The sterile male technique: Irradiation negatively affects male fertility but not male courtship

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**A B S T R A C T**

The sterile male technique is a common method to assign paternity, widely adopted due to its relative simplicity and low cost. Male sterility is induced by exposure to sub lethal doses of chemosterilants or irradiation, the dosage of which has to be calibrated for every species to provide successful male sterilisation, without affecting male physiology and behaviour. While the physiological effects of sterilisation are usually assessed for each study, the behavioural ones are rarely analysed in detail. Using the orb web spider Argiope keyserlingi as a model we first tested (1) the validity of the thread assay, which simulates male courtship behaviour in a standardised context, as a proxy representing courtship on a female web. We then investigated (2) the effectiveness of male sterilisation via irradiation and (3) its consequences on male courtship behaviour. Our results validate the thread assay and the sterile male technique as legitimate tools for the study of male courtship behaviour and fertilisation success. We show that these techniques are time and cost effective and reduce undesirable variation, thereby creating opportunities to study and understand the mechanisms underlying sexual selection.

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**1. Introduction**

Polyandry is common and widespread among animal taxa (Simmons, 2005; Taylor et al., 2014). This phenomenon has selected for multiple post-copulatory mechanisms based on optimising the number of ova fertilised by preferred males. Post-copulatory mechanisms can be classified as sperm competition (Parker, 1970), or cryptic female choice, both of which can result in differential fertilisation success. The proposed mechanisms under sperm competition include displacement, inactivation or mere dilution of rival sperm (Simmons, 2005). Under cryptic female choice, females allocate sperm from preferred males based on differences in male characteristics or behaviour (Eberhard, 1996).

Studying post-copulatory selection requires experimental techniques that quantify the paternity shares of different males. Two of the most common techniques currently used are genetic markers (e.g., Achmann et al., 1992; Simmons and Achmann, 2000) and the sterile male technique (e.g., Parker, 1970). The genetic marker technique requires the development of microsatellite markers, and hence can become relatively expensive and time consuming, particularly in taxa in which many eggs are produced. As a consequence, one of the most widely adopted techniques to assign paternity to individual males is the sterile male technique because of its simplicity and low-cost. Males are sterilised by exposure to sub lethal doses of chemosterilants or radiation from X-ray or γ sources. The doses are ideally optimised so that they do not affect condition, sperm viability or fertilisation capacity, but do induce complete sterility through chromosomal mutations that result in the early death of embryos sired by treated males (Parker, 1970). Females are mated to one male that is fertile and one male that is sterile. Paternity of the offspring from double matings is assigned on the basis of egg development (Boorman and Parker, 1976).

Despite the advantages associated with the sterile male technique, there remain some significant issues to be resolved. The sterilisation treatment, dosage and type, has to be calibrated for every species to ensure successful male sterilisation, while also limiting the potential effects of irradiation on male behaviour. Limiting the side-effects of irradiation is particularly important as irradiation may alter male behaviours, such as courtship performance, on which cryptic female choice might be based. However, while the effects of irradiation on male fertility are often assessed on a study-by-study basis, the effects of irradiation on male behaviour are rarely assessed in detail (but see Schneider et al., 2006; Schneider and Lesmono, 2009).
Argiope keyserlingi (Karsch 1878) is an excellent model for studying how male courtship performance influences male reproductive success. *A. keyserlingi* females are polyandrous and exhibit cryptic female choice via controlling copulation duration (Elgar et al., 2000). Moreover, male *A. keyserlingi* courtship is long, complex and primarily vibrational, including elements such as shuddering (comprising of several antero-posterior rocks), abdominal wags (comprising of several dorso-ventral abdominal pumps) and mating thread dances (comprising of plucks and bounces on the mating thread) (Robinson and Robinson, 1980; Wignall and Herberstein, 2013a). The male enters the female's web and spends the initial phase wandering in the web periphery performing shudders and abdominal wags. The male then approaches the hub at the centre of the web where the female is located, performing frequent shudders. After reaching the female, the male spends from several minutes up to over 2 h at the hub touching her legs and abdomen. This tactile courtship is regularly interspersed with shudders, abdominal wags, grooming sessions and rests. The male then builds a mating thread. Hanging upside down from it he then starts generating vibratory signals known as the mating thread dance (Wignall and Herberstein, 2013a). The female responds by moving onto the mating thread and entering a characteristic "acceptance posture" that allows copulation to take place (Wignall and Herberstein, 2013a).

