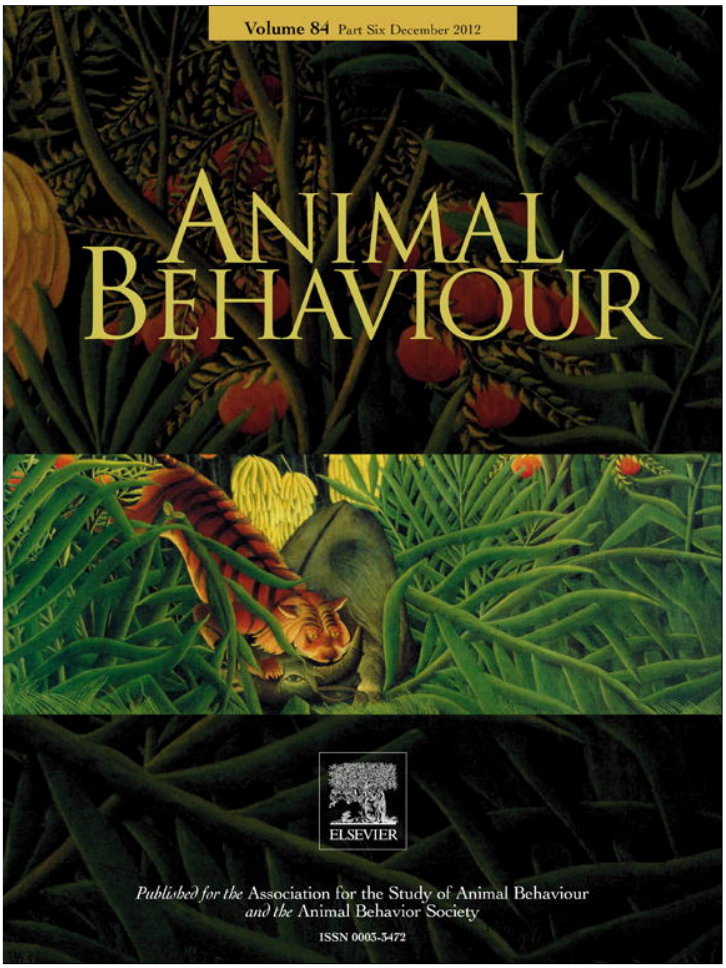

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Asymmetric reproductive isolation and interference in neriid flies: the roles of genital morphology and behaviour

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The processes underlying reproductive isolation, and the traits involved, are the subject of considerable debate in evolutionary biology. Studying the costly sexual interaction of species in secondary sympatry, a phenomenon known as reproductive interference, can help to shed light on past and present isolating mechanisms, as well as the implications of sympatry for individual fitness. We investigated the roles of two sets of traits, genitalic and behavioural, in reproductive isolation and interference in two species of Australian neriid flies, *Telostylinus lineolatus* and *T. angusticollis*. Surprisingly, although these species differ markedly in male but not in female genitalia, we found evidence that genital morphology resulted in asymmetric reproductive isolation: *T. lineolatus* males could transfer sperm to *T. angusticollis* females, but *T. angusticollis* males were unable to transfer sperm to *T. lineolatus* females. However, neither type of cross produced any viable hybrids. Behavioural responses also contributed asymmetrically to both reproductive isolation and reproductive interference. Males pursued both conspecific and heterospecific females. Females of both species discriminated against heterospecific males, but *T. lineolatus* females exhibited stronger discrimination than *T. angusticollis* females. Curiously, *T. angusticollis* males both fought and attempted to copulate with *T. lineolatus* males, resulting in reduced mating success for *T. angusticollis* males with conspecific females. Our findings show that both genitalic and behavioural traits can play important roles in reproductive isolation, but the consequences of interspecific divergence in these traits can be surprisingly complex, resulting in asymmetric effects on many aspects of interspecific interactions.

The ecological and evolutionary processes involved in the diversification of existing forms have been studied since Darwin's time, but important questions remain (Coyne & Orr 2004; Gröning & Hochkirch 2008). Even after species have become reproductively isolated, they can still encounter and interact with their congeners. When such interactions lead to reduced fitness, they are known as reproductive interference. Sexual interactions between species that cannot produce hybrid offspring are often not considered to play any significant role in evolution (Kishi et al. 2009). However, reproductive interference between species that cannot hybridize but have come into secondary contact can have contemporary importance, in that it can impose serious individual fitness costs through the expenditure of time, energy and gametes, as well as the risk of damage (Gröning & Hochkirch 2008), without any chance of producing offspring. Selection is therefore expected to favour discriminating mechanisms that minimize such costs. However, because sexual interactions between species that cannot produce viable hybrids have received relatively little study, the nature or causes of such reproductive interference remain poorly understood.

Reproductive interference is particularly likely to occur, and may be most costly, between closely related species because of similarities in phenotype, increasing the chance of mistaking heterospecifics for conspecifics (Gröning & Hochkirch 2008). In situations in which closely related or incipient species are found in sympatry, there is likely to be selection for traits that will reduce the likelihood of hybridization or reproductive interference, often resulting in divergence of reproductive characters or phenotypes. This phenomenon is known as reproductive character displacement (RCD; Crampton et al. 2011). RCD should result in fewer interspecific matings by facilitating discrimination between conspecifics and heterospecifics (Konuma & Chiba 2007). However, when species have been in secondary sympatry for a short time, or their distributions overlap only occasionally so that net selection for RCD is weak, RCD may be insufficient to prevent reproductive interference (delBarco-Trillo & Johnston 2010). The evolution of RCD may also be impeded by sexual selection within species, particularly when the same traits are used in both mate choice and species recognition (Ryan & Rand 1993; Higgie & Blows 2007). For example,

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reduced responsiveness to heterospecific traits by males may lead to reduced mating success with conspecific females.

The 'lock-and-key' hypothesis proposes that genitalia play a crucial role in reproductive isolation (Shapiro & Porter 1989): female genitalia function as a species-specific 'lock', while male genitalia are a species-specific 'key', preventing interspecific matings (Shapiro & Porter 1989). The theory has minimal support (House & Simmons 2003; Eberhard & Ramirez 2004; Andrade et al. 2008; Arbuthnott et al. 2010; but see Sota & Kubota 1998; Takami 2003; Tanabe & Sota 2008; Wojcieszek & Simmons 2012), in that male genitalia are typically much more variable across species than female genitalia; there are too many different keys and not enough different locks (Eberhard 1985). However, despite their apparent similarity across related species, female genitalia could diverge in subtle ways that contribute to reproductive isolation.

