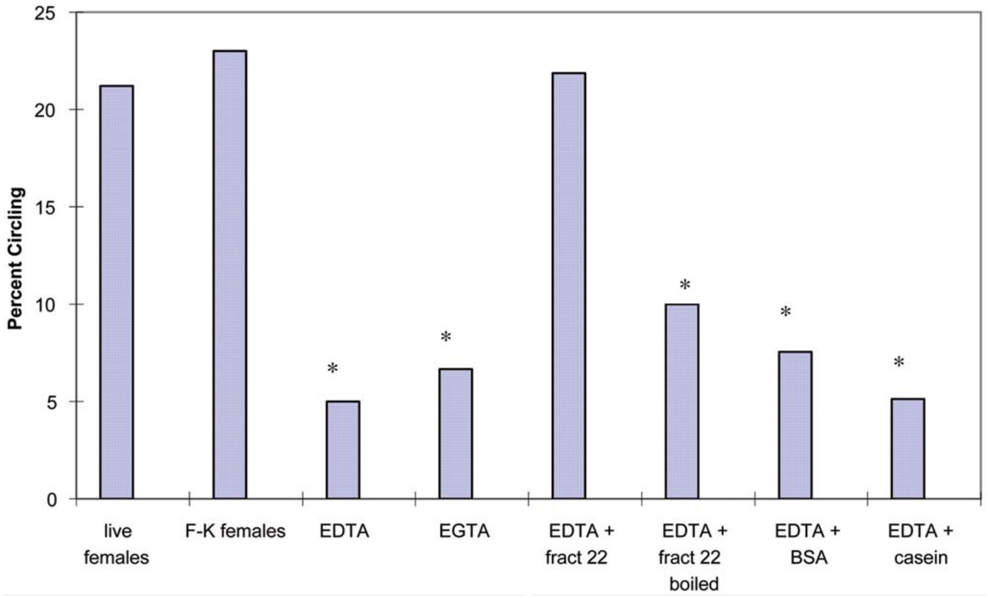

Figure 1. Effects of EDTA/EGTA extraction of female surface proteins on male mate recognition. F-K are freeze-killed females. BSA is bovine serum albumen. *indicates a significant difference from live female control, Fisher's exact test, $p < 0.05$. Percent circling is the proportion of male–female encounters that resulted in males initiating mating behavior.

responses was restored to that of live females. However, if fraction 22 was boiled for 10 min, it lost its activity. If EDTA treated females were exposed to the proteins BSA or casein, there was no restoration of their ability to elicit male mating responses.

The mating bioassay demonstrated that treatment of female rotifers with EDTA extracted surface proteins that are involved in mate recognition. This enabled us to attempt to re-attach these proteins to other EDTA treated females from different geographic populations and species (Fig. 2). Russian females served as the positive control against which all other strains were compared. Treatment of Russian females with EDTA significantly reduced by several fold their ability to elicit male mating responses, but female attractiveness was restored by exposure to ion exchange fraction 22 (Tables 1 and 2). Russian males attempted to mate with live GP females with the

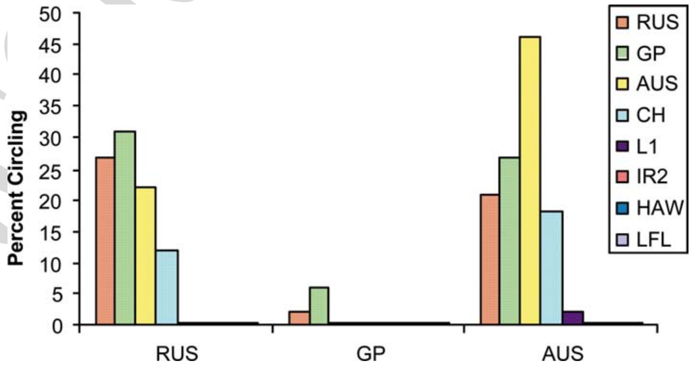

Figure 2. Comparison of EDTA extraction and reattachment of ion exchange fraction 22 to females of different clades and species. Live females were untreated, EDTA females were exposed to EDTA for 30 min, and EDTA + 22 females were exposed to EDTA then fraction 22. Percent circling is the proportion of male–female encounters that resulted in males initiating mating behavior. Results of statistical tests are presented in Table 1.

