

The Neuropeptide Sulfakinin, a peripheral regulator of insect behavioral switch between mating and foraging

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This **important** work investigates the mechanism that underlies the switch between feeding and mating behaviors in the oriental fruit fly, *Bactrocera dorsalis*. Using a variety of approaches, the authors show that this switch is mediated by the neuropeptide, sulfakinin, acting peripherally through the sulfakinin receptor 1 to regulate the expression of antennal odorant receptors. The evidence is **solid** in support of the hypothesis that sulfakinin signaling mediates changes in the periphery, although additional sites of action may also contribute to these changes.

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Abstract

Behavioral strategies for foraging and reproduction in the oriental fruit fly (*Bactrocera dorsalis*) are alternative options for resource allocation and are controlled by neuropeptides. Here we show that the behavioral switch between foraging and reproduction is associated with changes in antennal sensitivity. Starved flies became more sensitive to food odors while suppressing their response to opposite-sex pheromones. The gene encoding sulfakinin receptor 1 (*SkR1*) was significantly upregulated in the antennae of starved flies, so we tested the behavioral phenotypes of null mutants for the genes encoding the receptor (*skr1*^{-/-}) and its ligand sulfakinin (*sk*^{-/-}). In both knockout lines, the antennal responses shifted to mating mode even when flies were starved. This suggests that sulfakinin signaling via SkR1 promotes foraging while suppressing mating. Further analysis of the mutant flies revealed that sets of odorant receptor (OR) genes were differentially expressed. Functional characterization of the differentially expressed ORs suggested that sulfakinin directly suppresses the expression of ORs that respond to opposite-sex hormones while enhancing the expression of ORs that detect food volatiles. We conclude that sulfakinin signaling via SkR1, modulating OR

expressions and leading to altered antenna sensitivities, is an important component in starvation-dependent behavioral change.

Introduction

Foraging and mating behaviors are alternative options for resource allocation in insects. Starved and reproductively immature individuals intensify their foraging activity, whereas satiated and reproductively mature individuals increase their mating activity to facilitate reproduction (Chen et al., 2023 [↗](#); Cheriyaunkunel et al., 2021 [↗](#); Ebrahim et al., 2023 [↗](#); Ren et al., 2021 [↗](#)). Such behavioral changes involve complex neural pathways triggered by neuropeptide signaling (González Segarra et al., 2023 [↗](#); Lin et al., 2022 [↗](#); Nässel and Zandawala, 2022 [↗](#); Schoofs et al., 2017 [↗](#); Sun et al., 2023 [↗](#); Zhang et al., 2022 [↗](#)). The key regulators have been identified in the fruit fly *Drosophila melanogaster*, including the neuropeptides sulfakinin (Sk) (Wang et al., 2022 [↗](#); Wu et al., 2019 [↗](#)) and short neuropeptide F (sNPF) (Ko et al., 2015 [↗](#); Root et al., 2011 [↗](#)). Tyramine and diuretic hormone 31 then resolve the conflict between foraging and mating behavior in the central nervous system (Cheriyaunkunel et al., 2021 [↗](#); Lin et al., 2022 [↗](#)).

In mammals, cholecystokinin (CCK) transmits satiety by activating cholecystokinin receptor 1, leading to the expression of genes encoding other anorectic and orexigenic peptides and receptors in vagal neurons (Beutler et al., 2017 [↗](#); Burduga et al., 2008 [↗](#); Rachel et al., 2017 [↗](#)). CCK is also involved in the central integration of sensory inputs to regulate sexual behavior (Bloch et al., 1987 [↗](#); Micevych et al., 2004 [↗](#)). In insects, Sk, the ortholog of CCK, and its signals via two receptors, SkR1 (CCKLR-17D3 of *D. melanogaster*) and SkR2 (CCKLR-17D1), regulate sub-behavioral steps during foraging, food consumption, and mating in *D. melanogaster* (Fedina et al., 2023 [↗](#); Wang et al., 2022 [↗](#)). For example, Sk suppresses the expression of the *Gr64f* gustatory receptor gene, which inhibits feeding as a signal for the onset of satiety (Guo et al., 2021 [↗](#)). Sk also inhibits both male and female sexual behavior (Wu et al., 2019 [↗](#)). However, the complexity of Sk pathways, spatial and temporal dynamics of multiple ligands and receptors, is exemplified by cell-specific signaling via SkR1 for the promotion of mating receptivity in virgin females (Wang et al., 2022 [↗](#)), which is an opposite action of the inhibition of sexual behavior (Wu et al., 2019 [↗](#)). Modulation of peripheral sensory inputs, including odorant perception, is involved in the control of mating and foraging behavior in addition to the central neuronal circuits.