The nature and degree of behavioural discrimination between conspecifics and heterospecifics is also a key factor in the extent of reproductive interference. Selection may favour behavioural mechanisms that reduce the likelihood or costs of interspecific interactions. Even where species do not currently occur in sympatry, reproductive interference studies can reveal the possible consequences of any future overlaps in distribution.

We investigated the role of genitalia and behaviour in premating reproductive isolation and interference in two Australian species of neriid flies: *Telostylinus angusticollis* and *Telostylinus lineolatus* (Diptera: Neriidae). *Telostylinus lineolatus* occurs in northern Queensland and on a number of Pacific islands (Enderlein 1922; Hennig 1937), and typically appears to breed on rotten fruit (R. Bonduriansky, unpublished data). *Telostylinus angusticollis* is found in southern Queensland and New South Wales (NSW), and typically appears to breed on rotting tree bark (Bonduriansky 2006). However, both species are attracted to a diverse range of rotting vegetative material (R. Bonduriansky, personal observations), and would thus probably encounter each other on oviposition substrates if they occurred in sympatry. The ranges of these species may overlap in some locations between Brisbane and Cairns, although a zone of sympatry has not yet been located. However, it is clear that these species exist in allopatry throughout most of their ranges. No other neriid species are known to occur in Australia.

These species are similar phenotypically. *Telostylinus lineolatus* individuals have more distinct stripes on the dorsal surface of the thorax, whereas *T. angusticollis* individuals have a thicker orange stripe on the tibia of each leg. Also, *T. lineolatus* flies have one pair of dorsocentral bristles, while *T. angusticollis* flies generally have two (Bonduriansky 2009). These species also differ in the degree of plasticity of body size and shape. When reared on a rich larval diet, *T. angusticollis* males grow to be much larger than females and develop exaggerated secondary sexual characters, while males reared on a poor larval diet emerge around the same size as, or smaller than, females (Bonduriansky 2007). *Telostylinus lineolatus* exhibits less plasticity in body size and shape in response to variation in larval diet (E. Cassidy, E. Bath & R. Bonduriansky, unpublished data). *Telostylinus angusticollis* males reared on a rich larval diet are much larger, with longer legs and head capsules, than *T. lineolatus* males. Females of both species are less affected by larval diet, with *T. angusticollis* females consistently larger than *T. lineolatus* females. Males of both species will fight males that are of approximately the same size, but small *T. angusticollis* males tend to avoid fighting large males (E. Bath & R. Bonduriansky, personal observations).

To determine whether interspecific differences in genitalia prevent interspecific copulations, we investigated genital coupling in conspecific and heterospecific matings. To understand the contribution of behaviour to prezygotic reproductive isolation, as well as to reproductive interference, we also studied male–female and male–male interactions in conspecific and heterospecific contexts, as well as under varying sex ratios (one female with one male or one female with two males). We also asked whether the degree of reproductive interference was affected by phenotypic variation (generated through manipulation of larval diet quality) in the body size and shape of *T. angusticollis* males. *Telostylinus angusticollis* males raised on a poor larval diet are similar in body size to *T. lineolatus* males, so this treatment allowed us to establish whether the effect of male species resulted from a difference in body size. Finally, we investigated the potential for these species to produce viable hybrids.

METHODS

Source and Rearing of Flies

Telostylinus lineolatus were collected from the wild at Cow Bay, Cairns and Cape Tribulation, Queensland. *Telostylinus angusticollis* were collected from Fred Hollows Reserve, Sydney, NSW. Both species were reared as large (>200 individuals per generation), outbred populations in 15-litre population cages for several generations before the start of the present study. *Telostylinus angusticollis* eggs were collected from population cages and transferred to either high-quality ('rich') or low-quality ('poor') larval media, which differed three-fold in the concentration of protein and sugar (see Bonduriansky 2007 for details). The poor medium produced much smaller adult flies (comparable in size to average adults of *T. lineolatus*).

For investigation of behaviour and genital coupling, *T. angusticollis* eggs were collected from the population cage over a period of 7 days. Of these eggs, half were placed in rich larval medium and half were placed in poor larval medium. *Telostylinus lineolatus* eggs were collected from the population cage over a period of 4 days and all were placed in rich larval medium. Containers of larval medium were kept in a controlled temperature room at 25 °C and were watered regularly to prevent desiccation of the larval medium. Adult flies emerged around 3 weeks after eggs were collected. Males and females from each species were separated immediately after emergence and maintained in same-sex groups for 2 weeks prior to the behavioural study to ensure all flies were reproductively mature virgins. Adult flies were provided with a layer of moist coco peat as a source of water and a small petri dish containing brown sugar. The sugar was replaced regularly and the containers watered every second day.

Behavioural Interactions

To observe precopulation and copulation behaviour, virgin males and females of either the same or different species were placed in 250 ml plastic containers containing a layer of oviposition medium (rich larval medium, which had been allowed to grow mould to encourage mating and oviposition). Studies were conducted under two sex ratio treatments: one female with one male (experiment 1) and one female with two males (experiment 2). *Telostylinus angusticollis* males reared on rich and poor diets were included as separate treatments. *Telostylinus lineolatus* males, and females of both species, were raised only on rich diets.

Each replicate group of flies was observed for 45 min. We recorded the number of matings, duration of each mating, number of rejections by females, number of male–male mating attempts and number of fights between males. An interaction was recorded as a mating if it involved a male positioning himself above or behind a female, mounting her and remaining in this position for at least 10 s (Supplementary Fig. S1). Mate rejection by females was evidenced by females running away when males attempted to

position themselves above the females, females kicking males off before or after the male had attempted to mate or if females refused to raise their ovipositor (which is necessary for intromission). Fights between males consisted of males using their legs and bodies to strike one another. Male–male mating attempts were instances where one male would perform mating behaviour with another male instead of a female (i.e. positioning himself above the other male and attempting to initiate genital coupling). Durations were timed using a handheld timer. For each species × sex combination in experiment 1, we observed 20 replicates in two blocks. For each species × sex × larval diet combination in experiment 2, we observed 25 replicates. Both experiments were carried out at an ambient room temperature of 25 ± 3 °C, under fluorescent lighting.

