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Identification of sex, age and species-specific proteins on the surface of the harpacticoid copepod *Tigriopus japonicus*

Received: 22 July 1999 / Accepted: 21 March 2000

**Abstract** Behavioral experiments have shown that male copepods of the species *Tigriopus japonicus* (Nori) can distinguish species, sex, and developmental stage of potential mates using contact chemoreception. Lectin-binding patterns on the body surface of females have indicated that surface-bound glycoproteins may be important signals in mate choice. In the present study, the proteolytic enzyme trypsin was used to cleave surface proteins from females, reducing their attractiveness to males. The protein fragments released were used to make monoclonal antibodies. Three levels of screening were used to identify monoclonal antibodies that recognized proteins involved in mate recognition. One monoclonal antibody bound to the terminal urosome and lateral prosome of CV females, and its binding significantly decreased female attractiveness to males. Western blotting showed that this antibody bound the trypsin-cleaved fragment and proteins of homogenized CV females and virgin adult females, but did not bind proteins of homogenized males, CIH females, or females of *T. californicus* or *T. fulvus*. This antibody recognized proteins on the surface of females that may enable males to discriminate conspecifics, sex, and age. It is likely that this molecule has a central role in the evolution of reproductive isolation in this group.

**Introduction**

Reproductive isolation is central to the process of speciation. A variety of adaptations have evolved in marine species to prevent hybridization among closely related species (Palumbi 1994). Although molecular barriers to hybridization have been described for the gametes of broadcast-spawning marine invertebrates (Vacquier 1998), little is known about the barriers to hybridization in invertebrates that fertilize internally (Foltz 1995). In some animals with internal fertilization, gametic barriers to fertilization may be minimal if mating behavior is sufficiently discriminating to prevent contact between heterospecific gametes (Blows and Allen 1998).

In *Tigriopus japonicus*, a marine harpacticoid copepod with internal fertilization, species-selective mating behaviors are elicited in part by chemical signals on the surface of females (Kelly and Snell 1998). These copepods engage in pre-copulatory mate-guarding, the precocious grasping of juvenile females by adult males (Lonsdale et al. 1998). Mate-guarding can last for weeks (Ito 1970) and may represent a considerable cost to the male due to increased visibility to predators, reduced feeding, and loss of other mating opportunities. Males that guard juvenile females of later developmental stages will copulate sooner than those guarding females further away from their terminal molt. Significant benefit may be gained by assessing mate fitness before guarding a particular female, thus optimizing the number of copulations over the male’s lifetime.

*Tigriopus japonicus* males engage in a series of stroking behaviors with their antennules at the onset of guarding which enable them to discriminate species, sex and female developmental stage. Although males and females are morphologically indistinguishable to human observers until the fourth copepodite stage (CIV), Stage I and II copepodes removed from the grasp of males always developed into females, indicating that males can correctly identify the sex of early copepodites (Kelly et al. 1998). When presented with a choice between conspecific CV females and *T. californicus* females, *T. japonicus* males chose conspecifics significantly more often (Kelly and Snell 1998). Furthermore, males preferred females of advanced developmental stages to less mature females (Kelly et al. 1998).

Kelly and Snell (1998) used an array of lectins to examine surface glycoproteins that are thought to be...
important cues for male discrimination of copepods. Lectin-binding patterns differed vastly between adult males, adult females and Stage III females. Treatment of Stage III copepodite females with the proteases pronase E and chymotrypsin led to a significant decrease in their attractiveness to males. Based on these observations, it was hypothesized that *Tigriopus japonicus* male discrimination of species, sex and developmental stage in mate-guarding is mediated by surface glycoproteins.

In this paper, we were interested in elucidating the molecular basis of male discrimination in mate-guarding, as these molecules may play a role in the establishment and maintenance of reproductive isolation in copepods. We used the endoprotease trypsin to cleave proteins from the surface of *Tigriopus japonicus* CV copepodite females. Treatment with trypsin reduced attractiveness of these females to males, as measured by a reduction in mate-guarding. Trypsin cleavage produced a complex mixture of protein fragments; six bands were visible with sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS PAGE). Cleaved proteins were used for the production of a battery of monoclonal antibodies that allowed us to identify single epitopes from this complex mixture. To determine which antibodies recognized proteins involved in mate recognition, three criteria were evaluated: (1) hybridomas were screened for their ability to produce antibodies that bound to female body surfaces; (2) those antibodies that bound to the body surface were tested for their ability to reduce the attractiveness of females to males when bound; (3) we examined the ability of blocking antibodies to discriminate proteins from copepods of different species, sex and developmental stage.

### Materials and methods

#### Experimental conditions and species

Experiments were conducted at 25 °C in 15 ppt artificial seawater unless otherwise noted. Individuals of *Tigriopus japonicus* (Nori) were isolated from cultures maintained at these conditions, and *T. californicus* and *T. fulvus* individuals were isolated from cultures kept at 20 °C and 30 ppt. The copepods were allowed to acclimate to experimental conditions overnight prior to each experiment. All species were fed a mixed algal diet of *Tetraselmis suecica*, *Cryptomonas sp.*, and *Isochrysis galbana* twice each week.