The oriental fruit fly, *Bactrocera dorsalis*, is a destructive agricultural pest insect. Control of *B. dorsalis* includes the use of the behavioral responses to semiochemicals, such as the components in the sex pheromones and in the food smell (Jaffar et al., 2023 [↗](#)). Chemical components in the sex pheromone of this species includes 2,3,5-trimethyl pyrazine, 2,3,5,6-tetramethylpyrazine, ethyl laurate, ethyl myristate, ethyl cis-9-Hexadecenoate, and ethyl palmitate (Chen et al., 2023 [↗](#); Ren et al., 2021 [↗](#)) and in the food smells are ethyl benzoate, ethyl butyrate, and methyl eugenol, among others. (Kamala Jayanthi et al., 2014 [↗](#); Zhu et al., 2018 [↗](#)). The physiology and molecular mechanisms underlying the behavioral modulation involved in the different types of chemical attractants have not yet been studied in this species of insect.

We hypothesized that neuropeptide Sk-SkR and other neuropeptidergic systems are involved in the switch between foraging success and mating behaviors in the *B. dorsalis*. We were specifically interested in the alteration in odorant perceptions in the antennae leading to the success in foraging and/or mating depending on the physiological status. Sequential approaches, starting with the unbiased test for involvement of odorant perception in the behavioral switch, led us to further in-depth investigations of the role of neuropeptide and Sk-SkR systems. We demonstrate that sulfakinin signaling via SkR1 in the antennae alters the expression of sets of odorant receptor (OR) genes during starvation enhancing the foraging success rate. Our results thus provide insight into sulfakinin signal on peripheral olfactory system that is coordinated with the central neuronal circuits for modulation of behavior.

Results

Altered antennal sensitivity is associated with starvation-induced foraging and satiation-induced mating success

We developed an assay to investigate the behavioral switch between foraging and mating induced by the physiological state of *B. dorsalis* (Fig. 1A, B). Foraging success increased with the duration of the starvation period (Fig. 1D, and S1), whereas courtship and copulation rates decreased, also with the starvation length-dependent manner (Fig. 1G, H). Starved flies tended to move directionally toward the food source located in the center of the arena (Fig. 1C).

We therefore tested antennal sensitivities toward artificial food and opposite-sex body extracts using an electroantennogram (EAG). Flies of both sexes starved for 12 h showed a greater EAG response to food odor (Fig. 1E, F) but a weaker response to opposite-sex body extracts (Fig. 1I, J). Furthermore, starved flies also showed greater EAG responses to food volatile components and weaker responses to sex pheromone components (Fig. S2). This suggested antenna-mediated olfaction is associated with and required for successful foraging and mating.

Starvation-induced *SkR1* mRNA in the antennae is required for the switch from mating to foraging behavior

To identify signaling molecules responsible for the olfactory-dependent behavioral switch, we compared the expression profiles of neuropeptide signaling components in the antennae of fed and starved flies. We found that the components of three neuropeptide signaling systems were significantly upregulated in the antennae of starved flies, namely *short neuropeptide F receptor* (*sNPF*), *SIFamideR1*, and *SkR1*, the latter showing the strongest induction (Fig. 2A). *Sk* did not show the expression change in the antennae, but significantly increased in the head in comparison between those in starvation and after re-feeding, suggesting that the circulatory *Sk* released from the central nervous system could be the source for activation of *SkR1* in the antennae (Fig. S3).

We introduced a 7-bp deletion into the sulfakinin gene (*Sk*) using the CRISPR/Cas9 system, causing a frameshift that abolished the sequence region encoding two predicted mature peptides: *Sk-1* and *Sk-2* (Fig. 2B, S4A, B). We confirmed the absence of an anti-sulfakinin immunoreactive signal in the central nervous system of the *sk*^{-/-} flies (Fig. S4C). Only *SkR1* was upregulated in the starved flies among two predicted *Sk* receptor genes *SkR1* and *SkR2* (Fig. 2A, and S6). We therefore generated a *SkR1* null mutant (*skr1*^{-/-}) by introducing a 187-bp deletion using CRISPR/Cas9, resulting in a frameshift that resulted in a premature stop codon (Fig. 2B, and S5A–C). We confirmed the absence of the anti-*SkR1* immunoreactive signal in the antennae of the *skr1*^{-/-} flies (Fig. S5D).