Genital Coupling

The genitalia of males and females of both species were examined using dissections of individuals and mating pairs frozen in copula. Dissections were carried out under a Leica MZ16A stereoscope (Wetzlar, Germany). In uncoupled males, the epandrium and aedeagus were severed from the abdomen. In uncoupled females, the oviscape containing the female reproductive tract was severed from the abdomen. These structures were placed into a drop of saline solution on a microscope slide, and dissected using micropin probes and fine forceps.

To determine how male and female genitalia interacted during mating, one virgin male and one virgin female (in all possible sex × species combinations, each replicated 10 times) were placed in a mesh mating cage containing a petri dish of oviposition medium to encourage mating. Once pairs had begun to mate, they were flash frozen by quickly submerging the entire cage in liquid nitrogen. Flies were then transferred to a −20 °C freezer for storage. The pairs were later removed and allowed to thaw before being dissected in copula under a Leica MZ16A stereoscope. The abdomens of the male and female were severed from the flies' bodies using fine scissors and placed in saline solution, taking care to avoid separation of the male and female genitalia. A further cut was made, which separated the male's epandrium from the aedeagus, which was inserted into the female reproductive tract. The sclerotized outer casing of the female oviscape was then carefully removed to allow access to the reproductive tract. Any connective tissue was cleared away where it obstructed viewing of the reproductive tract.

Images of all specimens were made using a Leica DFC420 camera mounted on the Leica MZ16A stereoscope. Where necessary, additional images were made with the same camera mounted on a Zeiss Axioskop 40 (Göttingen, Germany) compound microscope, after a coverslip was placed over the dissected specimen.

Hybridization Experiment

In a separate experiment to test for the capacity of these species to hybridize, *T. lineolatus* eggs were collected from the Cow Bay, Cairns and Cape Tribulation stock cages and *T. angusticollis* eggs were collected from the Fred Hollows Reserve stock cage, and all eggs were transferred to rich larval medium. Adults that emerged were paired as virgins as follows: 25 pairs comprised a *T. angusticollis* male and *T. lineolatus* female (of which nine females were from the Cairns population, eight females were from the Cow Bay population and eight females were from the Cape Tribulation population), and 25 pairs comprised a *T. angusticollis* female and *T. lineolatus* male (of which eight males were from the Cairns population, nine males were from the Cow Bay population and eight males were from the Cape Tribulation population). All pairs were placed in 250 ml containers with a layer of moist coco peat at the bottom, small dishes of brown sugar and soy protein as food, and a petri dish of rich larval medium as oviposition substrate. Males that died prior to oviposition were replaced. Petri dishes were checked daily for oviposition, and eggs were transferred to 200 ml of fresh rich larval medium. Oviposition dishes and larval medium containers were watered regularly to keep the medium moist.

Data Analysis

Mating duration was analysed using ANOVA, with male species and female species as independent categorical factors and block as a random factor. ANOVA was also used to test for differences in mating duration between *T. angusticollis* males raised on rich and poor diets, with male diet and female species nominated as independent factors. Where the data did not meet the assumptions of parametric tests and could not be transformed to meet a normal distribution, nonparametric tests (Mann–Whitney *U* tests and Wilcoxon signed-ranks tests) were used. All statistical tests were carried out using STATISTICA 7 (StatSoft 2005). Data were initially analysed with *T. angusticollis* rich and poor males examined separately, but where there were no differences the data were pooled to allow an overall species comparison. Where there were no qualitative differences between blocks in experiment 1, data were pooled. Otherwise, results are reported separately for each block.

RESULTS

Behaviour

Experiment 1: one female, one male

There was no significant block effect on mating duration (ANOVA: $F_{1,88} = 0.07$, $P = 0.79$). Mean mating duration differed significantly between species. *Telostylinus lineolatus* males mated for longer than *T. angusticollis* males, regardless of the species of the female with which they were paired (ANOVA: $F_{1,69} = 74.82$, $P < 0.001$). *Telostylinus lineolatus* females mated for longer on average than *T. angusticollis* females, regardless of male species (ANOVA: $F_{1,69} = 18.39$, $P < 0.001$). There was also a significant interaction between male and female species, which appears to be primarily driven by a difference in *T. lineolatus* male mating duration influenced by female species: *T. lineolatus* males mated for longer with conspecific females than with heterospecific females and *T. lineolatus* females mated for longer with conspecific males than with heterospecific males (ANOVA: $F_{1,69} = 25.62$, $P < 0.001$).

There was a significant difference in the mean number of matings between blocks (Mann–Whitney *U* test: $Z = 2.21$, $N_1 = 41$, $N_2 = 32$, $P = 0.027$), with more matings in the first than in the second block. This difference was driven primarily by *T. lineolatus* males mating more often in the first block than in the second block, but the same was not observed for *T. angusticollis* males (Mann–Whitney *U* test: *T. lineolatus*: $Z = 2.33$, $N_1 = N_2 = 20$, $P = 0.02$; *T. angusticollis*: $Z = 1.66$, $N_1 = 40$, $N_2 = 20$, $P = 0.098$). In both blocks, however, *T. lineolatus* males mated more times than *T. angusticollis* males, regardless of female species (see Fig. 1; Mann–Whitney *U* test on pooled data: $Z = -3.45$, $N_{T.\ angusticollis} = 80$, $N_{T.\ lineolatus} = 40$, $P < 0.001$). This difference in mating frequency was most evident in cross-species matings, where *T. lineolatus* males mated more often with *T. angusticollis* females than *T. angusticollis* males mated with *T. lineolatus* females (Mann–Whitney *U* test: $Z = 3.01$, $N_{T.\ lineolatus\ \text{males}} = 20$, $N_{T.\ angusticollis\ \text{males}} = 40$, $P = 0.003$). This was also reflected in the significant difference in rejections by females in heterospecific treatments. Females of both species rejected heterospecific males more than males of their own species (Fig. 1; Mann–Whitney *U* test:

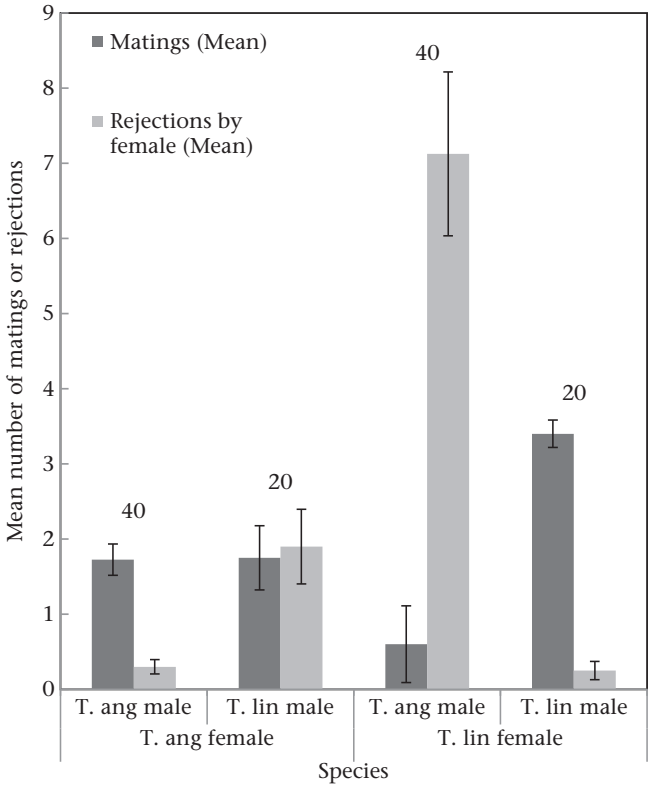

Figure 1. Effect of male and female species treatment on the mean number of matings and rejections in one 45 min observation period in experiment 1 (one female, one male). The dark bars indicate the mean number of matings, while the lighter bars indicate the mean number of rejections by females in a single observation period. Error bars represent the standard error of the mean. Numbers above the bars indicate sample size of each treatment.

T. lineolatus: $Z = 4.96$, $N_{\text{conspecific}} = 20$, $N_{\text{heterospecific}} = 40$, $P < 0.001$; *T. angusticollis:* $Z = -3.49$, $N_{\text{conspecific}} = 40$, $N_{\text{heterospecific}} = 20$, $P < 0.001$) but *T. lineolatus* females rejected heterospecific males more often than did *T. angusticollis* females (Mann–Whitney *U* test: $Z = -3.15$, $N_{T.\ lineolatus\ \text{female}} = 20$, $N_{T.\ angusticollis\ \text{female}} = 40$, $P = 0.002$).

There were no significant differences between *T. angusticollis* males raised on poor and rich diets in mating duration or number of matings (Mann–Whitney *U* tests: matings: $Z < -1.70$, $N_{\text{rich}} = 24$, $N_{\text{poor}} = 18$, $P > 0.089$). There was a near-significant interaction in mating duration (ANOVA: $F_{1,38} = 3.54$, $P = 0.068$), with poor-diet males experiencing a reduction in mating duration with *T. lineolatus* females relative to matings with *T. angusticollis* females, and rich-diet males experiencing an increase in mating duration with *T. lineolatus* females.

Experiment 2: one female, two males

Telostylinus angusticollis males achieved greater mating success with *T. angusticollis* females when paired with a conspecific male than when paired with a heterospecific male (Fig. 2; Mann–Whitney *U* test: $Z = 2.23$, $N_{\text{conspecific}} = 75$, $N_{\text{heterospecific}} = 50$, $P = 0.026$). In contrast, for *T. lineolatus* males, there was no significant difference in mating frequency when paired with a conspecific or heterospecific male (Mann–Whitney *U* test: $Z = -0.14$, $N_{\text{conspecific}} = 25$, $N_{\text{heterospecific}} = 50$, $P = 0.892$). In both species, however, the presence of a second male of either species significantly reduced the number of matings, relative to the number of matings that occurred when only one male was present (Mann–Whitney *U* test: over all treatments: $Z = 4.25$, $N_{1\ \text{male}} = 120$, $N_{2\ \text{males}} = 200$, $P < 0.001$; *T. lineolatus* same-species treatments: $Z = 4.03$, $N_{1\ \text{male}} = 30$, $N_{2\ \text{males}} = 25$, $P < 0.001$; *T. angusticollis* same-species treatments: $Z = 2.47$, $N_{1\ \text{male}} = 40$, $N_{2\ \text{males}} = 75$, $P = 0.008$).

There was also a significant difference in mating duration between experiments 1 and 2 for *T. lineolatus* males, which mated for longer when a second male of either species was present (Fig. 3; Mann–Whitney *U* test: $Z = -4.21$, $N_1 = 31$, $N_2 = 26$, $P < 0.001$). In contrast, *T. angusticollis* males did not mate for longer in the presence of a second male (Mann–Whitney *U* test: $Z = -1.47$, $N_1 = 42$, $N_2 = 63$, $P = 0.143$).

There was no significant difference in the mean conspecific mating duration or number of rejections by conspecific females between the mixed-species and same-species treatments in experiment 2 (Mann–Whitney *U* tests: $|Z| < 1.94$, $P > 0.06$). There was also no significant difference in the number of fights between males when there were two *T. angusticollis* males reared on rich larval diet, two *T. lineolatus* males or one male of each species in a container (Mann–Whitney *U* tests: $|Z| < 1.57$, $P > 0.117$). *Telostylinus angusticollis* males reared on poor larval diet rarely engaged in fights, regardless of the species or larval diet of the other male.

Telostylinus angusticollis males attempted to mate with heterospecific males more often than they attempted to mate with conspecific males (Mann–Whitney *U* test: $Z = -3.48$, $N_{\text{conspecific}} = N_{\text{heterospecific}} = 25$, $P < 0.001$). Moreover, *T. angusticollis* males reared on rich larval diet tried to mate more often with *T. lineolatus* males than with females of either species (Wilcoxon test: $T = 141$, $Z = 3.18$, $N = 50$, $P = 0.002$). There was no significant difference in the number of male–male versus male–female mating attempts made by *T. angusticollis* males reared on poor larval diet (Wilcoxon test: $T = 90$, $Z = 0.89$, $N = 50$, $P = 0.375$), or by *T. lineolatus* males (Wilcoxon test: $T = 23.5$, $Z = 1.54$, $N = 36$, $P = 0.124$).

