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Function, Developmental Genetics, and Fitness Consequences of a Sexually Antagonistic Trait

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expect that its loss of function leads to early pupariation. Homozygous *dilp8*$^{EX/EX}$ animals are viable, and their timing of pupariation is only slightly advanced (~4 hours) compared with that of control animals (Fig. 3E). This modest pupariation phenotype can be explained in the light of earlier genetic experiments showing that *discless* mutant larvae pupariate with normal timing (*5*). It suggests that the onset of metamorphosis relies on additional signals provided by other larval organs.

Our experiments suggest that Dilp8 relays the growth status of the discs to the central control of metamorphosis. This raises the possibility that Dilp8 travels from the discs, where it is emitted, to its target tissues. Consistent with this, when expressed in S2R+ cells, a myc-tagged full-length form of Dilp8 is recovered in the culture medium but not a truncated form lacking the signal peptide (Dilp8Δ-myc) (Fig. 4A and fig. S5A). Moreover, by using a specific Dilp8 antibody, we could observe Dilp8 in vesicular particles apical to the wing pouch as well as in the lumen separating the columnar epithelium from the peripodial cells in discs from *Rn>dilp8*, *Rn>avl-RNAi*, and *Rn>rpl7-RNAi* animals (Fig. 4, D to F, white arrows, and fig. S6, C to F) but not in *Rn>* discs where low levels of Dilp8 were only detectable in the lumen (Fig. 4C and fig. S6B). By contrast, a nonsecretable form of Dilp8 (Dilp8Δ-myc) is found perinuclear (fig. S5, C, F, and G), suggesting that it fails to enter the secretory pathway. When *dilp8* expression was targeted to a restricted domain of the disc, Dilp8 particles were detected in cells neighboring its expression domain, in the lumen, and in the basal part of the peripodial cells (Fig. 4G and fig. S6G). Therefore, Dilp8 is secreted from the disc epithelium and transits in the lumen and the peripodial cells, from where it may reach the hemolymph.

In addition, the secretion of Dilp8 is essential for its role in controlling developmental timing, because overexpression of the nonsecreted form of Dilp8 (*Rn>dilp8*Δ) is incapable of delaying pupariation (fig. S5, H and I).

What are the target tissues of Dilp8? The hormonal cascade for ecdysone production takes place in the brain (for PTTH production) and in the ring gland (for ecdysone production) (*19*). To test whether these tissues could be direct targets of Dilp8, we cocultured wild-type brains and attached ring glands (brain complexes) with discs expressing *dilp8* or *dilp8*Δ and tested whether Dilp8 produced by the discs could suppress ecdysone production in the brain complexes. As readout for ecdysone activity, we measured expression of *E75B* in brains before (98 hours AED) and after (120 hours AED) incubation with *dilp8* or *dilp8*Δ discs. In brain complexes cocultured with discs expressing nonsecreted Dilp8Δ (serving as a negative control), *E75B* was induced about eightfold, indicating that ecdysone activity can be detected in the brain and therefore that ecdysone production by the ring gland operates ex vivo (Fig. 4H). This induction was significantly suppressed upon coculture with discs expressing the secreted full-length Dilp8 (Fig. 4H). Although these experiments cannot rule out the existence of a secondary relay signal, they suggest that Dilp8 produced by the disc remotely acts on the brain complex to suppress ecdysone production and activity.

We have identified Dilp8 as a signal produced by growing imaginal tissues that controls the timing of metamorphosis. *dilp8* is induced in a variety of conditions that perturb the imaginal disc growth program. We propose that, in conditions of impaired growth, secreted Dilp8 acts on the brain complex to delay metamorphosis, allowing extra time for tissue repair and growth to occur. In addition, Dilp8 might serve to synchronize growth of undamaged tissues with delayed ones.

Our experiments also suggest that Dilp8 participates in a feedback control on growth during normal development, ensuring that animals do not progress to the next developmental stage before organs and tissues have completed adequate growth. Dilp8 shares some features with a distant insulin-like peptide family member, raising the possibility that peptides with similar roles may exist in vertebrates.