Starved *sk*^{-/-} and *skr1*^{-/-} flies showed the same locomotor activity and body size as wild-type (WT) controls when we measured their velocity and body length (Fig. 2C, and S7). However, both null mutants consumed more food than WT flies (Fig. 2D), consistent with previous results in *D. melanogaster* (Guo et al., 2021). Interestingly, *sk*^{-/-} and *skr1*^{-/-} male flies consumed more food at all times compared to the wild type. However, the corresponding mutant females consumed more food only at night. This may be due to the high basal feeding rate of females during the daytime, masking the increase in feeding in the knockout of *Sk* signaling. As shown in Figure 2D, WT females ate more food than males, and WT females consumed more food during the daytime. Another major difference between the null mutants and WT controls was that the mutant flies were less successful at foraging even after starvation, similar to the characteristics of satiated WT flies (Fig. 3A, B). However, foraging was not completely abolished. In addition, starved mutant

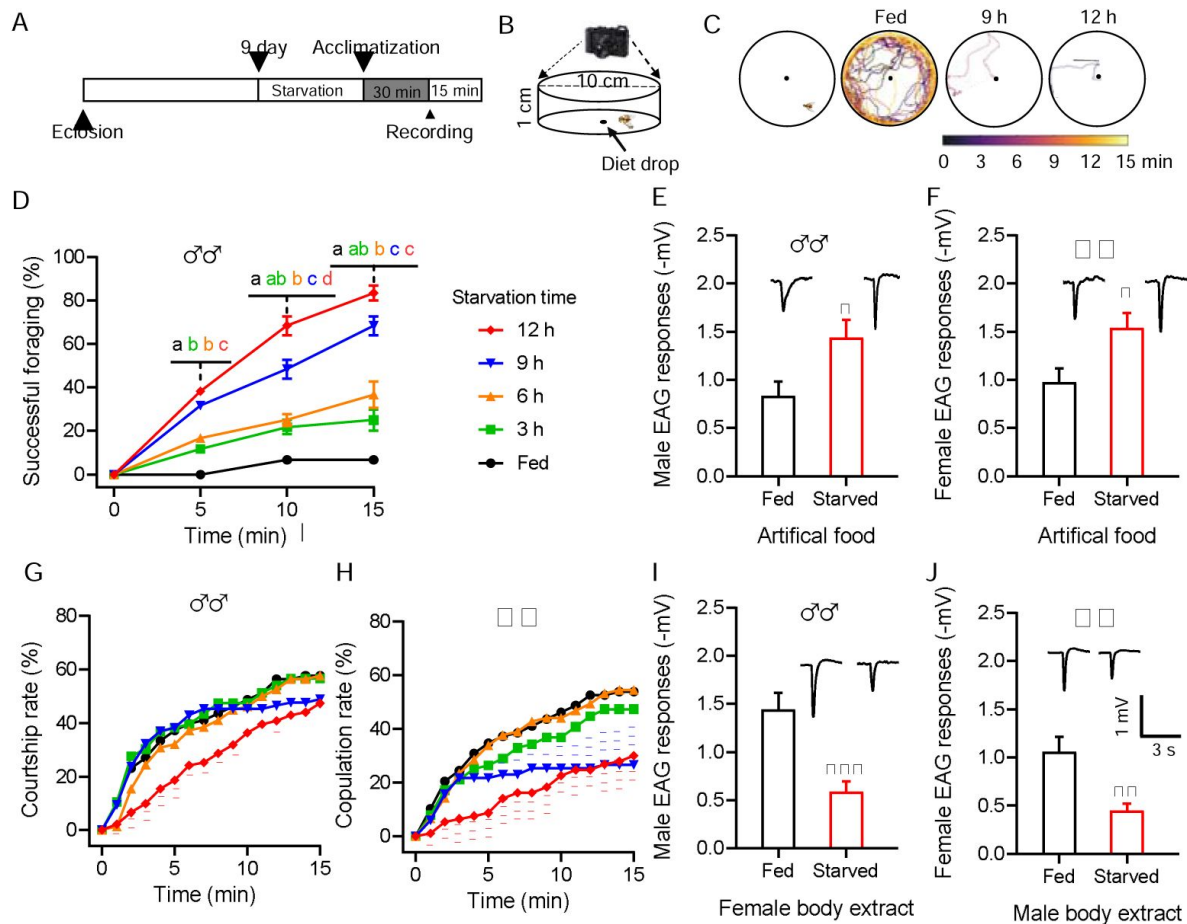


Figure 1.

Starvation alters the behavior and olfactory responses of *B. dorsalis*.

(A) Schematic illustration of the experimental design. (B) Schematic illustration of the mating behavior assay device. (C) Representative foraging trajectories in the 100 mm diameter arenas within a 15-min observation period of flies starved for different durations. (D) Cumulative successful foraging within a 15-min observation period of male flies starved for different durations. Data are means $\pm$ SEM, $n = 60$ flies for each condition. Different letters above the error bars indicate significant differences (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$). (E–F) EAG responses to artificial food in fed and starved male (E) and female (F) flies (unpaired t -test). (Top) Representative EAG recordings. (G) Cumulative courtship rate within a 15-min observation period of flies starved for different durations ($n = 78, 76, 78, 84$ and 91 , respectively, from fed to 12 h). (H) Cumulative copulation rate within a 15-min observation period of the flies starved for different durations ($n = 78, 76, 79, 83$ and 93 , respectively, from fed to 12 h). (G–H) Kruskal-Wallis and *post hoc* Mann-Whitney U tests were applied. (I–J) EAG responses to body extracts in fed and starved male (I) and female (J) flies. (Top) Representative EAG recordings. Data are means $\pm$ SEM, $n = 10$ –12 antennae per genotype (unpaired t -test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

