Using Rhodamine to tag mites for studies of pre- and post-copulatory sexual selection

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Abstract

Our understanding of sexual selection is advancing with new technologies that tag individuals or their sperm, revealing how females use post-copulatory processes to discriminate between competing mates. Many tagging methods have been devised primarily for model insect organisms like Drosophila or Gryllidae. Developing such novel methods, however, is expensive and requires intensive investment. In this experiment, we trial the use of Rhodamine B and Rhodamine 110 in a small arachnid, the bulb mite Rhizoglyphus echinopus, for pre and post copulatory observations as it is a relatively inexpensive and simple way to tag individuals and their ejaculate proteins. First, we tested whether Rhodamine B and Rhodamine 110 applied to food can be used as a tagging method to track and distinguish between individuals. Second, we explored whether Rhodamine applied in this way can be used to track sperm transfer. We found that both tagging probes worked well in tagging individuals and that we were able to distinguish between individuals using both LED and fluorescent microscopy. We also found that Rhodamine degraded rapidly in the animals, likely due to their fast metabolism. Due to the rapid degradation, we observed variable results in the sperm transfer trials. We suggest multiple uses for Rhodamine and highlight other invertebrates where this method may come into use for the study of sexual selection.

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Abstract

Our understanding of sexual selection is advancing with new technologies that tag individuals or their sperm, revealing how females use post-copulatory processes to discriminate between competing mates. Many tagging methods have been devised primarily for model insect organisms like *Drosophila* or Gryllidae. Developing such novel methods, however, is expensive and requires intensive investment. In this experiment, we trial the use of Rhodamine B and Rhodamine 110 in a small arachnid, the bulb mite *Rhizoglyphus echinopus*, for pre and post copulatory observations as it is a relatively inexpensive and simple way to tag individuals and their ejaculate proteins. First, we tested whether Rhodamine B and Rhodamine 110 applied to food can be used as a tagging method to track and distinguish between individuals. Second, we explored whether Rhodamine applied in this way can be used to track sperm transfer. We found that both tagging probes worked well in tagging individuals and that we were able to distinguish between individuals using both LED and fluorescent microscopy. We also found that Rhodamine degraded rapidly in the animals, likely due to their fast metabolism. Due to the rapid degradation, we observed variable results in the sperm transfer trials. We suggest multiple uses for Rhodamine and highlight other invertebrates where this method may come into use for the study of sexual selection.

Data availability statements: All data and code are available at here: https://anonymous.4open.science/r/Rhodamine_Methods-2A21/Rhodamine.Rmd

Introduction

Sexual selection results from differential access to gametes for fertilization (Shuker & Kvarnemo, 2021) and can be studied from multiple different angles. To truly understand how sexual selection functions, it is crucial to estimate its strength before and after mating (Lande & Arnold, 1983). Pre-copulatory sexual selection refers to the competition or mate choice processes occurring before mating, while post-sexual selection encompasses processes that happen after mating has occurred, such as sperm competition and cryptic female choice (Jones & Ratterman, 2009). Pre- and post-copulatory behaviours and the associated fitness of individuals expressing them are often used to infer the strength of sexual selection on sexual traits.

Most researchers estimate the strength of selection from pre-copulatory behaviours, as it only requires the observation of natural behaviours, such as the ability of an individual to secure and defend a territory or resource (Dubois & Giraldeau, 2005; Grant, 1993), the ability to outcompete a rival in direct competition (Parker, 1974), or the number of matings and order in which individuals mate (Jordan et al., 2014). However, while observing natural behaviours, the setting is often limited to a set number of individuals, which in many cases is not realistic for a natural population of invertebrate species. Successfully observing and identifying individuals across a wide variety of species and in more realistic scenarios is essential if we want to extrapolate these results to natural behaviours and conditions across the animal kingdom.

Despite the ubiquitous use of behavioural observations to estimate the strength of selection before copulation, our ability to identify and track individuals under

more natural circumstances can be difficult. For example, experiments aimed at identifying the traits that lead to a successful mating are often limited to pairs of interacting males and females (Wagner, 1998). If multiple males are used, individuals must be marked to distinguish between them (Jung et al., 2020). Although this is relatively simple in vertebrates, it is more difficult in invertebrates as they are smaller, have fewer identifying features, and often occur in large numbers. There are different tagging techniques developed for use in invertebrates (particularly insects), such as externally marking with fluorescent dusts, paint, ink, and body mutilation (see review Hagler & Jackson, 2001). These methods are usually inexpensive and relatively easy to apply, however can be toxic to the animal if applied incorrectly (Hagler & Jackson, 2001), can alter the animals' behaviour (Still et al., 2014), and some of the techniques are not permanent, meaning that individuals cannot be tracked for long periods (e.g. fluorescent dusts). A further problem is that if individuals are small and occur in large numbers, such as fruit flies (Grimaldi & Jaenike, 1984) or mites (Radwan, 1995), inexpensive and easy marking techniques can become expensive and complex to perform accurately for too many individuals.

Post-copulatory estimations involve measuring the number of offspring an individual produces, which is difficult when females mate with multiple partners, as it requires identifying the sires of the offspring through genetic markers (McClure et al., 2012) or sterile male techniques (Scott & Williams, 1994). For example, the green fluorescent protein (GFP) in *Drosophila melanogaster* (Manier et al., 2010) is often used to make post-copulatory observations. However, the costs of developing such techniques are high and may not be amenable to

small behavioural projects. Additionally, the observation of fluorescence can only be done under an expensive confocal microscope with fluorescent functions (Manier et al., 2010; Remington, 2011) and may have effects on protein function (Michaelson & Philips, 2006), which can affect sperm count and quality.

Colony-dwelling animals also often exhibit unique reproductive strategies, such as intense sperm competition (Simmons, 2005; Smith, 2012), which can also play into conflict or cooperation between or within sexes.

Some species of the class Arachnida are colony-dwelling animals, making them difficult to study under more natural circumstances. Arachnids are particularly interesting to study in regard to sexual selection and conflict because of their extreme sexual cannibalism (Schneider, 2014) and morphological dimorphism (McLean et al., 2018). However, sexual behaviours are difficult to study in arachnids for a few reasons. First, the prevalence of multiple mating opportunities makes it difficult to assess individual reproductive success of males (Smith, 2012). Second, the unique and variable genitalia and sperm of arachnids render post-copulatory processes cryptic (Eberhard & Huber, 2010). Lastly, large population numbers can obscure individual behaviours and interactions, posing challenges for detailed individual observations (Radwan, 1995). Most pre-copulatory behaviours of arachnids are usually observed in unnatural conditions where mating is limited to pairs or at most three individuals. Individuals are often identified with acrylic paint (e.g. Rypstra, 1985) and fluorescent dust (e.g. Still et al., 2014), both of which can have practicality issues (Evans & Gleeson, 1998) and can affect an animal's behaviour (Still et al., 2014). Additionally, post-copulatory observations are often limited by the extended generation time of

some arachnids (Murrell et al., 2005; Schmoller, 1970) or by female cryptic choice (Eberhard, 1997), therefore transgenic methods are not practical. This is why male sterilization through radiation is commonly used (Christenson et al., 1986), although this technique can alter feeding behaviour, reaction to light, decrease locomotion and chemoreceptivity (Langley et al., 1974), mating vigour and success, as well as the competitiveness of individuals (Oliva et al., 2012).