Table 1. Fisher's exact test comparing RUS male mating response (circling) to various homogamic and heterogamic females

Control female	Vs. Comparison female	Fisher's exact test p
RUS LIVE	RUS EDTA	<0.001
RUS live	RUS EDTA + F22	0.26
RUS live	GP live	0.35
GP live	GP EDTA	0.001
GP live	GP EDTA + F22	0.68
RUS live	AUS live	0.71
AUS live	AUS EDTA	0.001
AUS live	AUS EDTA + F22	0.061
RUS live	CH live	0.05
CH live	CH EDTA	0.008
CH live	CH EDTA + F22	0.45
RUS live	L1 live	<0.001
L1 live	L1 EDTA	>0.999
L1 live	L1 EDTA + F22	0.524
RUS live	LFL live	<0.001
LFL live	LFL EDTA	>0.999
LFL live	LFL EDTA + F22	>0.999

p is the probability of obtaining the result by chance.

same frequency as their own RUS females. Likewise, EDTA treatment significantly reduced GP female attractiveness, but it could be restored by exposure to fraction 22 (Table 1). A similar pattern was observed for AUS females, but exposure to fraction 22 seemed to render these females even more attractive than live AUS females, a result that is near significant by Fisher's exact test at $p = 0.061$ (Table 1).

RUS males initiated mating with live CH females at only one half the frequency as live RUS females. This response was eliminated by EDTA treatment, but restored by exposure to fraction 22. RUS males did not attempt to mate with live females of the L1, IR2, HAW, and LFL populations. More importantly, these females could not be made attractive to RUS males by exposure to fraction 22 (Table 2). We attempted to feminize RUS males by treatment with EDTA followed by expose to fraction 22. We hypothesized that conspecific males would detect them as 'females' and attempt to mate. All males thus treated failed to elicit any male mating responses.

The proteins extracted from the surface of RUS females by EDTA treatment were visualized on an SDS-PAGE gel stained with Sypro (Fig. 3). A few high molecular weight proteins (>66 kD) are present, but most of the approximately 17 prominent bands fall within the 66–12 kD range. These proteins were separated by ion exchange chromatography and the 40 one ml fractions were tested using the standard mating bioassay. Significant activity was found only in fractions 22 and 23 (Fig. 4), with Fisher's exact test $p < 0.05$. Visualization of these proteins on a SDS-PAGE gel stained with Sypro stain revealed about 10 prominent bands (Fig. 5). A 24 kD band was conspicuous in fractions 21, 22, and 23 and much reduced in other fractions.

Table 2. Mating bioassay of RUS males with females of various clades and species

Female	Live		EDTA		EDTA + F22		*Brachionus* morphospecies	COI Clade
	E	C	E	C	E	C		
RUS	230	59	303	7	206	43	*Plicatilis*	Manjavacas[a]
GP	67	21	48	3	45	12	*Plicatilis*	?
AUS	45	10	41	0	25	12	*Plicatilis*	Austria[a]
CH	43	5	66	0	85	15	*Plicatilis*	Austria[a]
L1	52	0	29	0	84	2	*Plicatilis*	*Plicatilis*[a]
LFL	76	0	65	0	34	0	*Ibericus*	Almenara[a]
IR2	30	0	34	0	49	0	*Ibericus*	Almenara[b]
HAW	34	0	53	0	55		*Rotundiformis*	*Rotundiformis*[b]

E – male–female encounters, C – circlings, F22 – ion exchange fraction 22, COI – cytochrome C oxidase subunit I gene.
[a]Gómez et al. (2002), [b]Snell & Stelzer, unpublished.