females (**Fig. 3C**) and males (**Fig. 3D**) showed high mating success rates, as same rates as fed WT flies. We tested the behavioral phenotypes of heterozygous mutant of *Sk* knockout flies. The results showed that foraging and mating behaviors of *Sk* heterozygous mutants were unaffected during starvation, suggesting the mutants are completely recessive (**Fig. S8**).

We next tested antennal sensitivity to food odors and body extracts by EAG in each genotype using both fed and starved flies in each case. The starved *sk*^{-/-} and *skr1*^{-/-} flies showed significantly weaker responses to food odors compared to the starved WT flies, although the response of *sk*^{-/-} flies was slightly stronger than that of fed WT flies (**Fig. 3E, F** and **S8**), which was similar to the behavioral response for a successful foraging rate (**Fig. 3A, B**). The antennae of starved *sk*^{-/-} and *skr1*^{-/-} flies showed the same EAG response to opposite-sex body extracts as fed WT flies (**Fig. 3G, H** and **S9**), which was significantly higher than that of starved WT flies.

Sets of ORs are differentially expressed when comparing satiated/starved WT and *sk*^{-/-} flies

To gain insight into the molecular basis of alteration of olfactory sensitivities by the *Sk*-*SkR1* system, we extracted RNA from the head epidermal tissues (including antennae) of fed and starved flies for sequencing (RNA-Seq). We compared starved WT and starved *sk*^{-/-} flies with satiated WT flies, focusing on the differentially expressed OR genes (**Fig. 4A**). We identified three OR genes (*OR7a.4*, *OR7a.8* and *OR10a*) that were upregulated in the starved WT flies but unchanged in starved *sk*^{-/-} flies compared to satiated WT controls, and two (*OR49a* and *OR63a*) that were downregulated in starved WT flies but unchanged in starved *sk*^{-/-} flies (**Fig. 4B**). *OR67d.3* and *OR74a* mRNA levels were similar in the satiated and starved WT flies. The differentially expressed genes identified by RNA-Seq were further validated by real-time reverse-transcription PCR (qRT-PCR) (**Fig. 4B**).

Sulfakinin regulates the expressions of starvation-induced ORs that detect food volatiles and starvation-suppressed ORs that detect pheromones

The OR genes identified in the RNA-Seq experiments were expressed in HEK 293 cells followed by the evaluation of functional responses to odorants using calcium mobilization assays. Tests with single high doses of volatiles representing food or sex pheromones allowed the functional categorization of ORs based on odorant specificities (**Fig. 4C**). *OR10a* was activated by ethyl benzoate (EBE), *OR7a.8* was activated by ethyl butyrate (EBU) or diethyl maleate (DM), and *OR7a.4* was activated by methyl eugenol (ME). The responses to these food odor volatiles were all dose-dependent, with *OR10a* showing the strongest response to EBE (**Fig. 4E**). In contrast, *OR63a* and *OR49a* showed dose-dependent responses to sex pheromone components but not to food odor volatiles (**Fig. 4C, D**). Therefore, the upregulated OR genes in starved WT flies, *OR7a.4*, *OR7a.8* and *OR10a*, were activated by the pheromonal components, while down regulated genes, *OR49a* and *OR63a*, were activated by food volatiles. Finally, we determined whether *SkR1* was expressed in OR neurons by testing the antennae for immunoreactivity to *SkR1* and odorant receptor co-receptor (*Orco*) antisera. A subset of clustered OR neurons showed immunoreactivity to *SkR1* (**Fig. 4F**), indicating that sulfakinin acts directly on *SkR1*-positive OR neurons.

Discussion

Dynamic changes in the olfactory sensitivity of insect antennae are associated with behavioral transitions in insects. Sensory inputs via olfactory perception often trigger certain behaviors, including foraging and mating (Anton and Rössler, 2021; Gadenne et al., 2016). Fine-tuning of