In *A. keyserlingi*, the shudders performed during the approach towards the female are important as a means for the male to reduce the risk of being attacked by the female (Wignall and Herberstein, 2013a,b). Females can also recognize differences in male courtship quality and express their preference by responding faster to preferred males and by reducing the incidence of post-copulatory sexual cannibalism (Wignall and Herberstein, 2013a). The importance of male shuddering behaviour for female mating decisions, and the ease with which it can be measured makes this system ideal for examining the effects of irradiation on male behaviour. We previously developed a simulated courtship thread assay for *Argiope radon* (Wignall et al., 2014). The thread assay consists of males walking and courting (shuddering) on a silk dragline collected from an adult virgin female. This assay simulates the male's approach from the periphery of the web to a female located at the hub. Male performance in this assay is highly consistent in *Argiope* (Wignall et al., 2014). This suggests that the thread assay is a good indicator of the intrinsic courtship quality of males.

Our study had three aims: first, we tested whether male courtship performance in the thread assay could be used as a reliable proxy for male courtship performance when interacting with a female in her web. Second, we tested whether a dosage of 40 Gy (16 Gy/min for 2.5 min) from a cobalt γ-emitter is sufficient to induce complete sterilisation in *A. keyserlingi*. Third, we tested whether sterilisation through irradiation affects male courtship behaviours in the thread assay. A dosage of 40 Gy (0.8 Gy/min for 50 min) has been used in related species (*Argiope lobata*: Welke and Schneider, 2009; *Argiope bruennichi*: Schneider et al., 2006; Schneider and Lesmono, 2009) in which it has been shown to be sufficient to induce complete sterilisation of males, while not affecting sperm viability (Schneider et al., 2006), courtship duration, copulation duration or cannibalism (Schneider and Lesmono, 2009).

2. Materials and methods

2.1. Study animals: Collection and care

The animals were collected between November 2012 and January 2013 from several suburban populations of metropolitan Sydney, Australia. All individuals were collected as juveniles and reared in the laboratory to ensure they were virgin and to standardize developmental conditions. This limited differences in experience and body condition, factors that are known to affect reproductive decision-making and aggression in some spiders (Wilder et al., 2009; Gibson and Uetz, 2012). Spiders were housed individually in 250 ml upturned plastic cups with a mesh floor for airflow and maintained in a temperature controlled room (26 °C) on a 12:12 h light:dark cycle. Males were fed with vinegar flies (*Drosophila melanogaster*) twice a week, females were fed with vinegar flies, sheep blowflies *Lucilia cuprina* (Calliphoridae) or house flies (*Musca domestica*) twice a week (see Zschokke and Herberstein, 2005) for rearing techniques. All spiders were sprayed with water every day. Individuals were checked for moults daily until they reached the adult stage, which can easily be recognised because of the differentiated secondary mating organs of males (the paired pedipalps) and the sclerotised epigynum of the females. Before the mating trials adult females were transferred into Perspex frames (50 × 50 × 10 cm), to build webs.