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Supplementary Materials
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Materials and Methods
Figs. S1 to S6

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Function, Developmental Genetics, and Fitness Consequences of a Sexually Antagonistic Trait

Abderrahman Khila,[1,2,3] Ehab Abouheif,[2] Locke Rowe[1]*

Sexual conflict is thought to be a potent force driving the evolution of sexually dimorphic traits. In the water strider *Rheumatobates rileyi*, we show that elaborated traits on male antennae function to grasp resistant females during premating struggles. Using RNA interference, we uncovered novel roles of the gene *distal-less* (*dll*) in generating these male-specific traits. Furthermore, graded reduction of the grasping traits resulted in a graded reduction of mating success in males, thus demonstrating both selection for elaboration of the traits and the role of *dll* in their evolution. By establishing developmental genetic tools in model systems where sexual selection and conflict are understood, we can begin to reveal how selection can exploit ancient developmental genes to enable the evolution of sexually dimorphic traits.

Sexual conflict, or sexually antagonistic selection, can influence the evolution and elaboration of novel sexually dimorphic traits in two distinct and potentially opposing ways (*1*). First, conflict over any interaction between the sexes (e.g., mating rate) may drive the evolution of novel or exaggerated antagonistic characters such as grasping and antigrasping structures involved in premating struggles (*2*). Second, the resulting sex-biased selection on these traits gen-

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erates sexual antagonism if the trait has a shared genetic basis in the two sexes (*3*). This intralocus conflict constitutes a major obstacle to the evolution of sexual dimorphism that can be overcome with the evolution of sex-specific regulatory mechanisms (*3*).

Our understanding of the role of sexually antagonistic selection in driving the evolution of dimorphisms in natural systems is rapidly increasing (*1*, *4*, *5*). However, we have little understanding of the developmental genetic mechanisms underlying these novel traits (*6*). For example, the extent to which highly conserved and pleiotropic developmental genes can evolve to resolve intralocus conflict and enable the evolution of sexual dimorphism remains unclear. In a few cases, such genes have been shown to play a role in the evolution of dimorphic traits (*6*–*9*); however, in no case have gene effects on the fine-scale structure and function of these traits, and their fitness consequences, been determined.

In water striders (Heteroptera: Gerridae), females resist costly superfluous mating through vigorous struggles aimed at rejecting male mating attempts (*2*, *10*). The genus *Rheumatobates* is distinguished by a diverse set of structural modifications of male appendages that are used to overcome this female resistance (*11*, *12*). Here, we focused on *Rheumatobates rileyi*, in which males have evolved spectacular appendage elaborations, particularly in the morphology of their antennae (*11*, *12*). We used fine-scale behavioral and mating performance analyses, coupled with pyrosequencing and RNA interference (RNAi) knockdown, to (i) reveal the structure and function of these traits, (ii) identify genes and genetic modifications underlying their evolution, and (iii) quantify their fitness consequences.

Using a combination of high-speed video (movies S1 to S3), flash-freezing of mating pairs, and scanning electron microscopy, we discovered four composite traits of male antennae that are used to gain a purchase on resistant females during premating struggles (Fig. 1, fig. S1, and movie S1). The first trait is the wrench-like shape of the male antennae that is formed by the curvature of the distal part of the first segment, the second segment, and the proximal part of the third segment (dashed outline in Fig. 1A), which fits precisely around the female eye (Fig. 1, E and F, fig. S1, and movie S1). The second trait is a "spike" formed by a set of 8 to 10 large bristles located on the ventral side of the most proximal segment (green in Fig. 1A). The spike fits in the groove formed by the intersection of the female's head capsule, first thoracic segment, and eye (Fig. 1, C, E, and F, and fig. S1). The third trait is a "pad" with a set of four or five internal setae on the second most distal segment (red in Fig. 1A). The pad rests underneath the female eye, and its internal setae may prevent male antennae from sliding sideways (Fig. 1, D to F). The fourth trait is a "hook" that is located on the most distal segment of male antennae (purple in Fig. 1, A and B). The hook fits into two alternative positions underneath the female's head (referred to as positions 1 and 2 in Fig. 1, D to F). In position 1, the hook fits into a groove at the intersection of the female's eye, head, and first thoracic segment (Fig. 1E), whereas in position 2 the hook sits between the first and second thoracic segments (Fig. 1F). Males alternate their antennae between positions 1 and 2 to leverage their body on top of the female in order to copulate. We found that the hook is equipped with a row of button-like structures (red in Fig. 1B), which may increase friction, enhancing the hook's grip. Thus, these elaborate traits are not simply generalized grasping structures, but are exquisitely shaped to the structural detail of the female head.