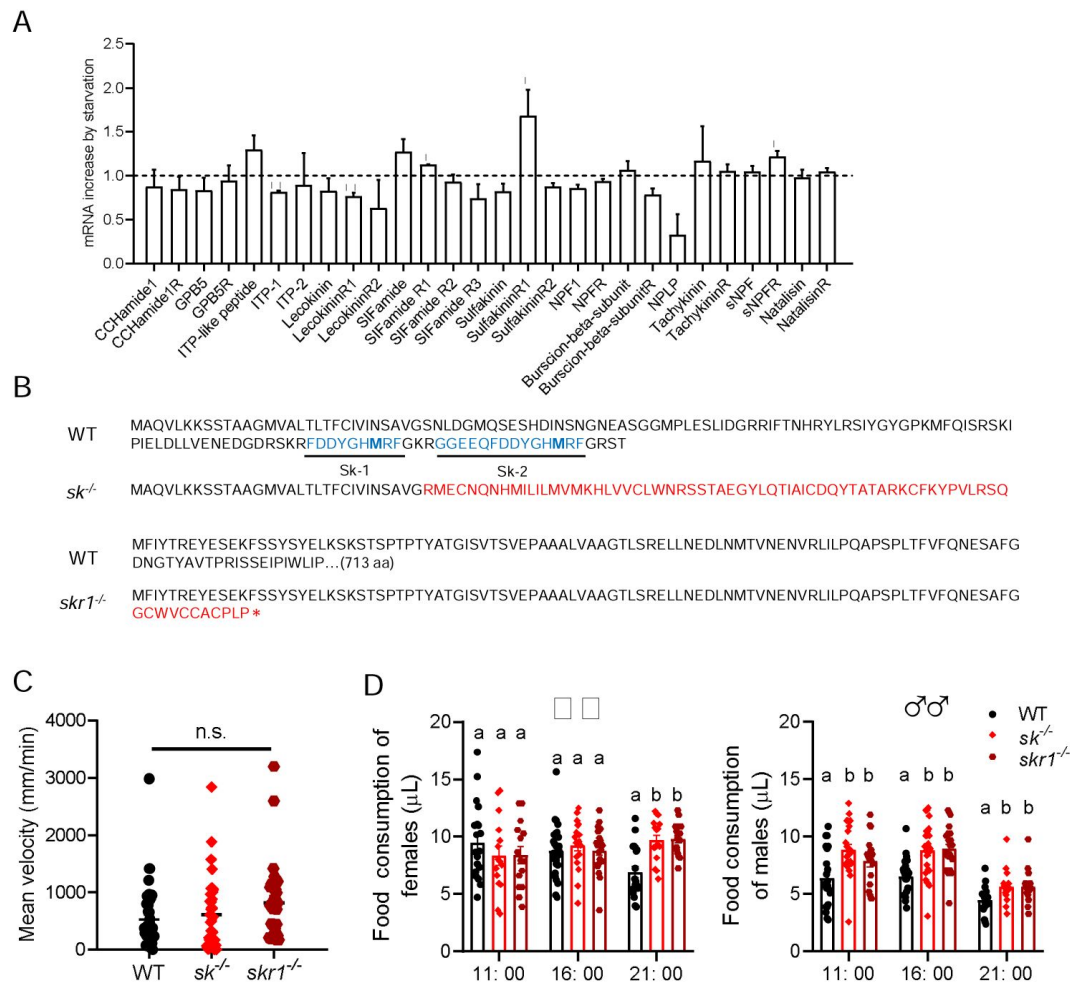


Figure 2.

Starvation-induced expression of neuropeptide system components and phenotypes of null mutant flies.

(A) Changes in the expression of neuropeptide system components in the antennae of starved flies. The relative expression values are the fold-changes compared to the control. Data are means $\pm$ SEM, $n = 3$ (unpaired t -test; $*P < 0.05$, $**P < 0.01$). (B) Altered amino acid sequence and mature peptides in the null mutants *sk^{-/-}* and *skr1^{-/-}* compared to wild-type (WT) flies. (C) Mean velocity of flies representing each genotype during a 15-min observation period. Data are means $\pm$ SEM (Kruskal-Wallis test). (D) Knockout of sulfakinin and SkR1 increases food consumption in *B. dorsalis* at different times of day. Each experiment consisted of one fly, and at least 15 flies were contained for the assay. Different lower-case letters indicate significant differences between treatments (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$).