In this study we trial Rhodamine for use in tracking individual bulb mites (*Rhizoglyphus echinopus*), a colony-dwelling arachnid. Rhodamine is a fluorescent probe that binds to proteins in the animal, including the ejaculate, which can be used as a marker for mating studies. Rhodamine has been used in invertebrates such as leafhoppers (Hayashi & Kamimura, 2002), fireflies (Reijden et al., 1997) and moths (Blanco et al., 2006; Sparks & Cheatham, 1973), to observe male ejaculate and spermatophores in a female's reproductive tract. Rhodamine is a cost-effective way to stain sperm or oocytes and can be observed under a LED light of a microscope, a fluorescent laser, or by the naked eye (Blanco et al., 2006; Hayashi & Kamimura, 2002; Sparks & Cheatham, 1973). Using Rhodamine is also time-efficient, as it does not require back-crossing individuals into a population as in a GFP approach (Manier et al., 2010) and can instead be injected into the animal (Sparks & Cheatham, 1973), spermatophore (Reijden et al., 1997), female reproductive tract (Hayashi & Kamimura, 2002) or fed to the animal by mixing the dye into their diet (Blanco et al., 2006; Sparks & Cheatham, 1973). Rhodamine has been shown to have little to no effect on the lifespan (Blanco et al., 2006) or mating behaviour (Reijden et al., 1997) of animals, although such studies with Rhodamine have been done in insects, and its potential to be used in arachnids

143 remains unknown.

144 *R. echinopus* male and females are polygynandrous (Radwan, 2009), live in
145 colonies, and the males are polyphenic (Radwan, 1995, 2001). Polyphenisms refer
146 to an extreme case of phenotypic plasticity where one gene can express multiple
147 discrete phenotypes triggered by an environmental cue (Yang & Pospisilik, 2019).
148 In the case of *R. echinopus,* the polyphenism is triggered by colony density
149 (Radwan, 2001) and juvenile size. When density is high, most males will moult
150 into the scrambler male morph and use a passive sneaker tactic. When the
151 density is low, males will moult into a fighter male morph and use a weaponized,
152 mate monopolizing tactic (Radwan, 2001, 2009). Because individuals are so
153 small, it is impossible to use traditional marking techniques used in other
154 invertebrates (Hagler & Jackson, 2001). This means that mating trials are usually
155 limited to observing single pairs, which can give an inaccurate representation of
156 mating preference, fitness, and intrasexual competition (Anderson et al., 2007;
157 Shackleton et al., 2005). This limits our ability to explore individual behaviours
158 and how the strength of selection may vary due to shifts in the relative density of
159 morphs. Rhodamine, however, offers the opportunity to tag individuals by
160 staining the food (yeast) that they eat. Our aims were thus to (1) test Rhodamine
161 B and Rhodamine 110 as a tagging method to track and identify individuals and
162 to (2) determine whether Rhodamine could be used to track sperm transfer.

163 **Methods & Methods**

164 Rhodamine Description

165 Rhodamine is a fluorescent probe used for the fluorescent labelling of proteins.
166 The fluorescence is created by the presence of a planar, multi-ring aromatic

xanthene core structure with nitrogen in place of oxygen atoms in the outer rings (Beija et al., 2009; Hermanson, 2008). Rhodamine B (RhB) contains two ethyl groups on each nitrogen and a carboxylate group at the 3rd position of its lower ring, while Rhodamine 110 (Rh110) contains no substituents on the upper nitrogens and the carboxylate on the lower ring. RhB has an excitation wavelength of 546nm and emission wavelength of 568nm, while Rh110 has an excitation wavelength of 500nm and emission wavelength of 522nm. Both reagents are water soluble (Hermanson, 2008).

Rhizoglyphus echinopus stock population

The stock populations of *Rhizoglyphus echinopus* used in this study are descendants of a population sourced off an infested organic onion purchased in August 2005 from a health food shop in Perth, WA (Buzatto et al., 2012). The descendants of these populations were subsequently maintained at UNSW Sydney in New South Wales from 2019. We housed the mites in six 90mm Petri dishes partially filled with Plaster of Paris, which were kept inside closed food containers. We placed the containers in dark incubators at a temperature of 22°C. Distilled water was regularly sprayed to maintain >90% humidity level. The mites were provided with Allinson's dried yeast as a food source and tissue paper as a substrate, *ad libitum*. To preserve genetic diversity within the cultures, a small proportion of mites were periodically transferred between Petri dishes. All individuals used to test the protocol were virgins sourced from a subset of females from the stock population. We isolated the larvae from these females and reared them individually in small cylindrical glass vials (diameter = 100mm and height = 14 mm; hereafter referred to as vial) with a Plaster of Paris

base (4-5mm thick) on top of damp filter paper in a 90mm Petri dish in a food container. We closed the vials with a small piece of cotton wool.

Rhodamine Set-Up

Concentration

We tested two solution concentrations for our protocol. We mixed 4.17mM of Rhodamine 110 and B, as used in Reijden, Monchamp, and Lewis (1997), referred to as the original concentration, and we doubled this concentration to 8.34mM of Rhodamine B and 110, referred to as the doubled concentration. We mixed the solution with 2mg of yeast, shaking the mixture well until it was homogenous. We pipetted 0.25mL of each solution into a vial with one individual mite – hence forth these vials are referred to as Rhodamine vials. We only fed the Rhodamine solution to male bulb mites, since female bulb mites would be the recipients of the Rhodamine-stained ejaculate. In total, 108 males were fed the Rhodamine solution for 24 hours minimum before each testing protocol (RhB original concentration n=27 males, RhB double concentration n=27 males, Rh110 original concentration n=27 males, Rh110 double concentration n=27 males). While some methods inject Rhodamine into the animal (e.g. Sparks and Cheatham 1973) we are unable to do this with *R. echinopus,* as they are too small and fragile.

Mounting Media

We tested four different mounting media for our protocol: distilled water, Fluoromount, Immu-mount, and phosphate buffer solution (PBS). We used the distilled water medium for the light-emitting diode (LED) illumination trials only, while we used Fluoromount, Immu-mount, and PBS for the fluorescent illumination trials. Before we placed an individual male into the mounting

medium, we washed them in a droplet of distilled water on a Petri dish to remove any excess Rhodamine solution stuck to its body. We mounted all individuals for the fluorescent trials ventral side up on a 76.2mm x 25.4mm microscope slide with the cover slip placed gently on top to avoid squishing the mites. We mounted 3 males in PBS for each Rhodamine treatment and concentration (total n=12), and 12 males in either Fluoromount or Immu-mount for each Rhodamine treatment and concentration (total n=96). We cured the slide for a minimum of an hour in a closed container to minimise light exposure. Alongside the Rhodamine male mites, we always mounted a negative control male, which consisted of a male that was not fed Rhodamine, for RhB (n=3) and Rh110 (n=3). The three negative control males were re-used since no treatment was used on them, and thus their fluorescence should not have changed between microscope viewings.

LED Illumination

To visually distinguish between individuals, we cleaned mites in a droplet of distilled water and visually observed 10 male mites from each Rhodamine treatment and concentration (total n=40) under an Optico ASZ-200 Stereo Microscope.

Fluorescent Illumination

To observe the fluorescence of the Rhodamine fed male mites, we used a Zeiss LSM 780 or Zeiss LSM 880 microscope with the 10x (0.45 DICII) objective. To assess RhB fluorescence, we used a laser with 514 wavelength and the range indicator from 525nm to 740nm. To assess Rh110 fluorescence, we used a laser with 488 wavelength and the range indicator from 499nm to 696 nm. We always

started with a positive control sample – a male mite that had been fed the highest concentration of Rhodamine and/or fed the solution most recently – and would therefore fluoresce the brightest. We set the laser gain so the fluorescence was visible but not over saturated. We then compared all subsequent males to the positive control laser gain. We took two images of each sample, the laser image with fluorescence and a Transmission-photo multiplier (TMP) image. The image was always focused on the genitalia of the mite.