Male antennal traits begin to appear in the third instar and are elaborated during the fourth and fifth instars (Fig. 2), followed by the most substantive change at final molt, where all four traits are completed (compare Fig. 2, E and F). In contrast to males, female antennae (Fig. 2, H to L) grow uniformly throughout development and are not elaborated. To identify candidate genes responsible for the development of these male grasping characters, we performed a small-scale sequencing of the transcriptomes of appendages from third-, fourth-, and fifth-instar males and females. Among these sequences, we found a transcript that corresponds to the *distal-less* (*dll*) gene. *dll* is known to play important roles in controlling appendage growth and patterning, as well as bristle formation throughout development (*13*–*16*). Furthermore, we found that *dll* transcripts in *R. rileyi* are shorter than those of other hemipterans (*17*) [including other closely related water striders (*18*)] in which male antennae are not elaborated (Fig. 3A and figs. S2 and S3). We therefore analyzed the expression and function of *dll* in developing male and female antennae.

We discovered that, although *dll* is expressed in both male and female antennae (fig. S4), it functions only in the males to generate all four antennal grasping traits (Fig. 3). *dll* RNAi also causes a subtle (2 to 9%) shortening of the legs in males (fig. S5A). Several other male grasping

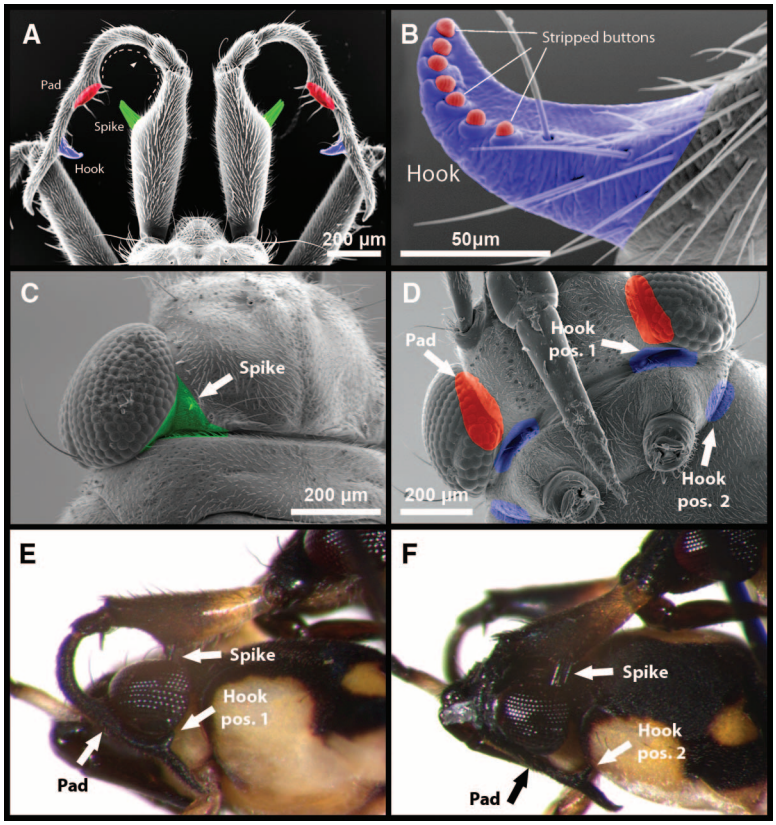

Fig. 1. Male and female structures involved in premating struggles. (**A**) Dorsal view of male antennae. (**B**) Close-up of the distal hook. (**C** and **D**) Dorsal (C) and ventral (D) views of female's head. (**E** and **F**) Positioning of antennae when males grasp females. In (A) to (D), the grasping structures in male antennae and the corresponding grasped structures on the female head are represented by the same colors.