2.2. Experimental design

2.2.1. Validity of the thread assay as a model for natural courtship

2.2.1.1. The thread assay. We tested whether male courtship during a controlled courtship assay (a thread assay) is a suitable proxy for male courtship in a female's web. A silk dragline thread was pulled from a mature virgin female's spinnerets and fixed to a wooden frame across a 30 cm span (Fig. 1) angled at ~45° (Wignall et al., 2014). The female on her web was placed beside the wooden frame to allow passive flow of volatile female pheromones, which, together with the contact chemicals present on the silk dragline, induce male courtship behaviour (Gaskett et al., 2004; Gaskett, 2007). A virgin male (*n* = 12) was weighed before the assay then placed in a plastic vial (30 ml). The male in the open vial was placed beside the female in her web to allow him to receive pheromones. The vial was then closed for 2 min and attached to the lower left side of the span for the male to acclimate. The vial lid was then removed, and the male was allowed to make his own way out and up onto the span. During a typical trial, a male walked up the dragline, performing the typical shudders observed during natural courtship along the way (Supplementary Video 1). The assay was considered over when the male first touched the wooden frame at the end of the dragline. Between every trial the dragline was removed, the frame was washed with fresh water and a
new silk dragline was fixed onto the wooden frame. Silk draglines were taken from one, randomly assigned female for each male's asssay. Three females were used in this experiment, such that each female was used for 4 different males. Each assay was recorded with high-speed video at 300 frames per second (Casio Ex-F1 digital camera; Casio America, Inc., USA).

We measured from each video recording: the total time for the male to travel across the silk dragline (from the male's first touch of the silk to the first touch of the wooden frame at the end), the number of shudders, each shudder duration (from the beginning of the first rock to the end of the last active one, not including the time he spent passively moving on the silk thread after a shudder due to momentum), the number of rocks within each shudder and the gap duration (time between each successive shudder). The mean shudder duration, number of rocks, shudder rate (total travel duration divided by number of shudders), shudder ratio (shudder duration divided by number of rocks; used as an indicator of how fast the male rocks) and gap duration were calculated and together considered to represent male courtship performance during the assay.

2.2.1.2. Male courtship within a female's web. Male courtship behaviour in the thread assay was compared to male courtship behaviour in a female's web. To measure male courtship in webs, we set up courtship tests in which we recorded each male's behaviour during his approach to a female in her web. The same 12 males tested in the thread assay were used for this experiment so that we could directly compare performance in the thread assay to performance in a successful mating scenario (males were allowed to copulate with the females).

All web courtship assays took place in the Perspex frames in which virgin females had built their webs. A plastic vial (30 ml) containing a male was placed into one of the lower corners of the frame and opened, allowing the male to enter the female's web of his own accord. The first stage of courtship (from the moment the male touched the silk until he reached the female at the hub) was recorded with high-speed video at 300 frames per second. The remainder of the web courtship assays were recorded at 25 frames per second (Casio Ex-F1 digital camera; Casio America, Inc., USA). From the high speed recordings we measured: the total time taken to reach the female (from the first shudder the male performs when he starts moving in the female's direction to when he arrived within touching distance of the female), the number of shudders, the number of active rocks in each shudder, the shudder duration, the shudder ratio and the gap duration. The mean shudder duration, number of rocks, shudder rate, shudder ratio and gap duration were calculated and together considered to represent male courtship performance.

2.2.2. Effects of irradiation on male fertility

Males were randomly assigned to either a normal control (C, n = 10) or irradiated (IR, n = 10) treatment. The IR males were placed in 3 ml plastic vials and irradiated with a dose rate of 16 Gy/min for 2.5 min, amounting to a dosage of 40 Gy from a cobalt γ-emitter. During the irradiation treatment of IR males, C males were handled precisely as for the IR males: individuals were placed in 3 ml plastic vials for the same duration as the irradiated males.

Males were used in double-mating trials at least 24 h after the irradiation/treatment. In the double-mating trials, virgin females (SS, n = 5) were mated to two, sterile IR males within 24 h in order to check the effectiveness of sterilisation. We also ran a second series of double mating trials in which virgin females (NN, n = 5) were mated to two, fertile C males within 24 h. These double-mating trials allowed us to estimate the proportion of undeveloped eggs in natural clutches.

Mated females laid their first eggs inside a protective egg sac within 3 weeks after being mated. In the following weeks each female laid up to 5 egg sacs. All egg sacs were removed from the female's frame as they were laid and placed individually in upturned plastic cups with a mesh floor for airflow. Egg sacs were kept in a temperature controlled room (26 °C) on a 12:12 h light:dark cycle and sprayed with water daily. After 4 weeks development, the egg sacs were opened and examined under a microscope to count the number of developed and undeveloped eggs.

