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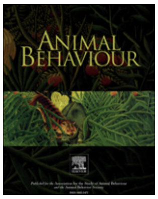

Asymmetric reproductive isolation and interference in neriid flies: the roles of genital morphology and behaviour

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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