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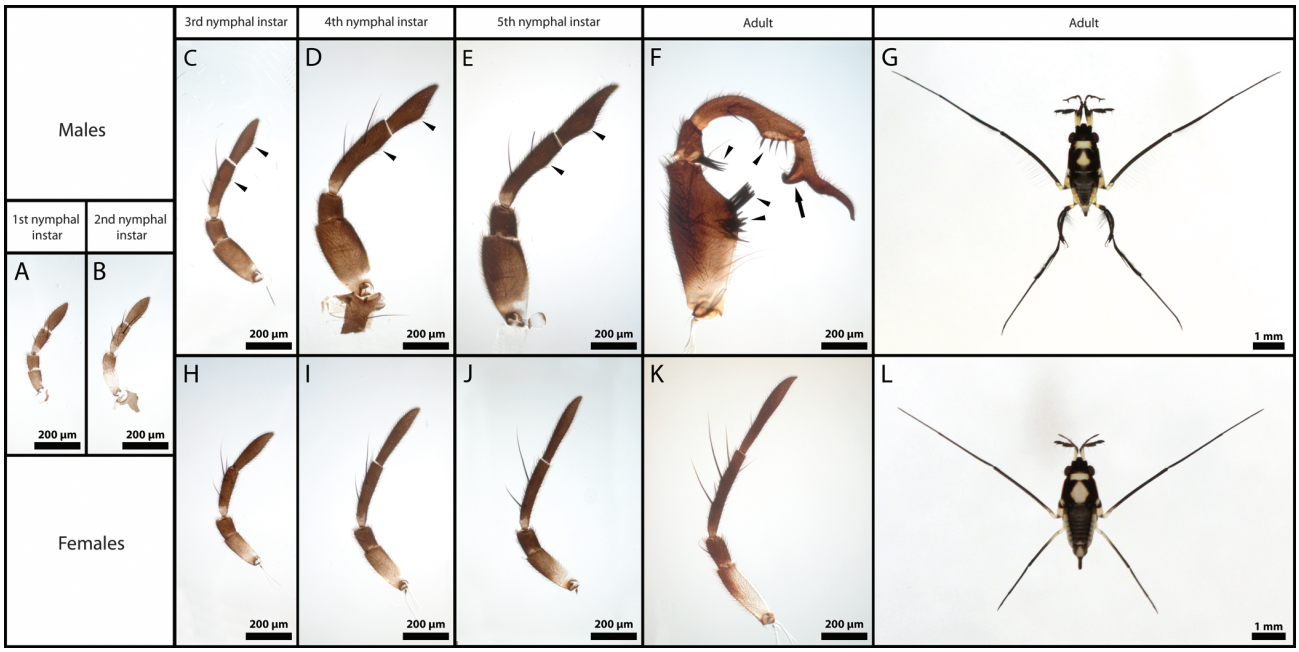

Fig. 2. Morphogenesis of male and female antennae. (**A** and **B**) Antennae of first and second nymphal instars. (**C** to **L**) Male [(C) to (F)] and female [(H) to (K)] antennae from third nymphal instar through adult; (G), adult male; (L), adult female.

Fig. 3. (**A**) Comparison of Dll protein sequence in *R. rileyi* (Rr_Dll), *Limnoporus dissortis* (Ld_Dll), and the human louse *Pediculus humanus* (Ph_Dll) (*18*). Note that Rr_Dll is shorter; missing sequence (dashes) is immediately downstream of the homeodomain (in bold). Black asterisks indicate sequence identity among the three species. Motifs conserved between Ld_Dll and Ph_Dll are highlighted in green. (**B** to **M**) Effect of *dll* RNAi on *R. rileyi* male antennae. (B) to (E): Normal male antennae with close-ups of the hook (C), spikes (D), and pad (E). (F) to (I): Antennae of males showing severe *dll* RNAi effect. (J) to (M): Antennae of males showing moderate *dll* RNAi effect. White asterisks indicate traits affected by *dll* RNAi treatment. Colors are as in Fig. 1.