2.2.3. Effects of irradiation on male courtship performance

To test for effects of irradiation on male courtship performance, we ran a thread assay for 20 adult, virgin males. Thread assays were conducted and analysed as described above (see Section 2.2.1.1). Males were then randomly assigned to either a normal control (C, n = 10) or irradiated (IR, n = 10) treatment and irradiated (40 Gy) or underwent controlled handling conditions as described above (see Section 2.2.2). After 24 h, all males were tested again in the thread assay.

To further test for effects of irradiation on male courtship behaviour, we conducted double mating trials to assess male performance when in female webs. This allowed us to examine the potential for differences in total courtship duration and differences in copulation success between irradiated and control males. Each virgin female (n = 24) was mated with one virgin IR male and one virgin C male within 24 h. Interactions were recorded at 25 fps (Casio Ex-F1 digital camera; Casio America, Inc., USA). Copulation duration for each male was measured, and whether the males were cannibalised or not.

2.3. Statistical analyses

2.3.1. Validity of the thread assay as a model for natural courtship

The five variables used to characterise male courtship in the thread assay and during the approach to the female in the web were reduced into principal components using NIPALS PCA in R using the ade4 package (`nipals` function). This technique simplifies the data set into principal components that represent holistic male courtship performance (specifically shuddering behaviour). This reduces the risk of Type I errors due to multiple hypothesis testing. NIPALS PCA is a modification of traditional PCA that allows rows containing missing values (e.g., assays in which a male did not shudder). We extracted principal component scores for each male for the thread assay and for the trial conducted in a female's web. These principal component scores were used to test whether male performance in the thread assay was correlated to his performance in a female's web using Pearson's correlations.

2.3.2. Effects of irradiation on male courtship performance

NIPALS PCA was used to reduce the male courtship performance variables into principal components as described above (see Section 2.3.1). Male courtship performances in the thread assays before and after the irradiation treatment were compared using an ANCOVA (R package 'lawstat'). Homoscedasticity was checked using Levene's test (R package 'lawstat'). Homogeneity of regression slopes was tested by re-running an ANCOVA, using an interaction term between the independent variable and covariate (R package 'lawstat'). All ANCOVA assumptions were met for both PC1 and PC2. Copula durations of irradiated (IR) and control (C) males were compared using a one-way ANOVA (R package 'irr', function 'icc'). Copula durations of the first males to mate with the females were analysed separately from copula durations of the second males to account for the expected difference in the mean copula duration of the two groups due to the tendency of mated females to be more aggressive toward males than virgin
females (Herberstein et al., 2002). The frequency of cannibalism between IR and C males was compared using a $\chi^2$-test, tested separately for first and second males.

3. Results and discussion

Our results establish the thread assay as a proxy with which to estimate male natural courtship performance. Our study also reveals that irradiation is a reliable technique with which to induce sterilisation without adversely affecting male courtship and copulation behaviour. These findings provide validation for a fundamental tool with which to simplify the quantitative study of courtship and its consequences on fertilisation success.

### 3.1. Validity of the thread assay as a model for natural courtship

Two principal components were extracted from the NIPALS PCA representing the 69.46% and the 30.54% of the variance in male courtship performance (Table 1). The loadings matrix for each principal component is shown in Table 1, with PC1 representing mainly shudder rate, shudder and gap duration, and PC2 representing mainly number of rocks and shudder ratio. Male courtship performance in the thread assay and on the female’s web showed a significant positive correlation for PC1 ($R = 0.75$, $p < 0.01$; Fig. 2a) and a similar, but non-significant trend for PC2 ($R = 0.42$, $p = 0.17$; Fig. 2b).

These results indicate that male courtship performance in the thread assay is highly correlated with male performance on a real web suggesting that the thread assay is a legitimate proxy for male courtship performance during a natural interaction on a female web. However, some differences were apparent in male courtship for PC2, which represented rocking behaviour during shuddering.