Genitalia

Male genital morphology

In both species, the aedeagus consists of a basal, middle and distal section (Fig. 4). The epandrium and the basal and middle sections of the aedeagus are similar in the two species (compare Fig. 4a, b). In both species, the basal and middle sections are connected by a complex hinge, which allows the aedeagus to bend at least 180°. The middle section also has a flexible spot in the middle that may allow the aedeagus to bend during intromission. The primary differences between the two species occur at the distal section of the aedeagus. Where the middle section joins the distal section, both species possess a rigid spike, but this spike is much larger in *T. angusticollis* (Fig. 4a, b). In *T. angusticollis*, the distal section is a long, unsclerotized, flexible tube (Fig. 4a), which is coiled up at the base of the epandrium when the genitalia are retracted. In *T. lineolatus*, the distal section is a sclerotized hook with a short, transparent tube at the tip (Fig. 4b). The hook can bend and straighten at a flexible spot in the middle.

Female genital morphology

The female genitalia appear to be very similar in the two species. A distal ovipositor leads to a highly elastic tube with a granular texture (Supplementary Fig. S2). This tube leads into a muscular bursa copulatrix, which is normally bent in an S-shape but can be straightened during mating (Supplementary Fig. S3). The only visible difference is at the proximal end of the bursa, where two thin spermathecal ducts extend from a common, muscular lumen: in *T. angusticollis* the anterior duct leads to one spermatheca and the posterior duct divides into two branches leading to two spermathecae (Fig. 4c). In *T. lineolatus*, there are only two spermathecae, one at the end of each spermathecal duct. The anterior duct leads to an enlarged spermatheca, while the posterior duct leads to

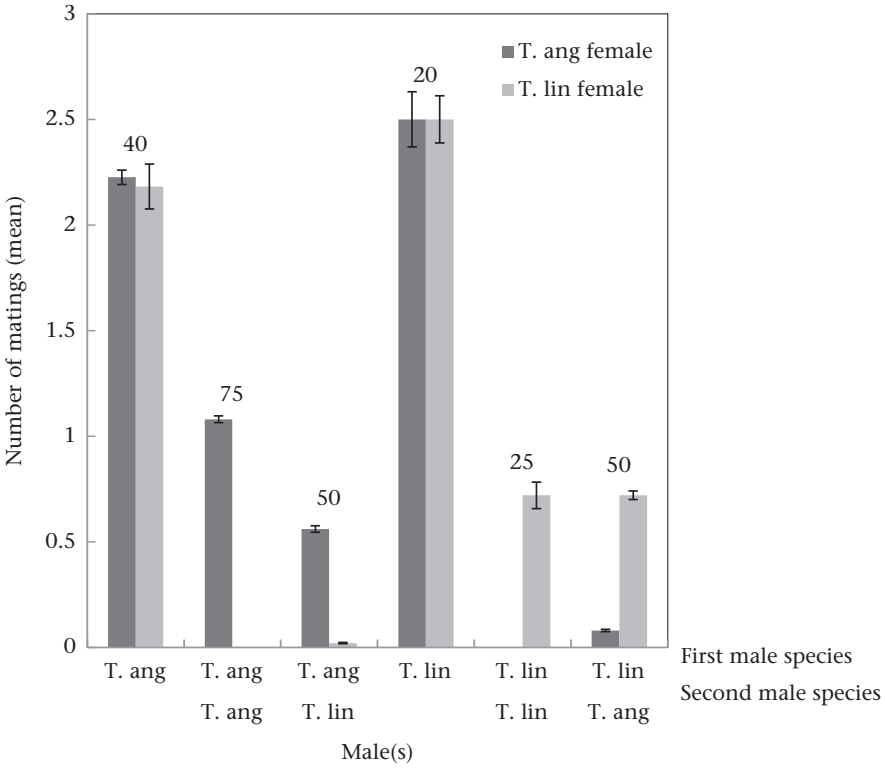

Figure 2. Mean number of matings in one 45 min time period in experiments 1 (one female, one male) and 2 (one female, two males). The number and species of males used in the experiment are shown below the plot. In treatments involving two males of different species, bars represent matings by the first male. In treatments involving two *T. angusticollis* males, data for males reared on rich and poor larval diets were pooled. The dark bars indicate experiments using *T. angusticollis* females, while the lighter bars indicate experiments using *T. lineolatus* females. Error bars represent the standard error of the mean. Numbers above the bars indicate sample size of each treatment.