A

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Rr_Dll    YPFPPMHHNTYPSYHLAPYPQPCPSPPKD-EKCGEEGG-LRVNGKGKKMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQN
Ld_Dll    YPFPPMHHNTYTGYHLASYAPQCPSPPKD-EKCGEEGG-LRVNGKGKKMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQN
Ph_Dll    YPFPPMHQNSYSSYHLGGYGPQSPPKESELDKIGVEDGPLRVNGKGKKMRKPRTIYSSLQLQQLNRRFQRTQYLALPERAELAASLGLTQTQVKIWFQN
          *******:*:*..***. *   .*.  .: :* * *.* ************************************************************

Rr_Dll    RRSKYKKMMKA-----------------------------------------------------------------------------------------
Ld_Dll    RRSKYKKMMKAAQQGGGGGGGQGGGAPHMLGGLPPQSPPPAAAILQGSGGSSSGSSQQHSPGGGGGGGGGPGYMSHVGTPTPSSTPQSDVSPHGM--SP
Ph_Dll    RRSKYKKMMKAAQNTGPGGQNNNGGGN--------GSPAPPGGATVGILGGNNSTGSQNSPNYG-------HHNQNQTSPSPSSTPVSDMSPHGLSGSP
          ***********

Rr_Dll    ---------------------------------PPHTAYMPQYSWYQTGAPPSEPNTAPGLLTVWPSV
Ld_Dll    PAA-WDMKP------VGPPAHPP-----HSAPPPPPHTAYMPQYSWYQAGPPPSEPNSTTGLLTVWPSV
Ph_Dll    PTMNWDMKPNINNLGVTPTHHTTGHPHHTPTHHHPTHHSYMPQYSWYNADT-ANQP-----LLTVWPAV
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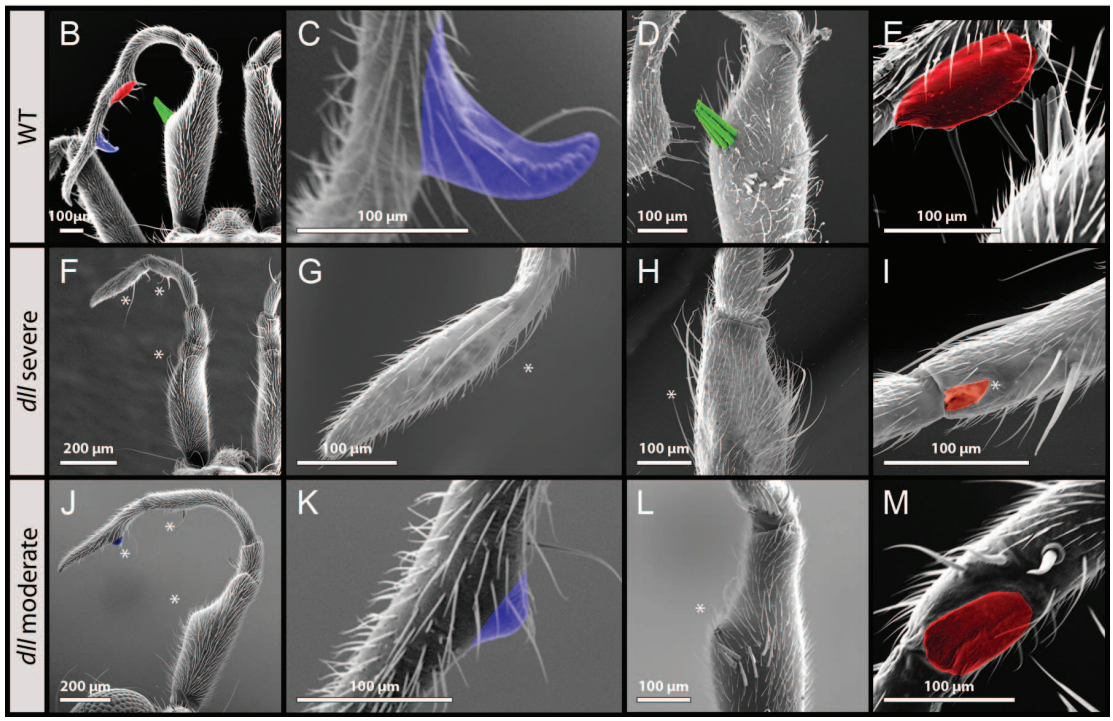