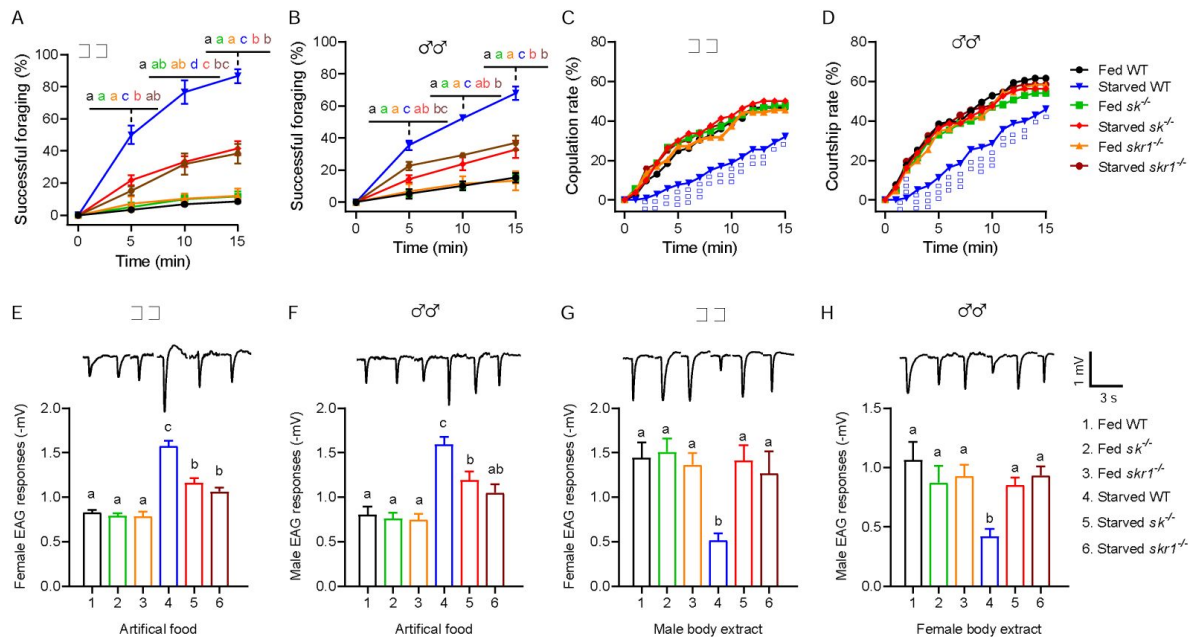


Figure 3.

Sk-SkR1 signaling is required for changes in behavior and olfactory sensitivity induced by starvation.

(A–B) Cumulative successful foraging within a 15-min observation period of fed and starved flies with different genotypes. The successful foraging of females (A) and males (B) was measured separately. Data are means $\pm$ SEM, $n = 60$ flies for each condition. Different letters above the error bars indicated significant differences (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$). (C) Cumulative copulation rates within a 15-min observation period of fed and starved flies with different genotypes ($n = 92, 86, 88, 106, 80$ and 76 , respectively, from fed WT to starved *skr1*^{-/-}). (D) Cumulative courtship rates within a 15-min observation period of fed and starved flies with different genotypes ($n = 91, 85, 86, 98, 80$ and 77 , respectively, from fed WT to starved *skr1*^{-/-}). (C–D) Kruskal-Wallis and *post hoc* Mann-Whitney U tests were applied ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (E–F) EAG responses to artificial food in fed and starved flies of different genotypes. The EAG responses to artificial food in females (E) and males (F) were measured separately. (Top) Representative EAG recordings. (G–H) EAG responses to body extracts in fed and starved flies of different genotypes. The EAG responses to body extracts of females (G) and males (H) were measured separately. (Top) Representative EAG recordings. Data are means $\pm$ SEM, $n = 10$ – 12 antennae for each condition. Different letters above the error bars indicated significant differences (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$).