Degeneration

We tested whether the Rhodamine degenerates in the male mite, and if so, how quickly. After males were fed their Rhodamine solution for at least 24 hours, we washed the male in a droplet of water and placed him into one of three treatments: a vial with the Rhodamine yeast solution (n=36 RhB males, n=36 Rh110 males), a vial with yeast containing no Rhodamine (n=33 RhB males, n=33 Rh110 males), an empty vial without yeast (n=36 RhB males, n=36 Rh110 males), and a negative control (n=3 RhB males, n=3 Rh110 males). After 24 hours in these vials, we mounted half of the males of each treatment in Fluoromount and Immu-mount, ventral side up. For this examination, we no longer used the PBS mount after examining the mounting medium results. After at least one hour of curing the slides in a closed container, we examined the slides under the microscope Zeiss LSM, where all males were compared to the positive control.

Mating Protocol

To determine if the Rhodamine is binding to the ejaculate of male mites and if it is transferred to females, we fed virgin males a Rhodamine solution for a

263 minimum of 24 hours. We then divided those individuals into five treatments:
264 virgin males left in Rhodamine vial (positive control; n=32 RhB males, n=32
265 Rh110 males), virgin males moved from Rhodamine vial and placed in an empty
266 vial (degeneration control; n=54 RhB males, n=54 Rh110 males), virgin males
267 that were allowed to mate with females in an empty vial (male treatment; n=46
268 RhB males, n=46 Rh110 males), females mated with Rhodamine treated males
269 (female treatment; n=33 RhB females, n=33 Rh110 females), and a negative
270 control (n=3 RhB males, n=3 Rh110 males). We made sure the males and females
271 mated by checking the vials every 10 minutes until a male mounted a female.
272 Mating would take approximately two hours and therefore we kept the virgin
273 males in the positive control and degeneration control in their vials for the same
274 amount of time as the mating treatment. After the pair was finished mating, we
275 mounted all individuals from the five treatments ventral side up in Immu-mount.
276 We observed the individuals by examining the positive control first and
277 comparing all subsequent samples to the positive control using the same laser
278 gain settings.
279 To determine whether the Rhodamine from male ejaculates was integrated into
280 the eggs, we isolated four females in total (two females mated with an RhB fed
281 male and two males mated with an Rh110 fed male) and collected their eggs
282 three days after mating. We mounted a subset of the eggs in Immu-mount and
283 observed them under the microscope. There were Rhodamine yeast particles on
284 the slide which we used as a positive control.
285 Analysis
286 We used Fiji, an extended version of the biological image analysis program

ImageJ (Schindelin et al., 2012), to measure the specimens' fluorescence for concentration, mounting medium, degeneration and mating protocols. For each laser image, we took three replicate measurements with the circle tool: area of the selection, integrated density of the selection in the image of the mites' body near the genitalia and three replicate measurements of the background (see Figure S1 for reference). The TMP image of the mite was used for reference when measuring the fluorescence (or lack thereof) near a sample's genitalia. We then calculated the Corrected Total Cell fluorescence (CTCF) of each sample through the formula:

$$CTCF = Integrated\ Density - (Area\ of\ selection \times flourescence\ of\ background)$$

To test for CTCF differences due to the solution concentration or the mounting medium, we used a linear model including the concentration, mounting medium and their interaction as fixed effects. To test for CTCF degeneration, we used a linear model with treatment, mounting medium and their interaction as fixed effects. To test for CTCF variations we ran a linear model with mating status as a fixed effect. Mating status was defined with the following groups: virgin control (virgin male not fed Rhodamine), positive control (Rhodamine-fed virgin male left in the vial with Rhodamine), Rhodamine fed virgin male moved to an empty vial for 24 hours, Rhodamine fed mated male, and mated females (mated to a Rhodamine fed male). All models had a Gaussian error distribution. We used R version 4.1.3 (R Core Team, 2022) for all our analyses. We obtained all estimated marginal means, and Tukey contrasts with the "emmeans" package (Lenth, 2022).

Results

311 All our *Rhizoglyphus echinopus* mites survived the 24 hours in the feeding

312 Rhodamine treatments.

313 Individual Tagging

314 *LED Illumination*

315 We were able to visually assess male mites fed RhB and Rh110 under the stereo

316 microscope. Males fed Rh110 (Figure 1A) had a dark orange tint and males fed

317 RhB (Figure 1B) had a purple tint inside their bodies when compared to

318 unmarked mites (Figure 1C).

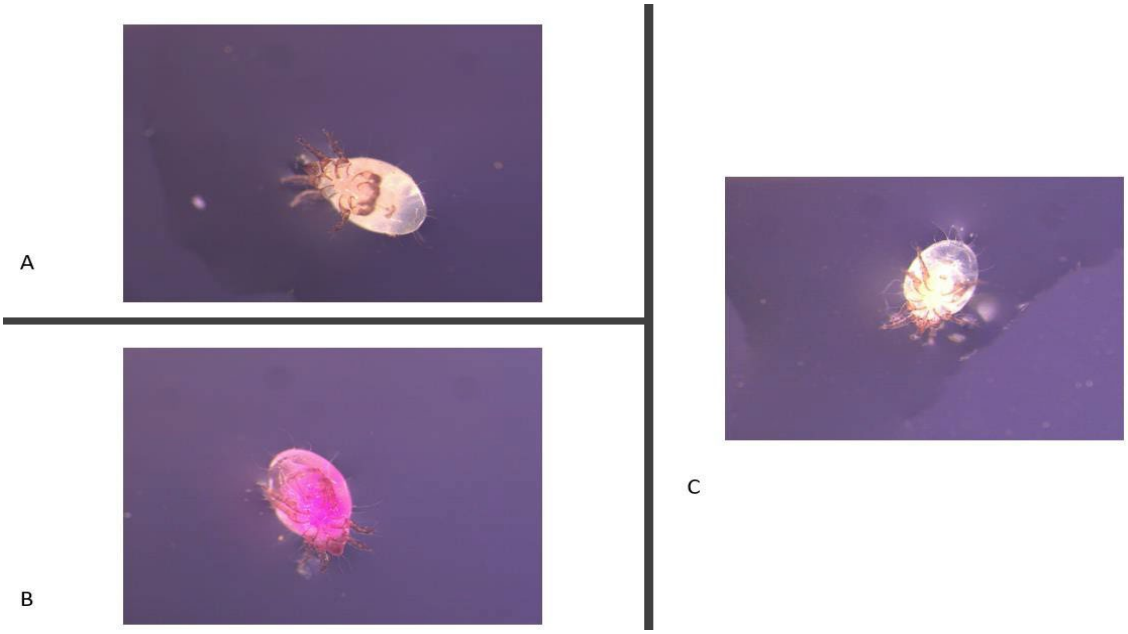

Figure 1. Male mites fed A) Rh110 solution with yeast for 24 hours, B) RhB solution with yeast for 24 hours, and C) yeast with no Rhodamine.