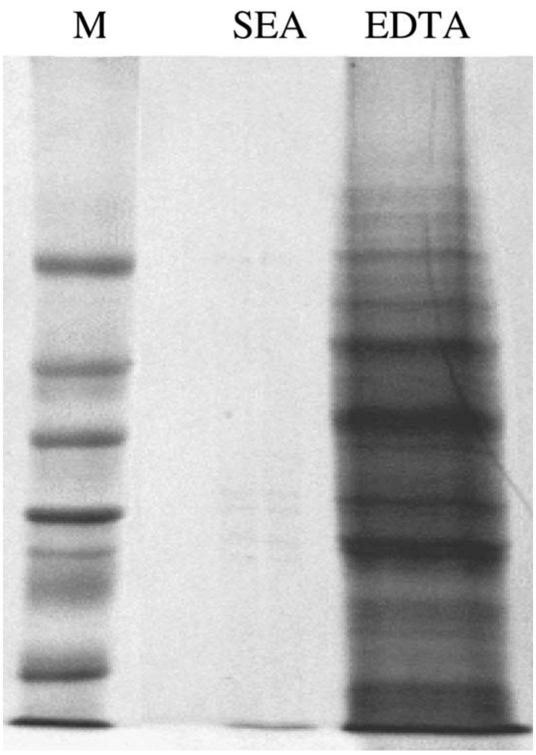

Figure 3. SDS-PAGE of EDTA-extractable surface proteins from *B. plicatilis*. M is Mark VII molecular weight markers 66–14 kD (Sigma Chemical Company). SEA is extraction with seawater, and EDTA is extraction with seawater containing 100 mM EDTA.

Discussion

The abundance of glycoproteins on the surface of rotifers was demonstrated by the binding of several fluorescently labeled lectins (Snell & Nacionales, 1990; Snell et al., 1993). A variety of lectins was tested, but only those with glucose/mannose affinity like Con A, *Lens culinaris, Vicia fava*, and *Pisum sativum* bound to females. Localization was primarily in the corona region where they bound to ciliary membranes. When these lectins were bound, females elicited significantly fewer mating responses from males. This lectin blocking of mate recognition demonstrated the functional significance of these surface glycoproteins as signals used by males to recognize mating partners. Cleavage of surface proteins by proteinase K also rendered females significantly less attractive to males (Snell et al., 1988), as did cleavage of N-linked oligosaccharrides by the glycohydrolase N-glycanase (Snell et al., 1995). These observations clearly implicated surface glycoproteins as having a key role in rotifer mate recognition.

Treatment of *B. plicatilis* females with the detergent CHAPS and EDTA removed surface proteins and eliminated the male mating response (Snell & Nacionales, 1990). The EDTA effect was especially interesting because it often did not kill the females, yet removed surface proteins critical for mate recognition. How EDTA removes proteins from membranes is not well understood, yet it is routinely used in extraction procedures for isolating active membrane proteins (Nomura & Suzuki, 1995; Ziola et al., 2000). As a strong chelator of Ca^{++} and Mg^{++} ions, EDTA disrupts electrostatic binding between peripheral membrane proteins and proteins more firmly anchored in the membrane. This selectively releases and solubilizes the peripheral membrane proteins,

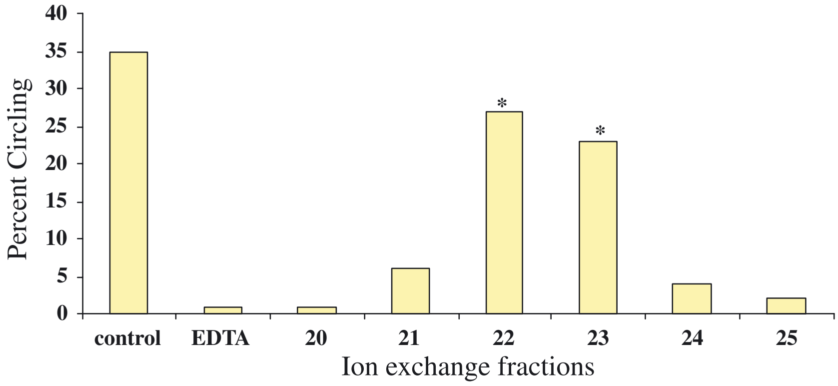

Figure 4. Ion exchange chromatography of EDTA-extractable rotifer proteins. Control is live females, numbers 20–25 refer to the ion exchange fractions. * indicates a significant difference from EDTA treated females, Fisher's exact test, $p < 0.05$. Percent circling is the proportion of male–female encounters that resulted in males initiating mating behavior.