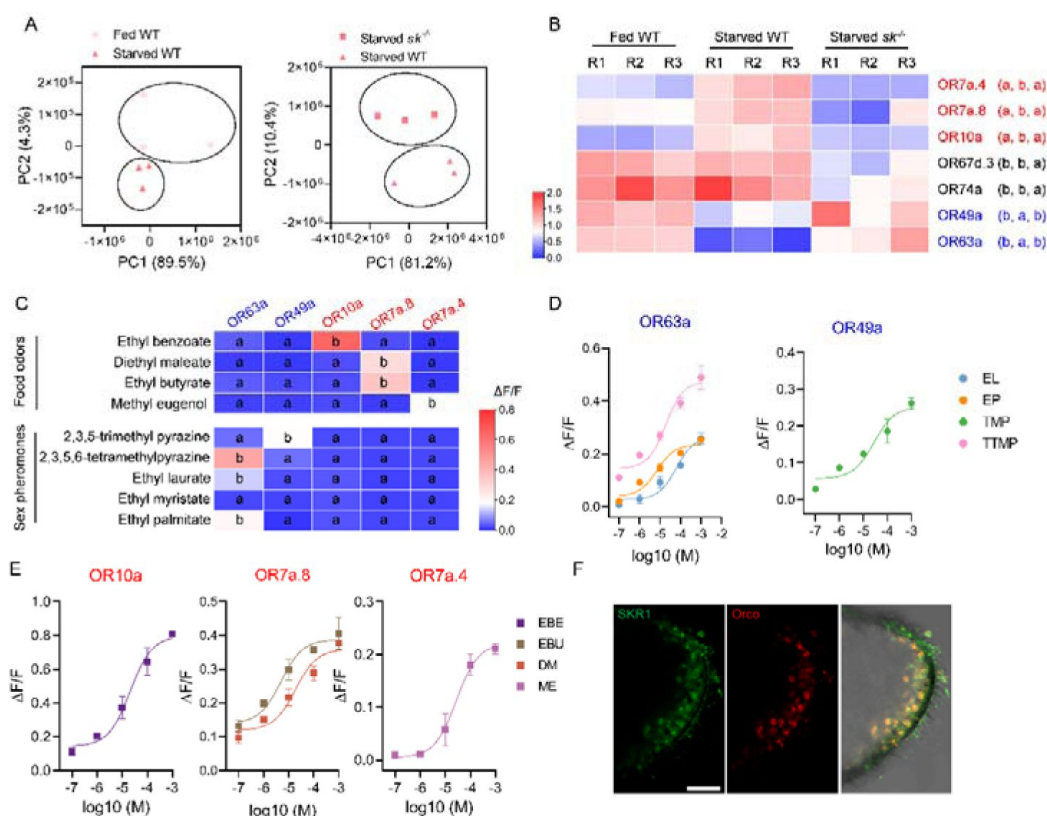


Figure 4.

The transition of olfactory responses is associated with the expression of different sets of ORs in OR neurons induced by Sk-SkR1 signaling.

(A) Principal component analysis (PCA) using differentially expressed genes obtained from pairwise comparisons between different treatments. (B) The expression profiles of the candidate OR genes in wild-type (WT) and *sk^{-/-}* flies determined by qRT-PCR. The OR names with red fonts represent the set of ORs for food odor and blue fonts represent the set of ORs for sex pheromone. Data are mean relative expression levels $\pm$ SEM. R1–R3 represent biological replicates. Different lower-case letters indicate significant differences between treatments (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$). (C) Quantification of calcium levels following the response of candidate ORs to sex pheromones and food odors at a concentration of 10^{-4} M ($n = 3–6$). Different lower-case letters indicate significant differences between treatments (one-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$). (D) Dose–response curves of candidate ORs to sex pheromone components such as ethyl laurate (EL), ethyl palmitate (EP), 2,3,5-trimethyl pyrazine (TMP), and 2,3,5,6-tetramethylpyrazine (TTMP). (E) Dose–response curves of candidate ORs to food odorants such as ethyl benzoate (EBE), ethyl butyrate (EBU), diethyl maleate (DM), and methyl eugenol (ME). In both cases, $n = 5$. (F) Co-localization of Orco (red) and SkR1 (green) neurons in *B. dorsalis* antennae. Scale bars = 50 μ m.

the antennal OR repertoire would be an efficient way to co-opt behavioral outputs (Anton and Rössler, 2021 [DOI](#); Xu et al., 2024 [DOI](#)) as we have shown here for the oriental fruit fly *B. dorsalis*. Changes in odorant sensitivity during transitions between foraging and mating have been reported in previous studies. For example, the antennae of female *D. suzukii* become significantly more sensitive to the host fruit odor isoamyl acetate after mating (Crava et al., 2019 [DOI](#)). Similarly, starved *Aedes aegypti* mosquitoes downregulate the sensitivity of lactic acid receptor neurons in grooved peg sensilla, which are used during host search (Davis, 1984 [DOI](#); Oliveira et al., 2017 [DOI](#)), whereas satiated mosquitoes increase the sensitivity of OR neurons in trichoid sensilla to compounds representing the oviposition site (Siju et al., 2010 [DOI](#)). In this study, we determined the mechanisms underlying the changes in antennal sensitivity when switching between foraging and mating behavior. Sk-SkR1 signaling was required to increase the success of foraging and to suppress mating during a period of starvation. The activation of SkR1 on the antennal OR neurons upregulates the expression of ORs that detect food volatiles while suppressing those that detect pheromones.

The coordinated actions of sulfakinin on multiple downstreams, the antennae and other parts of the peripheral and central nervous system in *B. dorsalis*, are not yet fully understood. Sulfakinin is generally known as a satiety signal that reduces food intake and suppresses the digestive system in *D. melanogaster* and a number of different invertebrate species (Nässel and Wu, 2022 [DOI](#)), whereas we found that the Sk-SkR1 signal is required to promote foraging by making the antennae more sensitive to food odorants during starvation. In *D. melanogaster*, sulfakinin inhibits the sugar receptor GR64 in the proboscis and proleg tarsi, thus reducing food ingestion (Guo et al., 2021 [DOI](#)). Our data also support the role of sulfakinin as a satiety signal because feeding rate was increased in *sk*^{-/-} and *skr1*^{-/-} null mutants (Fig. 2D [DOI](#)). Sulfakinin was shown to inhibit both male and female sexual behavior in *D. melanogaster* (Wu et al., 2019 [DOI](#)), in agreement with our results. In addition to the previous study showing the roles of central nervous system in Sk-mediated inhibition of sexual behavior in *D. melanogaster*, we described a Sk direct action on the peripheral olfactory system in *B. dorsalis*. The underlying mechanism is the secretion of the mature peptide Dsk-2 (but not Dsk-1) by Dsk-MP1 and Dsk-MP3 neurons, which then triggers downstream neurons expressing SkR1 in the central nervous system to suppress male mating behavior (11). However, there was a contradictory report showing the role of sulfakinin promoting the receptivity in mating in virgin females (Wang et al., 2022 [DOI](#)). The activity of sulfakinin peptides Sk1 and Sk2, through the receptors SkR1 and SkR2, is therefore likely to be complex, with spatially and temporally dynamic effects in multiple downstream systems.