319 *Fluorescence Illumination*

320 RhB fluorescence did not differ significantly between the original or doubled

321 concentration within the same mounting medium (Figure 2; Table S1). We did,

322 however, see a difference between the negative control and the concentrations

323 within the same mounting medium. Both the original and doubled concentration

324 of RhB fluoresced significantly more than the negative control in Fluoromount

(Figure 2A; Table S1). In contrast, only the doubled concentration fluoresced more than the negative control in Immu-mount (Figure 2B; Table S1). No treatment differed in fluorescence from the negative control in PBS (Figure 2C; Table S1; See Figure S2 for images of fluorescence).

Rh110 fluorescence differed significantly between the original and doubled concentration in Immunomount (Figure 2B; Table S1). Only the original concentration of Rh110 fluoresced significantly more than the negative control in both Fluoromount (Figure 2A; Table S1) and Immu-mount (Figure 2B; Table S1). In PBS, no treatment differed in fluorescence from the negative control (Figure 2C; Table S1; See Figure S2 for images of fluorescence).

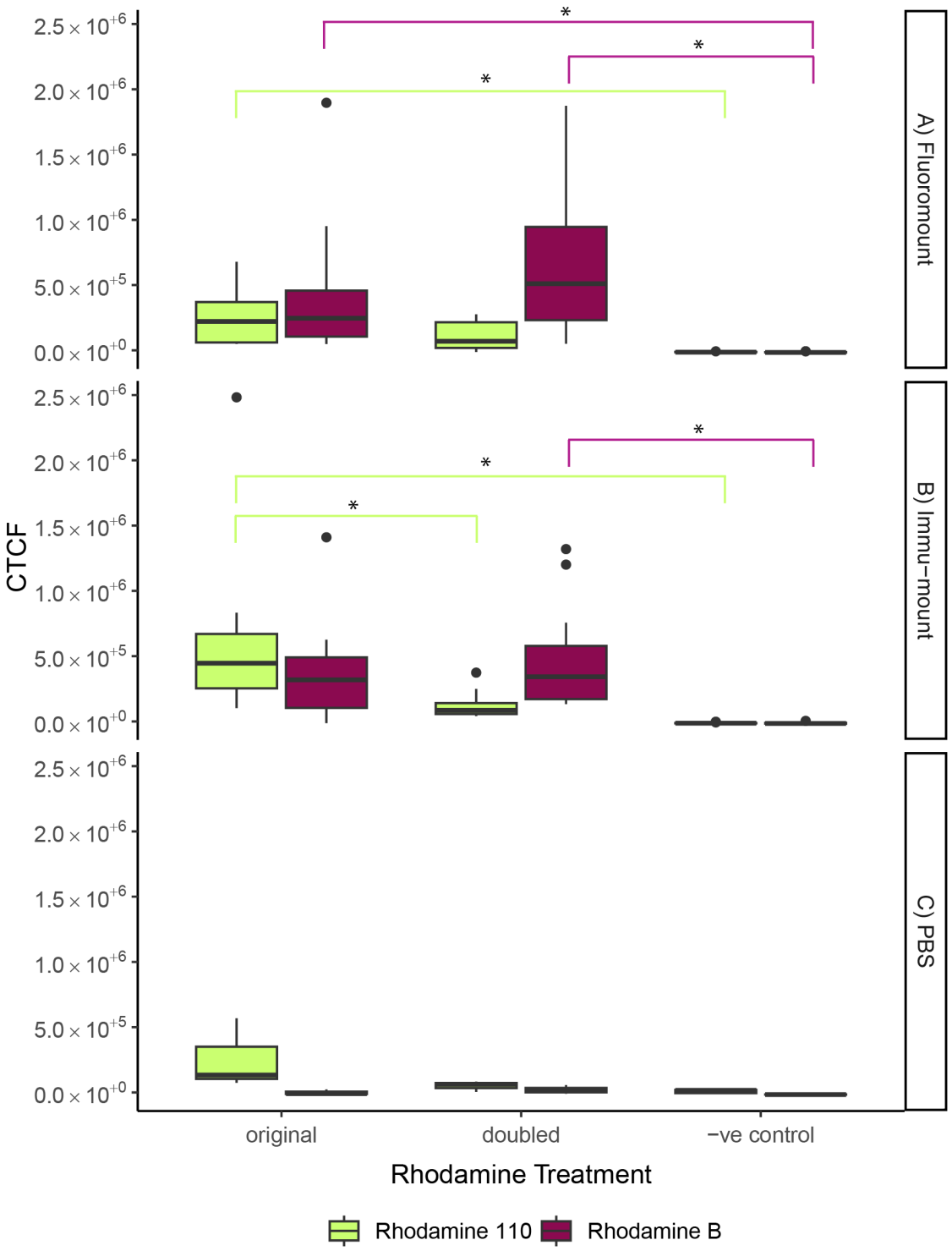

Figure 2. Corrected total cell fluorescence (CTCF) of the Rhodamine treatment for original (4.17mM) and doubled (8.34mM) concentrations with the negative control in mounting medium A) Fluoromount, B) Immu-mount, and C) PBS. Significant differences are outlined using an asterisk and lines matching the colours of the treatment.

335 Degradation of Fluorescence

336 Next, we explored how samples degraded once the male mite was fed the
337 solution. RhB fed males that were either fed yeast or put into empty vials for 24
338 hours fluoresced similarly to the negative control and significantly less than the
339 positive control in Fluoromount (Figure 3A; Table S2). In Immu-mount, the
340 males that were left in an empty vial for 24 hours fluoresced similarly to the
341 positive control (Figure 3B; Table S2), while the males that were fed yeast
342 fluoresced significantly less than the positive control with no difference from the
343 negative control (Figure 3B; Table S2). The negative control fluoresced significantly
344 less than the positive control in both Immu-mount and Flouromount (Figure 3; Table
345 S2).

346 Rh110 fed males that were either fed yeast or put into empty vials for 24 hours
347 did not fluoresce differently from the negative control in both Fluoromount and
348 Immu-mount and were significantly lower in fluorescence than the positive
349 control in Immu-mount (Figure 3; Table S2). The negative control fluoresced
350 significantly less than the positive control only in Immu-mount (Figure 3; Table S2).