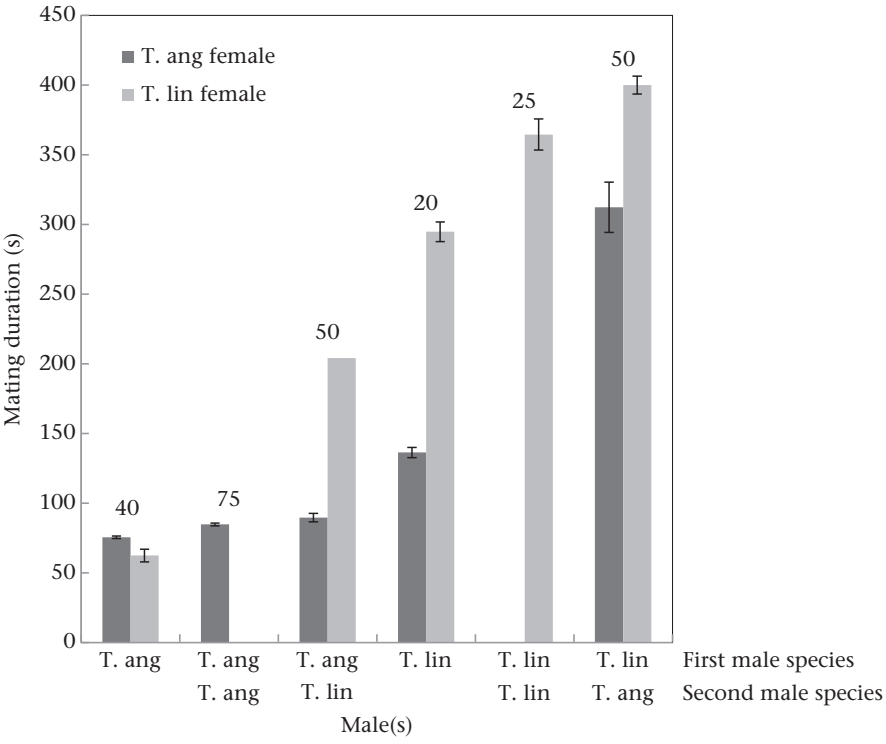

Figure 3. Mean duration of observed copulations in experiments 1 and 2. The number and species of males used in the experiment are shown below the plot. In treatments involving two males of different species, bars represent matings by the first male. In treatments involving two *T. angusticollis* males, data for males reared on rich and poor larval diets were pooled. The dark bars indicate experiments using *T. angusticollis* females, while the lighter bars indicate experiments using *T. lineolatus* females. Error bars represent the standard error of the mean. Numbers above the bars indicate sample size of each treatment.

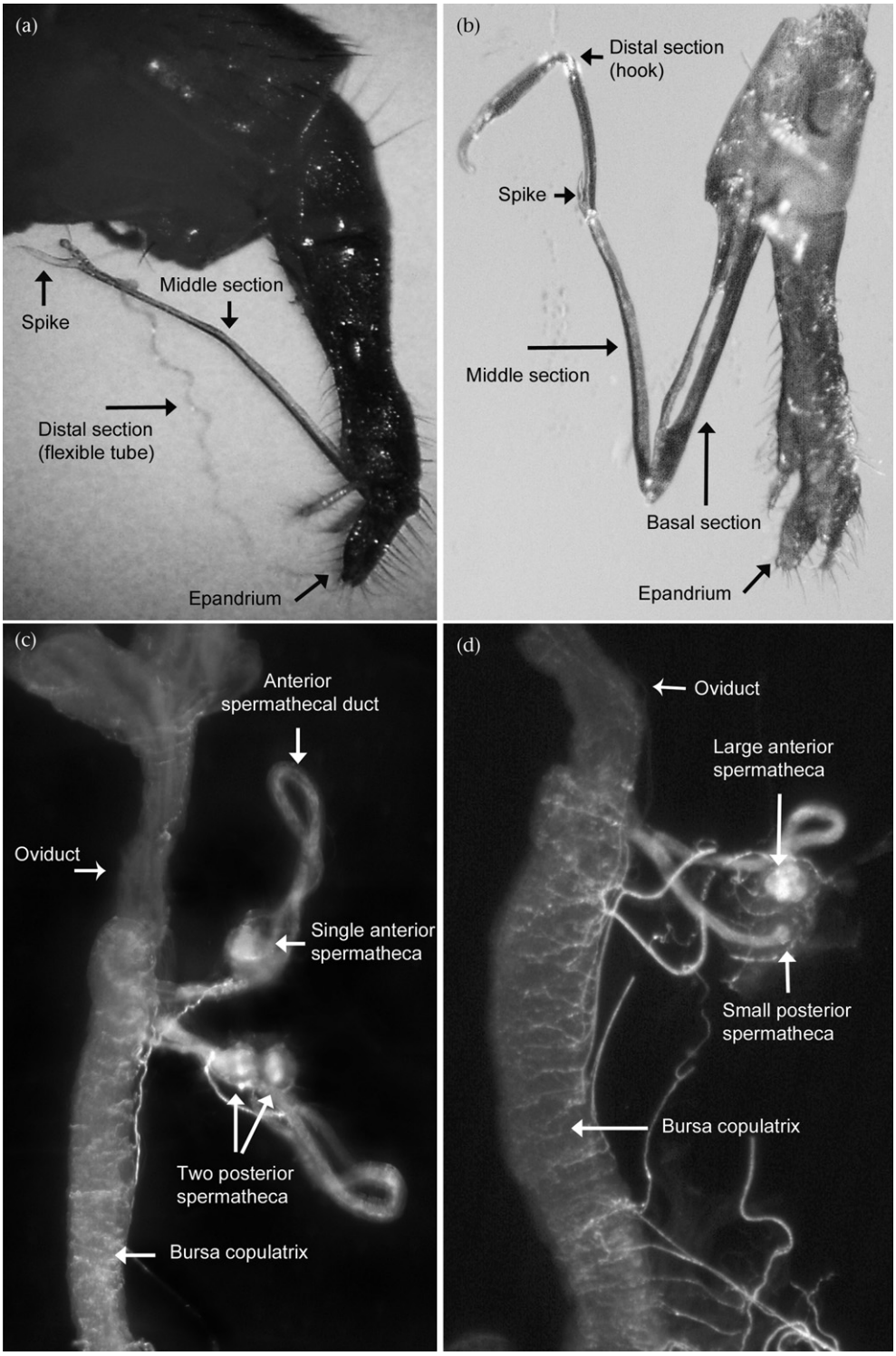

Figure 4. Male and female genitalia: (a) *T. angusticollis* male epandrium and aedeagus; (b) *T. lineolatus* male epandrium (severed from abdomen) and aedeagus; (c) *T. angusticollis* female bursa copulatrix and spermathecae; (d) *T. lineolatus* female bursa copulatrix and spermathecae (note sperm in the spermathecal ducts and spermathecae of both females).

a reduced spermatheca (Fig. 4d). All spermathecae are spherical and surrounded by muscle.

Copulation and genital coupling in conspecific and heterospecific pairs

There was little precopulatory courtship visible in either species under observation. Males would approach females and position themselves above the female (Supplementary Fig. S1). Males would use their epandrium to raise the female's oviscape so that it was extended laterally, and then attempt to insert their aedeagus. Males sometimes tapped the oviscape with their epandrium in the first 20 s of mating. Occasionally males would withdraw their aedeagus from the female after 20 s and reinsert it (more frequently in *T. lineolatus*). In both conspecific and heterospecific

matings, females could reject males by running away from the male as he approached, refusing to raise the oviscape, or kicking the male with the rear legs. A female could also end a mating after copulation had begun by pushing out the male's genitalia with her legs. Once the male had inserted his aedeagus, there was no evidence of other forms of stimulation reported in other neriids (Eberhard 1998).