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Principal component 1</th>
<th>Principal component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shudder duration</td>
<td>0.53</td>
<td>0.12</td>
</tr>
<tr>
<td>Number of rocks</td>
<td>0.41</td>
<td>0.61</td>
</tr>
<tr>
<td>Gap duration</td>
<td>0.50</td>
<td>0.11</td>
</tr>
<tr>
<td>Shudder ratio</td>
<td>0.21</td>
<td>0.75</td>
</tr>
<tr>
<td>Shudder rate</td>
<td>0.50</td>
<td>0.19</td>
</tr>
<tr>
<td>Eigenvalues</td>
<td>3.14</td>
<td>1.38</td>
</tr>
<tr>
<td>% variance explained</td>
<td>69.46%</td>
<td>30.54%</td>
</tr>
</tbody>
</table>

One possible explanation for the difference between the two tests could be that males face highly variable conditions in natural female webs. Female A. keyserlingi vary in their aggressiveness (Wignall and Herberstein, 2013a), in the size of their webs (Herberstein et al., 2000) and in their attractiveness/quality (Gaskett et al., 2004). Males may respond to these differences by adjusting their rocking behaviour within each shudder. This is the most likely explanation for the observed results, rather than assuming the thread essay measured a different aspect of male behaviour, or that males were consuming energy reserves, as shuddering behaviour is highly repeatable within individuals even after multiple tests (Wignall et al., 2014).

Despite small differences in male courtship behaviour between the thread assay and the female’s web, our results demonstrate that male performance is matched between the tests for shudder duration, gap duration and shudder rate. This is particularly important given that earlier work has demonstrated that shudder duration and rate are important for female mating decisions in this species (Wignall and Herberstein, 2013a). Signalling rate is often a target for female decision-making, and are also often the signal components that are the most energetically costly (Kotiaho et al., 1998; Cady et al., 2011). Nevertheless, the indication of some variation in rocking between the test contexts suggests caution should be applied when using the thread assay to examine this behaviour.

The use of the thread assay has multiple advantages for experimental work. Firstly, the assay conditions are highly standardised. Hence, variables that could influence male courtship on the web, such as the distance for the male approach to the female at the hub (which can start from any part of the web periphery), the characteristics of the web (architecture, tension, presence of prey) and, in particular, the female’s response to the male do not need to be factored into the protocols. The use of the thread assay also significantly reduces the time required for analysis as the thread assay records male courtship for a maximum of 1 min (max. recording time in this experiment: 54.25 s) while recordings of courtship in the female’s web run until the male mates with the female which can take up to 3 h. Courtship behaviour is often lengthy and complicated by many interacting factors, which make quantitative analysis difficult. The thread assay developed here can help solve some of these issues.

### 3.2. Effects of irradiation on male fertility

No developed eggs were found in any of the clutches laid by the five females double mated with two sterile males (mean egg
development = 0 ± 0%; SS, n = 19 egg sacs). One female doublemated with two fertile males died before she could lay any eggs. The remaining four females laid multiple egg sacs (range 1–4). The hatching success for the first 3 egg sacs was 100 ± 0% (CC, n = 8 egg sacs). Two females laid a fourth egg sac: mean egg develop-oment 47 ± 33% (CC, n = 2 egg sacs).

Our findings demonstrate that male irradiation from a cobalt γ-emitter at a dosage of 40 Gy is an efficient and effective method to induce complete sterility in A. keyserlingi. Many studies adopting the sterile male technique require the implementation of a correction factor to account for the proportion of unfertilised or undevel-oped eggs that might be present in natural clutches, due to insufficiency of sperm to allow fertilisation, disease or genetic incompatibility (Boorman and Parker, 1976; Schneider and Lesmono, 2009). In the present study, there were no undeveloped eggs in the first 3 egg sacs of females double mated with fertile males. These results show that, at this dosage of radiation, it is not necessary to apply a correction factor to the first egg sac laid by an A. keyserlingi double mated female. However, while this study indicates that A. keyserlingi has low levels of infertility, it is advised to include in every study a control treatment to check natural rates of fertilisation, particularly when studying successive egg sacs.