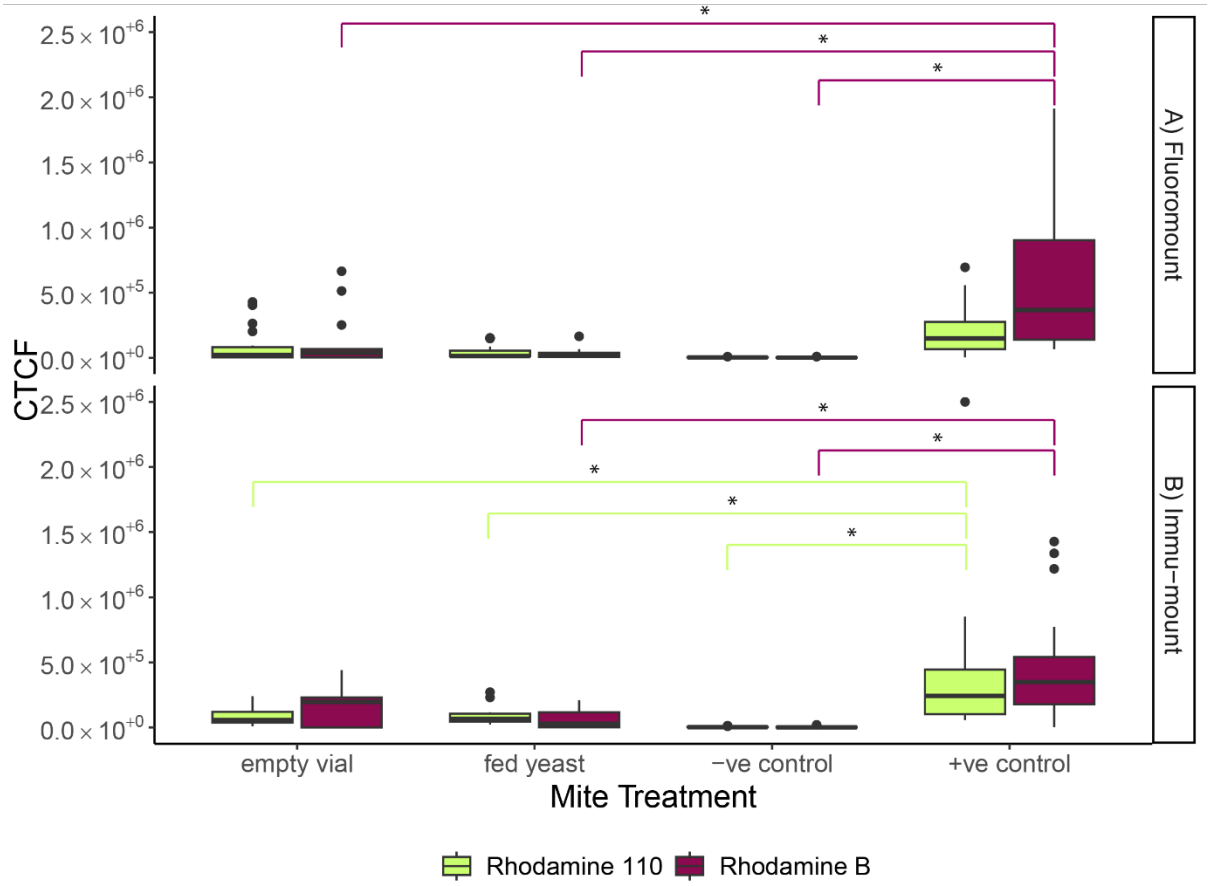

Figure 3. Corrected total cell fluorescence (CTCF) of the Rhodamine degradation treatment for males kept in empty vials for 24 hours after treatment, fed yeast for 24 hours after treatment, negative control male that was never fed Rhodamine, and positive control male that was kept in a Rhodamine vial for 24 hours, in mounting medium A) Fluoromount and B) Immu-mount. Significant differences are outlined using an asterisk and lines matching the colours of the treatment.

Sperm Transfer

Males fed RhB fluoresced less after mating compared to the positive control (i.e. virgin males left in a vial that could continue to feed on Rhodamine) and virgin males left in an empty vial (Figure 4; Table S3). Virgin males left to feed on Rhodamine fluoresced the most compared to all other treatments (Figure 4; Table S3). Additionally, females that mated with RhB fed males fluoresced, although the level of fluorescence is not significantly different from the negative control and significantly less than any of the male treatments (Figure 4).

Rh110 fed males fluoresced similarly after mating compared to virgin males left in a vial that could continue to feed on Rhodamine and virgin males left in an empty vial (Figure 4; Table S3). Additionally, females that mated to males fed Rh110 fluoresce less than males left to feed on Rhodamine 110, although the level of fluorescence was not significantly different from the negative control (Figure 4; Table S3). The eggs of females mated to RhB and Rh110 fed males did not fluoresce (See Figure S3 for reference).

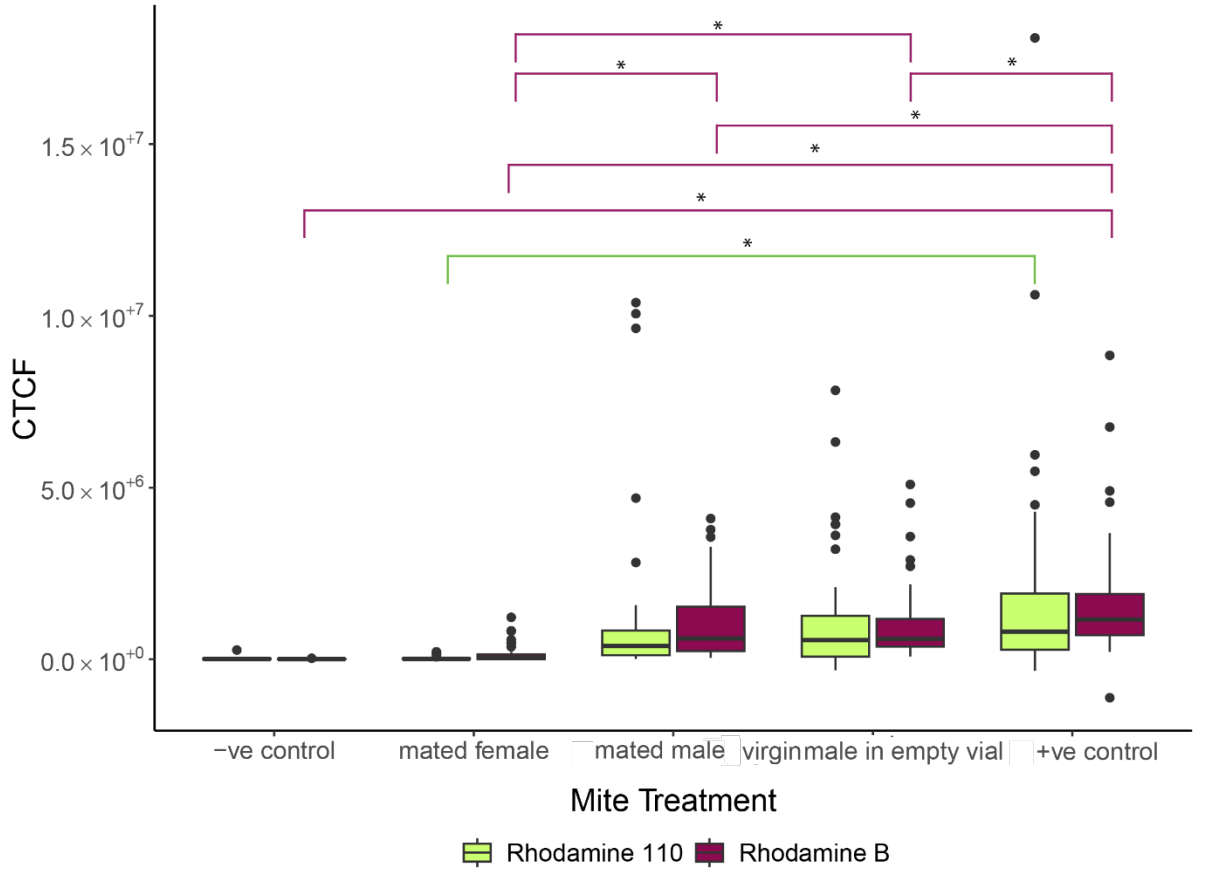

Figure 4. Corrected total cell fluorescence (CTCF) of the sperm transfer treatment for negative control male that was not fed Rhodamine, female mated to a Rhodamine treated male, Rhodamine treated male mated with a virgin female, Rhodamine treated male left in an empty vial, and a positive control male that was kept in a Rhodamine vial in Immu-mount. Significant differences are outlined using an asterisk and lines matching the colours of the treatment.