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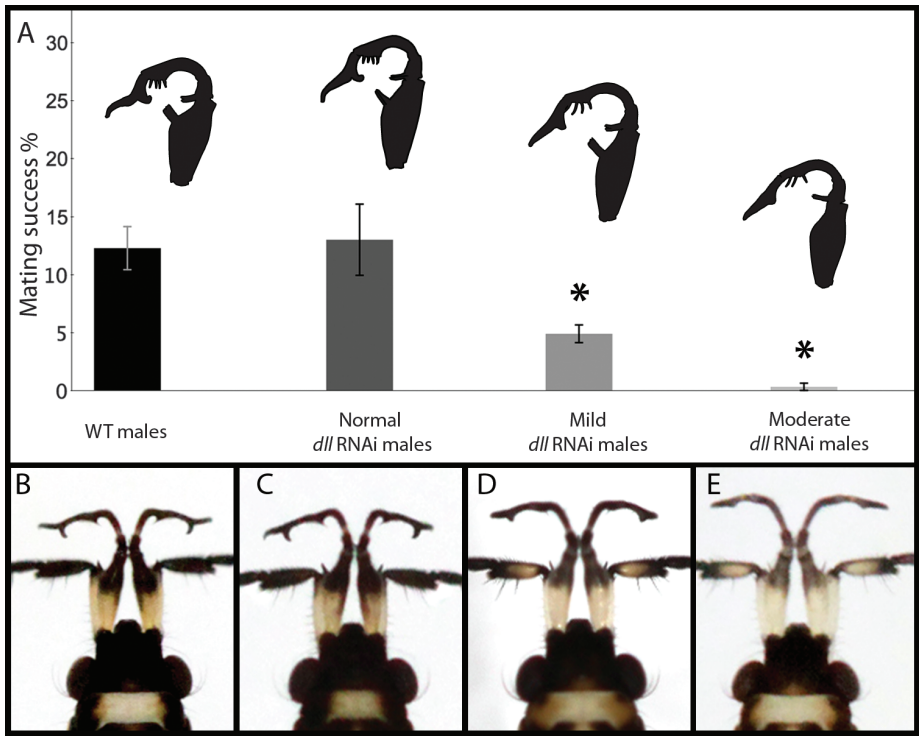

Fig. 4. Effect of *dll* RNAi on male antennae and its consequences for mating performance. (**A**) Graded reduction of antennal grasping structures results in a graded reduction in male mating performance. Mating success is measured as the percentage of the total number of premating struggles in which the male immobilizes and successfully copulates with the female. There is a significant overall effect of treatment (analysis of variance; $F = 32.6$, df = 3, $^*P < 0.001$) on mating success, and post hoc contrasts reveal that mating success differences in the treatments can be summarized as wild type = normal > mild > moderate (Tukey's honestly significant difference, $\alpha = 0.05$). Mean numbers of mating attempts per male: wild type, 11.02 ± 6.85 (SE); normal, 13.00 ± 1.04; mild, 16.6 ± 5.49; moderate, 36.91 ± 12.55. Antennal diagrams for each treatment illustrate the degree of *dll* RNAi effect on antennal grasping structures. (**B**) Untreated male showing antennae morphology. (**C** to **E**) *dll* RNAi males with varying degrees of trait reduction: normal (no obvious reduction) (C), mild (D), and moderate (E).

structures, like those on the rear legs, are elaborated at developmental stages similar to those seen with the antennae (fig. S6, A to F) but are not affected by *dll* RNAi (compare fig. S6, F and G). This indicates that *dll* is not responsible for elaborating male grasping structures on other appendages, which suggests that they have a different genetic basis. Finally, we observed no effect on female appendages other than a small (2 to 4%) and insignificant reduction in appendage length, similar to what we saw in males (fig. S5B). Therefore, *dll* has evolved a novel function during late nymphal development to generate grasping traits specifically on male antennae.