It is interesting to note that, while the first 3 egg sacs laid by females double mated with control males contained no undevel-oped eggs, a proportion of undeveloped eggs were present in the fourth egg sac. This may be explained by the depletion or degrada-tion of sperm stored by females. The depletion of sperm stored by females has been noted in several species (e.g., D. melanogaster: Pyle and Gromko, 1978; Panurus biarmicus: Sax et al., 1998; Araneae: Austad, 1984; Hexapoda: Thornhill and Alcock, 1983). Female A. keyserlingi only store between 2000 and 6000 sperm per copulation (Herberstein et al., 2011). If sperm depletion or degradation does occur in A. keyserlingi while females are still capa-ble of producing viable offspring, then polyandry may have a selective advantage for females. Indeed, the underlying benefits of polyandry are contentious, particularly for species in which costs to females related to courtship and copulation are significant (e.g., A. keyserlingi: Herberstein et al., 2002; D. melanogaster: Holland and Rice, 1999; Stegodyphus lineatus: Maklakov and Lubin, 2004, 2006; Hexapoda: see Snook, 2014; Callosobruchus maculatus: Power and Holman, 2014; Passer domesticus: Hsu et al., 2014).

3.3. Effects of irradiation on male courtship performance

Two principal components were extracted from the NIPALS PCA representing 70.77% and the 29.23% of the variance in male courtship performance (Table 2). The loadings matrix for each principal component are shown in Table 2, with PC1 representing mainly total duration and shudder number, and PC2 representing mainly number of rocks and shudder duration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Principal component 1</th>
<th>Principal component 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shudder duration</td>
<td>0.42</td>
<td>−0.51</td>
</tr>
<tr>
<td>Number of rocks</td>
<td>0.33</td>
<td>−0.61</td>
</tr>
<tr>
<td>Gap duration</td>
<td>0.06</td>
<td>−0.31</td>
</tr>
<tr>
<td>Shudder ratio</td>
<td>0.34</td>
<td>0.24</td>
</tr>
<tr>
<td>Total duration</td>
<td>0.56</td>
<td>0.27</td>
</tr>
<tr>
<td>Shudder number</td>
<td>0.52</td>
<td>0.38</td>
</tr>
<tr>
<td>Eigenvaules</td>
<td>2.73</td>
<td>1.13</td>
</tr>
<tr>
<td>% variance explained</td>
<td>70.77%</td>
<td>29.23%</td>
</tr>
</tbody>
</table>

Irradiation treatment had no effect on male courtship performance (PC1: F3,17 = 0.03, p = 0.98; PC2: F3,17 = 0.35, p = 0.71). Further, there was no significant difference in copulation duration between irradiated and control males (first males to mate with the female, F1,22 = 0.41, p = 0.53; second males to mate with the female, F1,22 = 0.84, p = 0.37). There was no significant difference in the frequency of cannibalism between irradiated and control males (first males to mate with the female, χ2 = 0.96, p = 0.33; second males to mate with the female, χ2 = 0.02, p = 0.89).

These results demonstrate that male courtship performance in the thread assay does not change after irradiation and nor do copu-latory behaviours, specifically copulation duration and frequency of cannibalism. There has been great interest in the effects of irradiation on whole-organism performance. This is particularly true in species of bio-control significance for agriculture and disease transmission (e.g., Bactrocera tryoni: Collins et al., 2009; Weldon et al., 2010; Aedes albopictus: Oliva et al., 2012). The major-ity of these studies have concentrated on comparisons between the performance of irradiated and non-irradiated males for the develop-ment and assessment of biocontrol programs. Few to date, how-ever, have tested the effects of irradiation within individuals, and even fewer have compared behaviours of reproductive signifi-cance, such as courtship performance, in detail. Further, our under-standing of the effects of irradiation in species that have the potential of becoming models for sexual selection theory, such as in spiders, lags considerably behind. The results presented here demonstrate the potential for adopting the sterile male technique and the thread assay for future studies such as comparing male paternity shares.

4. Conclusions

We have developed and validated the thread assay as an appro-priate tool with which to simplify and standardise the study of courtship behaviour in A. keyserlingi, an important model species for sexual selection. Importantly, we also demonstrate that irradiation treatment does not affect male courtship behaviour. This opens up huge potential for adopting the sterile male technique for paternity assignment in studies investigating the relationship between male behaviour and fertilisation success.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.02.014.

References