Discussion

We found that Rhodamine can be used in *Rhizoglyphus echinopus* to tag individuals for behavioural observations. The best concentration to use for the Rhodamine treatment is the same one used by Reijden, Monchamp, and Lewis (1997) in fireflies. When the mites were fed the original concentration of 4.17mM of Rhodamine mixed with yeast, we found that we could still identify the individuals under a regular LED light of a stereo microscope (Figure 1). It is even easier to identify individuals under a fluorescent microscope, except that this requires individuals to be sacrificed to be mounted (Figure 2). Doubling the concentration increased fluorescence in RhB (Figure 2), but in Rh110 the doubling of the concentration lowered the fluorescence in the specimen (Figure 2), most likely because Rh110 was less soluble if too much product is used, and therefore fewer particles are ingested by the individuals.When mounting the specimen, we found that PBS was not a viable mounting medium as there was too much autofluorescence for RhB and Rh110 treatments. In contrast, the best mounting medium was Immu-mount, as the fluorescence was less variable for RhB treatments and more visible for Rh110 treatments (Figure 2B-C).

Our trials suggest that there is still some troubleshooting to be done for the use of Rhodamine in post-copulatory trials of *R. echinopus*. We found that RhB and Rh110 degenerate quickly in the mites unless they are continuously fed more Rhodamine while being left in the Rhodamine vial (Figure 3). The degeneration is even quicker if the male is fed yeast for 24 hours after a Rhodamine feeding (Figure 3), which suggests that Rhodamine is likely binding to digestive proteins. Additionally, we found that degeneration occurs when a mite is removed from the Rhodamine treatment and placed in an empty vial for the same amount of time that it took a Rhodamine-treated male mite to mate, approximately two hours (Figure 3). RhB looks more promising for post-copulatory observations than Rh110, as the degeneration of RhB is slower

than that of Rh110, but the differences between mated males and virgin males are too variable (Figure 3). Since the Rhodamine degeneration in the body of the mite is so quick, we did not run any longevity trials.

We found that mated females fluoresced variably and so dimly that it was impossible to compare ejaculate tailoring of males in females. The light fluorescence that we see is most likely due to some fluorescent ejaculate transferring occurring, but not enough to make conclusive comparisons. The lack of bright fluorescence may be due to the degradation of Rhodamine occurring in the male (Figure 4) and the majority of the Rhodamine binding to the digestive proteins of the male rather than the seminal proteins. Since we may be seeing much of the Rhodamine protein binding occurring in the digestive system of the mite, rather than their seminal proteins, there is not enough transfer of Rhodamine to the female to make any conclusive comparisons of ejaculate tailoring or transfer. The rapid degeneration would also explain why the eggs do not fluoresce at all after a female has been mated with a Rhodamine-treated male. However, the dull fluorescence in the females may also be due to the makeup of the ejaculate of the male. Male mites may not have many ejaculate proteins compared to fruit flies (Sirot et al., 2009) and therefore there are fewer proteins for the Rhodamine to bind to in mite ejaculate. Additionally, the mechanisms underlying sperm production and replenishment in bulb mites remains poorly understood. Further research is required to explore sperm production in bulb mites and whether our approach could be used to explore sperm competition in this species.

We found that Rhodamine can be used to mark individuals of *R. echinopus* and believe that this can be useful in studies of female mate choice, male-male competition, and social environmental effects on mating behaviours. *R. echinopus* males are polyphenic, and weapon expression and consequent mating strategies are determined by colony density. Given current

research on *R. echinopus,* we have a general picture of male and female mating behaviours in this system, but much information is still missing. Females are likely polyandrous in this system (Radwan, 2009), yet we do not know whether they exercise female choice. Fighter males potentially monopolize females by killing rival males (Radwan, 2001), but whether this strategy is always exercised by the fighter male may depends on the environmental context – such as the number of females available or the number of rival males. By marking the individuals with RhB and Rh110 we can observe mating strategies of different morphs in several social contexts, including more natural ones. In addition, by marking multiple individuals we eliminate the need to constantly observe the animals and remove them from stable environmental conditions as we can check on the vials less frequently. This eliminates the risk of behavioural changes or mating disturbances associated with experimental design. It is necessary to explore whether Rhodamine affects the animals behaviourally through controlled experiments to ensure coloured individuals are not discriminated against in mate-choice experiments. It would also be important to explore whether Rhodamine affects fecundity or sperm quantity in mites, as this can have negative effects on fitness for both sexes.

Many invertebrate studies are constrained by the number of pairings for mating experiments, resulting in false or unrealistic claims about female choice, male choice, same sex competition, and male and female behavioural interactions (Andersson, 1994; Andersson & Iwasa, 1996). Rhodamine has the potential to be used to mark invertebrates and study them in environments more similar to their natural social environment. In invertebrates, behaviours of interest are prevalent in large social constructs. Model systems such as fruit flies, mites, crickets, beetles, and moths are good candidates for the Rhodamine method as these animals are easy to keep in the lab and to feed or inject with Rhodamine. They also have translucent

438 abdomens, pupae, eggs, or spermatophores, which can be used to identify a Rhodamine tagged
439 individual. Using the Rhodamine method to tag these individuals is more practical than
440 fluorescent dusts that can be easily transferred between individuals or cleaned off by the
441 individuals (Still et al., 2014) and is more cost and time effective than GFP (Manier et al.,
442 2010).
443 While in our system we found that there is still more troubleshooting to be done with
444 Rhodamine and its use in post-copulatory experiments, the method might work better with
445 animals of slower metabolism or animals with larger sperm or ejaculate. While most animals
446 are not as transparent as mites, Rhodamine may still be observed if the animals' genitalia are
447 dissected and mounted, or in the pupae or eggs of the animal. Additionally, because
448 Rhodamine is easily mixed into food, animals that are fed to small invertebrate carnivores
449 such as spiders may be used to explore questions regarding female cryptic choice.
450 In conclusion, we found that Rhodamine is a cost-effective and simple way to tag small
451 invertebrates to study pre-copulatory mating behaviours in a more natural social context.

Supplementary Material

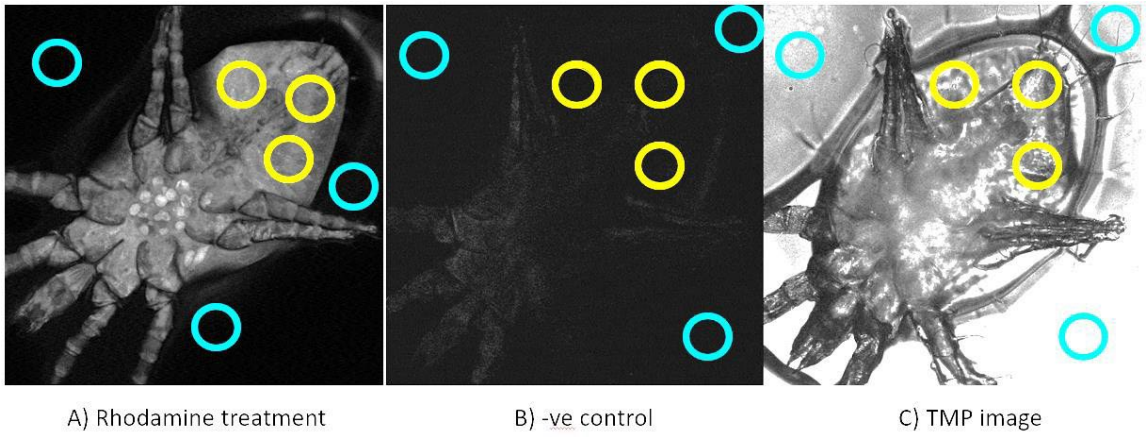

Figure S1. Reference of how the measurements of CTCF were taken using the circle tool in Fiji. Blue circles represent the background measurements and yellow circles represent the body measurements for A) Rhodamine treated fluorescent image, B) negative control image of the negative control for guidance

Table S1. Corrected Total Cell Fluorescence (CTCF) estimate contrasts, including standard error (SE) and the degrees of freedom (df), from the linear model comparing two concentrations (original, doubled) and three mounting media (Fluoromount, Immu-mount, Phosphate Buffer Solution (PBS)) for Rhodamine B (RhB) and Rhodamine 110 (Rh110) treatments. Bolded rows are significant.