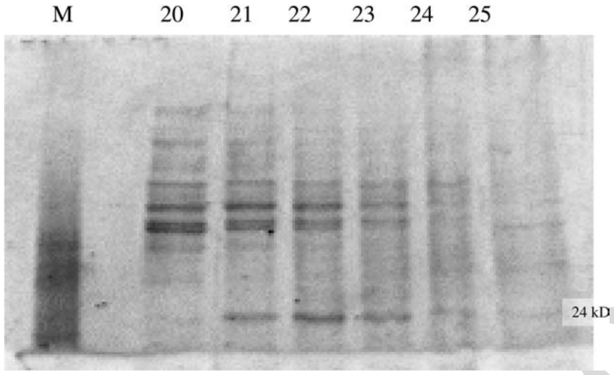

Figure 5. SDS-PAGE of ion exchange fractions of EDTA-extractable surface proteins from *B. plicatilis*. M is molecular weight markers 66–14 kD. Numbers 20–25 refer to the ion exchange fractions.

leaving the remaining membrane intact. The activity of this class of proteins in mating bioassays has demonstrated their role in rotifer mate recognition. EGTA is a more selective chelator than EDTA, chelating only Ca^{++}. EGTA's effectiveness in removing peripheral membrane proteins critical in mate recognition demonstrates that Ca^{++} and not Mg^{++} mediates the binding of these proteins to rotifer body surfaces. It further emphasizes that these proteins are only loosely associated with the body surface and are not covalently linked or transmembrane proteins.

Surface glycoproteins were removed from *B. plicatilis* RUS females by our EDTA treatment and successfully transferred to other *B. plicatilis* strains, but not all. RUS females are in the Manjavacas clade (Table 2) and their mate recognition proteins could be transferred to AUS and CH strains which are in the Austrian clade. The Manjavacas and Austrian clades are closely related phylogenetically, separated by only about 18 ITS nucleotide substitutions and about 150 (21%) COI nucleotide substitutions (Gómez et al., 2002). However, RUS mate recognition proteins could not be transferred to the L1 strain which is in the *B. plicatilis sensu strictu* clade. The *B. plicatilis* clade is separated from the Manjavacas clade by about 32 ITS nucleotide substitutions and about 235 (33%) COI nucleotide substitutions. Furthermore, no successful transfers of mate recognition proteins were made from the Manjavacas clade to any *B. ibericus* and *B. rotundiformis* species. The conclusion is that successful transfer of mate recognition proteins among rotifer species is possible only when they are quite closely related.

What mechanism limits the transfer of mate recognition proteins to only very closely related species? The model that we envision is one where a primary signal glycoprotein on females interacts species-specifically with a male receptor. This triggers the male mating response when the stimulus is sufficiently intense. This signal glycoprotein is only loosely bound to the body surface of females, but it is oriented so that its oligosaccharrides are accessible to males. Surrounding surface proteins on females provide anchor sites and hold this primary signal glycoprotein in the right orientation to be detected by male receptors. These surrounding proteins are important because their structure determines the affinity of the body surface for the signal glycoprotein and its orientation to the external environment. As species diverge, small changes in the structure of their surface proteins can reduce affinity for the signal glycoprotein or alter its orientation. Likewise, small changes in the structure of the signal glycoprotein itself could modify its ability to interact appropriately with the other body surface proteins on females.

Transfer of fraction 22 proteins to EDTA treated RUS females restored their attractiveness to conspecific males. However, we were unable to transfer these proteins to EDTA treated males and render them attractive to other conspecific males. *B. plicatilis* males almost never attempt to mate with other males and this aversion could not be overcome by transferring female proteins to males. This suggests a sexual dimorphism in the body surface proteins of *B. plicatilis* males and females.

Snell et al. (1995) described a rotifer glycoprotein called gp29 that seemed to be a prime candidate for the primary signal glycoprotein. It was purified from *B. plicatilis* using lectin affinity chromatography and its activity was probed using a polyclonal antibody raised against this protein. Unfortunately, not enough protein was isolated to obtain any amino acid sequence and the polyclonal antibody has been expended. Consequently, there is no way to relate gp29 to the EDTA extracted proteins described in this paper that have clear activity in mate recognition. The proteins described here should be regarded as an independent approach to mating protein isolation that hopefully will lead to the characterization and sequencing of proteins and genes responsible for mate recognition in rotifers.

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