The *dll* RNAi phenotypes we obtained range from complete loss to a subtle reduction of male antennal grasping traits (Fig. 3, Fig. 4, and fig. S7). We categorized these male antennal phenotypes into four broad classes: (i) "severe," in which all four antennal grasping traits are lost or nearly lost, such that male antennae become similar to those of females (Fig. 3, F to I, and fig. S4); (ii) "moderate" (Fig. 3, J to M, and Fig. 4E); (iii) "mild" reduction of antennal grasping traits (Fig. 4D and fig. S7); and (iv) "normal" antennal grasping traits (Fig. 4C and fig. S7). All classes of *dll* RNAi males are fully viable and repeatedly attempt to mate with females, which suggests that there are no deleterious pleiotropic effects when *dll* RNAi is applied at late nymphal development. The observed graded reduction of antennae and viability of *dll* RNAi males provided us with a unique opportunity to determine the consequence of their reduction on mating performance.

In mating performance tests, the experimentally graded reduction of antennal grasping structures resulted in a corresponding graded reduction in mating success. The mating success of males with moderate trait reduction was significantly reduced below that of wild-type males (Fig. 4). Examination of premating struggles revealed that these moderate males tended to fail during the initial flip of the female because their antennae failed to maintain their position around the female's head (fig. S8 and movie S2). The mating success of males with a mild reduction of antennal traits was significantly higher than for moderate males but lower than for wild-type males (Fig. 4). Males with mild reduction tended to fail later in the behavior sequence (movie S3) than did moderately reduced males. In contrast to both mild and moderate males, the mating success of *dll* RNAi males with normal antennae was not significantly different from that of wild-type males (Fig. 4). *dll* RNAi males, including those with normal antennae, tended to have mild reductions in leg length (fig. S7). Although we cannot exclude the possibility that the mild reduction in leg length contributes to the reduced mating success of *dll* RNAi males, we could not detect any effect on leg grasping structures (figs. S6 and S7) or any failure of their function in the videos. Even in wild-type males, only 12% of mating attempts were successful (Fig. 4). Such a large proportion of failures is a measure of the effectiveness of female resistance, which in turn accounts for the remarkable elaboration of antennae (*19*). Therefore, these data demonstrate both selection for elaborating male antennal grasping traits and the role of *dll* in their evolution.

Collectively, our results link an evolutionary change in morphology, the fitness advantage of this change, and its underlying genetic basis. The co-option of Dll late in development, after all the appendages have been specified (*20*, *21*), may have been an important factor that facilitated the evolution of male antennae elaboration without any apparent deleterious pleiotropic consequences (*22*). It remains unclear, however, how Dll function in elaborating the antennae is restricted to males. It is possible that proteins differentially expressed between the sexes, such as those encoded by sex determination genes, may act as cofactors to limit Dll's role to males (*9*, *23*–*25*). Furthermore, *R. rileyi dll* transcripts are shorter than those of other insects, including close relatives whose antennae are not sexually dimorphic (Fig. 3 and figs. S2 to S4). In these species, Dll does not seem to play any role in elaborating nymphal antennae (*20*) (fig. S9), raising the possibility that changes in *R. rileyi dll* coding sequence may be associated with the evolution of its novel function.