		Concentration Contrast	Estimate	SE	df	t value	P value
RhB	Fluoromount	original – doubled	-234670	158203	66	-1.48	0.31
		original – (-ve) control	**434187**	**170879**	**66**	**2.54**	**0.04**
		doubled – (-ve) control	**668857**	**170879**	**66**	**3.91**	**0.0006**
	Immu-mount	original – doubled	-94581	158203	66	-0.60	0.82
		original – (-ve) control	390995	170879	66	2.29	0.06
		doubled – (-ve) control	**485576**	**170879**	**66**	**2.84**	**0.02**
	PBS	original – doubled	-19573	316405	66	-0.06	1.00
		original – (-ve) control	16316	316405	66	0.05	1.00
		doubled – (-ve) control	35889	316405	66	0.11	1.00
	Fluoromount	original – doubled	149356	108069	75	1.38	0.36
		original – (-ve) control	**268776**	**108069**	**75**	**2.49**	**0.04**
		doubled – (-ve) control	119420	108069	75	1.11	0.52

Rh100	Immu-mount	**original – doubled**	**486713**	**108069**	**75**	**4.50**	**0.0001**
		original – (-ve) control	**622771**	**108069**	**75**	**5.76**	**<0.0001**
		doubled – (-ve) control	136058	108069	75	1.26	0.42
	PBS	original – doubled	208408	216138	75	0.96	0.60
		original – (-ve) control	249571	187181	75	1.33	0.38
		doubled – (-ve) control	41163	187181	75	0.22	0.97

Table S2. Corrected Total Cell Fluorescence (CTCF) estimate contrasts, including the standard error (SE) and the degrees of freedom (df), from the linear model comparing different degradation treatments (empty vial, yeast fed, positive control, negative control) and mounting media (Fluoromount, Immu-mount) for Rhodamine B (RhB) and Rhodamine 110 (Rh110). Bolded rows are significant.

		Treatment Contrast	Estimate	SE	df	t value	P value
RhB	Fluoromount	**Empty vial – (+ve) control**	**-451670**	**97090**	**115**	**-4.65**	**0.0001**
		Fed yeast – (+ve) control	**-523393**	**93043**	**115**	**-5.63**	**<0.0001**
		Empty vial – (+ve) control	99853	127121	115	0.79	0.86
		Fed yeast – (-ve) control	28129	124057	115	0.23	1.00
		Empty vial – fed yeast	71723	100018	115	0.72	0.89
		(+ve) control – (-ve) control	**-551522**	**121709**	**115**	**-4.53**	**0.0001**
	Immu-mount	Empty vial – (+ve) control	-279536	121709	115	-2.30	0.10
		Fed yeast – (+ve) control	**-377145**	**121709**	**115**	**-3.10**	**0.01**
		Empty vial – (-ve) control	158750	146786	115	1.08	0.70
		Fed yeast – (-ve) control	61141	146786	115	0.42	0.98
		Empty vial – fed yeast	97609	146786	115	0.67	0.91
		(+ve) control – (-ve) control	**-438285**	**121709**	**115**	**-3.60**	**0.0026**
	Fluoromount	Empty vial – (+ve) control	-124360	62596	145	-1.99	0.20
		Fed yeast – (+ve) control	-156132	66672	145	-2.34	0.09
		Empty vial – (-ve) control	69738	77412	145	0.901	0.80
		Yeast fed – (-ve) control	37966	80743	145	0.47	0.97
		Empty vial – fed yeast	31772	64920	145	0.49	0.96

Rh110		(+ve) control – (-ve) control	-194098	78887	145	-2.46	0.07
	Immu-mount	**Empty vial – (+ve) control**	**-298380**	**69572**	**145**	**-4.29**	**0.0002**
		Fed yeast – (+ve) control	**-295358**	**73440**	**145**	**-4.02**	**0.0005**
		Empty vial – (-ve) control	81034	8315	145	0.98	0.76
		Fed yeast – (-ve) control	84056	86416	145	0.97	0.77
		Empty vial – fed yeast	-3022	78006	145	-0.04	1.00
		(+ve) control – (-ve) control	**-379414**	**78887**	**145**	**-4.810**	**<0.0001**

Table S3. Corrected Total Cell Fluorescence (CTCF) estimate contrasts, including the standard error (SE) and the degrees of freedom (df), from the linear model comparing between different sperm transfer treatments (negative control, mated female, mated male, virgin male left in empty vial, positive control) all mounted in Immu-mount for Rhodamine B (RhB) and Rhodamine 110 (Rh110) fed individuals. Bolded rows are significant.

	Treatment Contrast	Estimate	SE	df	t value	p value
RhB	Mated female – (-ve) control	-135157	427884	172	-0.316	1.00
	Mated male – (-ve) control	-1049530	427884	172	-2.453	0.11
	Virgin male – (-ve) control	-1019221	423668	172	-2.41	0.12
	Pos. control – (-ve) control	**-1856277**	**451029**	**172**	**-4.12**	**0.0006**
	Mated female – mated male	**-914373**	**247039**	**172**	**-3.70**	**0.003**
	Mated female – virgin male	**-884064**	**239663**	**172**	**-3.69**	**0.003**
	Mated female – (+ve) control	**-1721119**	**285256**	**172**	**-6.03**	**<0.0001**
	Mated male – virgin male	30309	239663	172	0.126	1.00
	Mated male – (+ve) control	**-806746**	**285256**	**172**	**-2.83**	**0.04**
	Virgin male– (+ve) control	**-837056**	**278893**	**172**	**-3.00**	**0.03**
	Mated female – (-ve) control	211	907074	166	0.00	1.00
	Mated male – (-ve) control	-1160637	827006	166	-1.40	0.63
	Virgin male– (-ve) control	-041100	816632	166	-1.28	0.71

Rh110	Pos. control – (-ve) control	-2032961	848490	166	-2.40	0.12
	Mated female – mated male	-1160849	595672	166	-1.95	0.30
	Mated female – virgin male	-1041312	581183	166	-1.79	0.38
	Mated female – (+ve) control	**-2033173**	**625157**	**166**	**-3.25**	**0.01**
	Mated male – virgin male	119537	446015	166	0.268	1.00
	Mated male – (+ve) control	-872324	501973	166	-1.74	0.41
	Virgin male – (+ve) control	-991861	484692	166	-2.05	0.25