Genital coupling appeared to begin in both species with males inserting the closed joint between the basal and middle section of the aedeagus into the female's genital opening, and then unfolding the aedeagus inside the female reproductive tract until fully extended (Supplementary Figs S3, S4). The differing morphology of the male genitalia resulted in a difference in how far up the reproductive tract males of each species could reach. *Telostylinus angusticollis* males uncoiled the flexible tube that constitutes the distal section of the aedeagus and inserted it into the posterior spermathecal duct, which leads to two spermathecae (Supplementary Fig. S4). It is not known how tube uncoiling and insertion are achieved. Males then released sperm into the spermathecal duct, and the sperm moved rapidly through the branches of the duct and into the spermathecae (Supplementary Fig. S5). *Telostylinus lineolatus* males extended the sclerotized hook that constitutes the distal section of the aedeagus, and inserted the tip of the hook into the base of the posterior spermathecal duct (Supplementary Fig. S6). Once sperm had been deposited inside the spermathecal duct, *T. lineolatus* males removed their aedeagus from the duct and deposited a mass of sticky accessory gland material (probably a mating plug) inside the bursa.

In heterospecific pairs, genital coupling was achieved successfully in one combination but not in the other. In all 10 pairs composed of a *T. lineolatus* female and *T. angusticollis* male that were frozen in copula, the closed basal-middle joint of the male's aedeagus was inserted into the female's genital opening, but the aedeagus was not unfolded. Of the six *T. lineolatus* females dissected following copulation with a *T. angusticollis* male in experiment 1, none had sperm inside the reproductive tract. In contrast, in the 10 pairs composed of a *T. angusticollis* female and a *T. lineolatus* male, genital coupling appeared to progress as in conspecific *T. lineolatus* pairs: males were able to unfold the aedeagus fully inside the female reproductive tract, insert the tip of the distal hook into the posterior spermathecal duct, transfer sperm into the duct, and then deposit a mass of gelatinous accessory gland material inside the bursa copulatrix.

Hybridization Experiment

All females oviposited. However, none of the eggs hatched.

DISCUSSION

Our results provide evidence of both reproductive isolation mechanisms and reproductive interference between *T. angusticollis* and *T. lineolatus*. Divergence in sexual behaviour and genital morphology both played a role in reducing the frequency of interspecific copulations. However, similarity between these species in genital structure, as well as in behaviour, also resulted in heterospecific copulations, combat and homosexual mounting that may impose substantial fitness costs on individuals encountering heterospecifics, if these species were to come into secondary sympatry. Intriguingly, both the premating isolating mechanisms and the reproductive interference were substantially asymmetrical in their effects, a pattern that could have important evolutionary implications. Although *T. lineolatus* males were able to transfer sperm to *T. angusticollis* females, no viable hybrids were produced, indicating the existence of postmating isolating mechanisms.

Genitalia as Reproductive Isolating Mechanisms

The lock-and-key theory proposes that differences in genital morphology between species serve as a physical barrier to prevent interspecific mating (Shapiro & Porter 1989). We found that male genital morphology differed significantly between species in the distal section of the aedeagus, raising the possibility that these differences could act in reproductive isolation. Female genital morphology in the two species, on the other hand, was so similar that it was difficult to distinguish between species based solely on female genitalia, and there appeared to be no consistent differences between females of different species that would act as a mechanical barrier to heterospecific males. However, whereas *T. lineolatus* males had little trouble mating with *T. angusticollis* females, the reciprocal cross was never observed to result in full intromission or sperm transfer. This did not reflect a lack of sexual interest by males: when *T. angusticollis* males were paired with *T. lineolatus* females (experiment 1), mating attempts exceeded the average copulation duration for *T. angusticollis* conspecific pairs. *Telostylinus angusticollis* males also attempted to mate more often with *T. lineolatus* females (three or four mating attempts/pairing) than they did with *T. angusticollis* females (one or two mating attempts/pairing). This suggests that *T. lineolatus* females possess some mechanism that prevents intromission by *T. angusticollis* males. Unfortunately, female genitalia are difficult to study because they are internal, soft and membranous (Shapiro & Porter 1989). Thus, we may have overlooked differences between species in female musculature and sensory organs that could account for asymmetric reproductive isolation. Differences in structure may also be lost in the process of dissection, or may only be evident in live specimens (Cordoba-Aguilar 2005).

One testable prediction of the lock-and-key hypothesis is that the degree of genetic divergence between closely related species should be correlated with the frequency of potential reproductive contacts (Eberhard 1985): populations in sympatry should show greater divergence than those in allopatry. As the populations we studied were allopatric, but still differed significantly in male genital characters, it seems unlikely that reproductive isolation played a major role in the evolution of the current morphological differences in male genitalia. However, divergence in genitalia may have been initiated in the early stages of speciation, when the ancestors of these populations may have been more likely to encounter each other, and selection may have favoured genitalic isolating mechanisms. Although our results suggest some support for genitalia acting in reproductive isolation, they differ from the predictions of the lock-and-key theory in that there appears to be only one 'lock' that is species-specific (*T. lineolatus* females).

The females' long, coiled spermathecal ducts and the long, flexible tube of male *T. angusticollis* suggest male–female coevolution. Depositing sperm closer to the spermathecae could increase male fertilization success (van Lieshout & Elgar 2011), and may also reduce female control over sperm use (Marchini et al. 2009). Selection on males may therefore favour sperm deposition as close as possible to the spermathecae. If reduction in control over sperm use is costly for females, then there will also be selection on females to impede males' access to the spermathecal ducts (Cordoba-Aguilar 2005). In these neriids, selection on females to maintain control of sperm use (i.e. sexual conflict) may therefore have driven the evolution of long, coiled spermathecal ducts. It is also possible that the coiled ducts evolved to function in cryptic female choice, where only the fittest males are able to transfer sperm that is capable of reaching the spermathecae (Eberhard 1985; Eberhard & Ramirez 2004).