The graded effect of *dll* RNAi on male antennal grasping traits and its graded consequence on male mating performance suggests that even a slight elaboration of these traits from an unmodified ancestral state would be favored by female premating struggles. Therefore, in *R. rileyi*, selection resulting from female resistance may have favored the continuous elaboration of male antennae through this novel function of Dll. The elaboration of male antennae into grasping structures is not unique to *R. rileyi* but has evolved repeatedly within the genus *Rheumatobates* (*11*, *12*). This raises the possibility that variation in *dll* expression and function may underlie the diversity and degree of modification observed in the clade (*11*, *12*). By combining an understanding of the elaborated morphologies generated through sexual antagonism with the power of developmental

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genetics and genomics tools, water striders provide a model to reconstruct the genetic and adaptive paths to morphological diversity.

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Supplementary Materials

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Materials and Methods
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Impacts of Biodiversity Loss Escalate Through Time as Redundancy Fades

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Plant diversity generally promotes biomass production, but how the shape of the response curve changes with time remains unclear. This is a critical knowledge gap because the shape of this relationship indicates the extent to which loss of the first few species will influence biomass production. Using two long-term (≥13 years) biodiversity experiments, we show that the effects of diversity on biomass productivity increased and became less saturating over time. Our analyses suggest that effects of diversity-dependent ecosystem feedbacks and interspecific complementarity accumulate over time, causing high-diversity species combinations that appeared functionally redundant during early years to become more functionally unique through time. Consequently, simplification of diverse ecosystems will likely have greater negative impacts on ecosystem functioning than has been suggested by short-term experiments.

Experiments in grasslands regularly indicate strong plant diversity effects on biomass production (*1*–*16*), and some analyses indicate that relatively few of the species in any diverse mixture promote productivity (*1*, *5*). However, many important temporal aspects of the diversity-productivity relationship remain obscure, including whether and how its shape changes over time, because most biodiversity experiments have been short (≤4 years typically, median ≈2 years) (*10*).

Prior studies have characterized the response of plant biomass to species richness as saturating (*1*, *7*) or as continuously increasing, but in a decelerating manner (*6*, *8*, *9*). A recent meta-analysis (*1*) showed that saturating Michaelis-Menten curves were often the best statistical fit to the diversity-productivity relationship, suggesting losses of one or a few species at high richness levels could have minimal consequences for productivity.

Several experiments showed that the positive effects of species richness on biomass and productivity increased over time (*6*, *8*, *10*, *12*–*15*), but implications of such changes for the shape of the diversity-productivity relationship are unknown. Two possibilities can be distinguished: (i) differences between low and intermediate diversity levels grow, resulting in an elevated but saturating response curve (fig. S1, scenario I); or (ii) diversity effects become progressively greater at higher diversity, resulting in an elevated and more linear response curve (fig. S1, scenario II, nonsaturating response curve). These scenarios have fundamentally different implications for the consequences of biodiversity loss. If response curves become less saturating over time, losses of even very few species from diverse assemblages could cause substantial declines in productivity.

Several studies have assessed how biodiversity influences multiple functions within 1 year or across multiple years (*2*, *4*, *5*), but identifying what proportion of the available species pool contributes to multifunctionality does not address the question of whether species are redundant with respect to a single function, such as productivity, in a single year, let alone how that changes with time. We examined this latter issue by considering how the influence of biodiversity on productivity within each year changes from the early to the late years of two long-term experiments.

We present data from two long-running (≥13 years) grassland biodiversity experiments in Minnesota, USA: the "Cedar Creek Biodiversity Experiment" (BioDIV), planted in 1994–1995, and the "Biodiversity, CO_2, and N Experiment" (BioCON), planted in 1997 (*6*, *17*–*21*). These independent, comparable experiments allow us to assess temporal variation in the response of biomass to planted and realized species richness. Species pools differed somewhat in the two experiments, although both include many of the same C3 and C4 grasses, and nitrogen (N)–fixing and nonfixing dicotyledonous herbs. BioCON included elevated carbon dioxide and enriched N treatments (*18*, *19*), but only the ambient plots are considered here to facilitate comparison with BioDIV. In both experiments, the species richness across treatments ranged from 1 to 16 species, spanning the typical range observed from disturbed to native grassland (*17*).

In both experiments, the number of planted species explained an increasingly greater fraction of the variance in total plant biomass over time and had a larger effect on total plant biomass over time (Fig. 1, Table 1, and table S1). More-

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