#### Trypsin treatment

Male-choice experiments were performed to determine if female attractiveness to males was altered by the treatment of females with the serine protease trypsin. *Tigriopus japonicus* CV females were treated with trypsin (Sigma, EC 3.4.21.4, porcine pancreas, 15,200 U mg⁻¹) in 15 ppt seawater at 37 °C for 1 h. Control individuals were incubated under similar conditions but without the enzyme. Mating experiments were performed in 1 ml of 15 ppt artificial seawater per well of a 48-well cell-culture plate. Two CV females and one adult male were placed in each well; in control wells, both females were untreated while treatment wells contained one treated and one untreated female. To enable visual discrimination among morphologically identical individuals, half the females in each treatment were dyed with 10 μg ml⁻¹ Neutral Red dye for 1 h (Ansensrud 1989). Reciprocal treatments with Neutral Red demonstrated that the dye alone did not affect male-choice. At least 20 replicate wells were tested for each control and enzyme treatment. Male-choice of females was recorded as the number of treated vs control females mate-guarded after 1 and 2 h. Male choices in treatment wells were compared to those of control wells with a G-statistic contingency test (Sokal and Rohlf 1995, p. 726).

Isolation of proteins released by trypsin treatment

To isolate cell-surface proteins liberated by treatment with trypsin, 1600 CV *Tigriopus japonicus* females were removed from mate-guarding pairs in culture, held overnight, and then incubated in 320 μl of 10 μg ml⁻¹ trypsin (5 nM) in 10 mM phosphate buffered saline, pH 7.4, for 1 h at 35 °C. The resulting solution was frozen immediately and lyophilized to halt trypsin activity. To determine the complexity and yield of proteins released by trypsin cleavage, a sample was run on a 10% SDS PAGE gel and stained with silver. As a control, spontaneously shed protein was collected from the same number of untreated females incubated for the same time and temperature as trypsinized females. This protein was also run on a 10% SDS PAGE gel immediately after collection and after treatment with 10 μg ml⁻¹ trypsin.

Monoclonal antibodies raised against lyophilized protein were produced by the Monoclonal Antibody Facility at the University of Georgia. Approximately 1 μg of the lyophilized protein was injected directly into the surgically exposed spleen of an anaesthetized mouse both 14 and 3 days before spleen cell-fusion with immortal myeloma cells. Cell-fusion and cell-culture protocols were as described by Harlow and Lane (1988).

#### Monoclonal antibody screening

Antibodies produced by ≈220 hybridoma cells (fusion products of myeloma cells and spleen cells) were initially screened for their ability to bind to the surface of *Tigriopus japonicus* CV females. CV females were isolated from culture, and killed with a few drops of 10% 3-aminobenzoic acid ethyl ester (methanolsulfonate salt) in 1 ml seawater. The copepods were rinsed in phosphate-buffered saline (PBS). Two to four individuals were added to each well of a 96-well cell-culture plate and frozen until use. Thawed copepods were treated with ≈50 μl cell hybridoma supernatant for 2 h at room temperature, and then washed three times with 200 μl PBS. Samples were then exposed to FITC (fluorosothiocyanate)-labeled sheep, anti-mouse secondary antibody (Sigma F-6257) diluted 1:32 in 5% powdered milk in PBS for 1 h. After three washes with 200 μl PBS, the copepods were examined for surface binding of antibody with a Nikon OVR inverted microscope under fluorescence, with excitation and emission filters of 495 and 525 nm, respectively, at the Center for Ultrastructural Research at the University of Georgia. Cells that produced active antibodies were serially diluted until all cells in a culture were progeny of a single parent cell; thus, all cells produced the antibody of interest.

Preliminary experiments were performed to test whether the antibodies produced by these cultures reduced male mate-guarding when bound to females. Live *Tigriopus japonicus* CV females were incubated in a 1:1 solution of antibody-rich hybridoma supernatant with seawater for 1 h and then rinsed, and mate-guarding tests were performed. Controls were incubated in 1:1 solution of cell-culture media and seawater. Experimental conditions were as described earlier, with 20 control wells and 20 treatment wells per antibody. Male mate-guarding of females was observed at 1 and 2 h. Male choices in control wells were compared to those of males in treatment wells with a G-statistic contingency-table analysis (Sokal and Rohlf 1995, p. 726).

Based on the ability of their antibodies to affect mate-guarding, three hybridoma cell lines were expanded for large-scale antibody production; 3F11, 10B12, and 9G11. These antibodies were precipitated with ammonium persulfate, then reconstituted to a final volume approximating 40 ml in TBS (50 mM Tris, 0.3 M NaCl,