Behaviour as Reproductive Isolating Mechanism

Telostylinus lineolatus females appeared to be able to identify and reject heterospecific males consistently. This was not due to a significant difference in male body size. *Telostylinus lineolatus* females were equally likely to reject *T. angusticollis* males raised on a poor larval diet (and similar in body size to *T. lineolatus* males) as they were to reject *T. angusticollis* males raised on a rich larval diet. In contrast, *T. angusticollis* females rejected heterospecific males more often than conspecific males, but also mated more often with *T. lineolatus* males than with *T. angusticollis* males in experiment 1. The increased number of rejections and matings is probably more closely related to male effort than female species recognition, as *T. lineolatus* males attempted to mate more often than *T. angusticollis* in all treatments.

There was no obvious precopulatory courtship behaviour in either species, with males generally approaching females from behind. This makes it unlikely that premating behaviour is a character used by *T. lineolatus* females to identify conspecific males. It is possible that other phenotypic differences, such as coloration, chemical cues or tactile cues, were detected by females. Chemical cues can be important in species recognition in insects (Sota & Kubota 1998; Arbuthnott et al. 2010), copepods (Thum 2007) and salamanders (Verrell 1994). In *Drosophila serrata*, females choose males partially based on cuticular hydrocarbons (CHCs). CHCs play a role in both within-species mate choice and species recognition, with sexual selection and species recognition sometimes favouring different female responses (Higgie & Blows 2007, 2008). The use of CHCs or other chemical cues has not been investigated in neriid flies. The substantial difference between these species in male genitalia, while not sufficient to prevent sperm transfer from *T. lineolatus* males to *T. angusticollis* females, may none the less allow females of both species to identify and attempt to reject heterospecific males.

The apparent existence of species recognition abilities only in *T. lineolatus* females suggests that heterospecific mating may be especially costly in this species (Gröning & Hochkirch 2008; Crowder et al. 2010). *Telostylinus lineolatus* females are much smaller than *T. angusticollis* males reared on a rich larval diet, and may therefore suffer from increased damage in mating attempts with these males. Moreover, *T. lineolatus* females are smaller than *T. angusticollis* females, and as body size is often linked to fecundity in insects, may carry fewer eggs (Bonduriansky 2001). This may increase the proportional cost of mating with a heterospecific male if a certain number of eggs are fertilized but fail to hatch, as there are then fewer eggs remaining to be fertilized by a conspecific male than would be the case for a *T. angusticollis* female that mated with a *T. lineolatus* male.

Reproductive Interference

Reproductive interference is typically deemed to occur when reproductive interactions adversely affect the fitness of at least one of the species involved (Gröning & Hochkirch 2008; Kishi et al. 2009). This is most often seen in reduced mating frequency, shortened copulations or reduced conspecific success rate (Verrell 1994; Ben-david et al. 2009; Kishi et al. 2009). There was evidence of reproductive interference between *T. angusticollis* and *T. lineolatus*. Males of both species attempted to mate with heterospecific females, resulting in rapid rejections as well as prolonged mating attempts (*T. angusticollis* males with *T. lineolatus* females) and copulation with sperm transfer (*T. lineolatus* males with *T. angusticollis* females). *Telostylinus angusticollis* males also attempted to mate with *T. lineolatus* males, often more frequently than with females of either species.

T. angusticollis males (regardless of larval diet) had a reduced number of matings when in the presence of a *T. lineolatus* male. This is because *T. angusticollis* males expended much of their time and effort on fighting and attempting to mate with *T. lineolatus* males. This may result in substantial costs of lost opportunities and wasted energy for *T. angusticollis* males (Gröning & Hochkirch 2008). *Telostylinus lineolatus* males may also pay a cost from harassment in mistaken mating attempts, but we found no obvious costs to *T. lineolatus* individuals in terms of mating frequency or duration. These findings seem to run counter to the typical pattern of reproductive interference observed in other studies, which generally report higher costs for the species that is smaller or more discriminating (Takafuji et al. 1997; Ben-david et al. 2009; Kishi et al. 2009).

Interspecific matings and mating attempts may impose a variety of costs. Males may waste time, energy and ejaculate materials mating with heterospecific females (Gröning & Hochkirch 2008). Females may face even greater costs. As *T. angusticollis* males appear not to produce mating plugs, and their aedeagus terminates in an unsclerotized tube, it seems unlikely that they would be able to remove or bypass plugs deposited in the reproductive tract of *T. angusticollis* females by *T. lineolatus* males. The presence of mating plugs may thus be extremely costly for *T. angusticollis* females, in that it would prevent conspecific mating and fertilization. *Telostylinus lineolatus* accessory gland proteins may also have functions that are detrimental to females (Avila et al. 2011).

Another manifestation of reproductive interference for *T. lineolatus* males was increased duration of mating in the presence of a *T. angusticollis* male. More time spent on a mating leaves less time for other matings or gathering resources, possibly reducing overall fitness (van Lieshout 2010). Perhaps most importantly, mating may substantially increase the risk of predation in neriid flies, as it does in other insects (Sih et al. 1990). The primary predators of *T. angusticollis* are skinks, which see flies from below or from the side (Kawasaki et al. 2008). Mating pairs may thus attract the attention of predators much more than individual flies do. The presence of heterospecific males is therefore likely to be costly for males and females of both species.

The relationship between mating duration, mating frequency and reproductive success varies across species and is often complex (Siva-Jothy & Tsubaki 1989; Yasui 1994; Vermette & Fairbairn 2002). Further studies examining the relationship between behavioural measures and offspring number and viability would make it possible to quantify the costs of reproductive interference in these species. It is also unclear how closely laboratory conditions reflect those found in the wild. In particular, encounter rates in experimental containers may be unrealistically high, and females are limited in their ability to escape from males (Gröning & Hochkirch 2008).

The Potential for Hybridization

Because *T. lineolatus* males were able to inseminate *T. angusticollis* females, premating isolation between these species is incomplete, potentially allowing hybridization to occur. However, in heterospecific pairings, no viable offspring were produced, indicating the existence of effective postmating isolating mechanisms. It is not clear whether fertilization was achieved, or whether the eggs laid by females were unfertilized. Even if eggs were fertilized, cytological evidence suggests a difference in karyotype between these species (R. Bonduriansky, unpublished data), which is likely to prevent the formation of viable zygotes.

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Supplementary Material

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