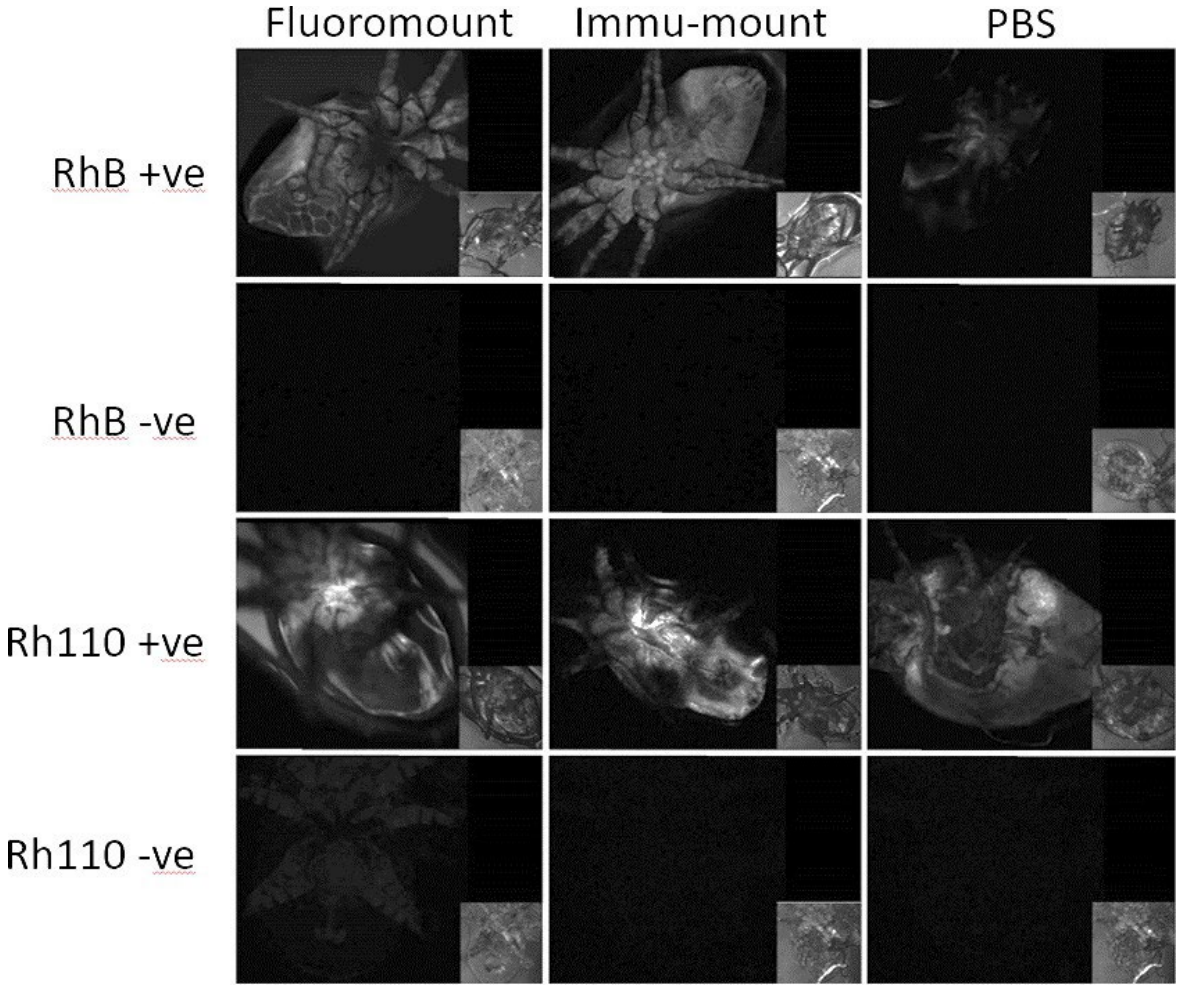

Figure S2. Zeiss LSM 880 images of Rhodamine treated mites and the negative controls, with all corresponding TMP images, in three different mounting media (Fluoromount, Immu-mount, PBS).

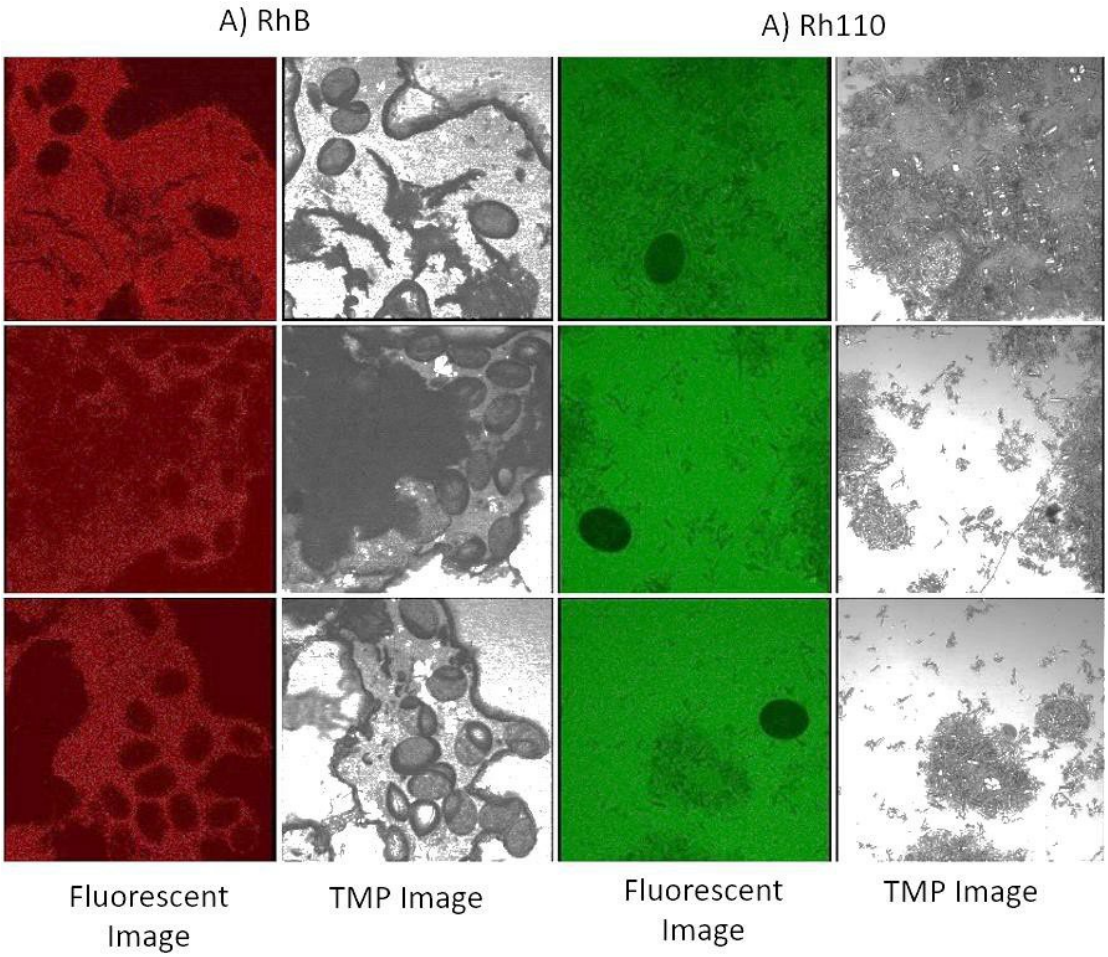

Figure S3. Zeiss LSM 880 images of eggs laid by females mated with A) RhB treated males and B) Rh110 treated males. The left image is the fluorescent image with corresponding wavelength for each Rhodamine type and the right image is the TMP image for reference.

Equation S1. Male fitness calculation in a social context treatment

$$Male\ Fitness = \frac{Total\ egg\ count\ from\ one\ female}{(Males\ that\ mounted\ female)}$$

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