Although successful foraging and EAG responses to food odors were strongly reduced in both null mutants, there was still a greater response than observed in fed WT controls (Fig. 3A, B, E, F [DOI](#)). This incomplete suppression suggests that other mechanisms, in addition to the main Sk-SkR1 signal, are relevant to the response. Indeed, sNPF and tachykinin signaling have both been described in the modulation of olfactory sensitivity in *D. melanogaster*, with sNPF enhancing sensitivity to food odors and tachykinin inhibiting sensitivity to aversive odors (Ignell et al., 2009 [DOI](#); Ko et al., 2015 [DOI](#); Root et al., 2011 [DOI](#)). Furthermore, neuropeptide F (NPF) and SIFamide also regulate foraging behavior at the level of the olfactory circuit (Jennifer and Yi, 2013 [DOI](#); Martelli et al., 2017 [DOI](#); Wang et al., 2013 [DOI](#)). Notably, NPF and tachykinin also regulate mating behavior at the peripheral level. Tachykinin inhibits courtship behavior by mediating the perception of an anti-aphrodisiac pheromone in male *D. melanogaster*. (Shankar et al., 2015 [DOI](#)). NPF neurons are also required to detect female sex pheromones (Gendron et al., 2014 [DOI](#); Kuo et al., 2012 [DOI](#)). Similarly, SIFamide also regulates mating behavior, although it is not yet clear whether it acts via the olfactory circuit (Sellami and Veenstra, 2015 [DOI](#); Terhzaz et al., 2007 [DOI](#)). We have not yet investigated the roles of other neuropeptides in *B. dorsalis* foraging and mating behavior, but we found that starvation-induced the neuropeptide receptor genes SkR1, sNPF, and SIFamideR1 ($P < 0.05$). In addition, tachykinin, SIFamide, and ion transport peptide (ITP)-like peptide were also

induced by starvation, although the increase was not statistically significant. Therefore, other neuropeptides in addition to sulfakinin are likely to be involved in the switch between different sets of ORs in the antenna as a means to control behavior.

The evolutionary origin of CCK/sulfakinin signaling can be traced back to the common ancestor of the Bilateria (Mirabeau and Joly, 2013 [↗](#); Nässel and Wu, 2022 [↗](#); Staljanse et al., 2011 [↗](#); Tinoco et al., 2021 [↗](#); Yu and Smagghe, 2014 [↗](#)). Most coelomate and pseudocoelomate phyla produce CCK/sulfakinin peptides and orthologs of the corresponding receptors. Sulfakinin is involved in foraging, satiety, locomotion, development, aggression and reproductive behavior in insects (Fedina et al., 2023 [↗](#); Nässel and Wu, 2022 [↗](#); Nässel and Zandawala, 2019 [↗](#)) although some insect lineages have lost both the peptide and its receptors (Consortium, 2010 [↗](#); Hauser et al., 2010 [↗](#)). CCK is also involved in satiety, memory, anxiety, and depression in mammals (Jens et al., 2007 [↗](#); Lau et al., 2023 [↗](#); Rana et al., 2022 [↗](#); Shen et al., 2019 [↗](#)). The many functions of CCK/sulfakinin in diverse taxonomic groups frequently include signaling between the digestive system (endoderm) and the neuronal system (ectoderm) (Nässel and Wu, 2022 [↗](#)). This suggests an ancestral function associated with coelomic development, and that additional complexity has evolved in each lineage.

In summary, we found that the neuropeptide sulfakinin mediates the transition between foraging and mating behavior by activating the receptor SkR1 and reprogramming the peripheral OR repertoire. We propose a working model to characterize this mechanism, in which Sk-SkR1 regulates the expression of different sets of ORs, leading to the behavioral switch (Fig. 5 [↗](#)). The modulation of peripheral sensory systems by sulfakinin, coordinating with the central neural circuit also controlled by sulfakinin, is the major trigger for a successful behavioral switch. We have thus provided new insight into the control of behavior by sulfakinin in *B. dorsalis*, which is one of the most widely distributed, fecund, and invasive insect pests to threaten global agriculture (Wan and Yang, 2016 [↗](#); Zhao et al., 2024 [↗](#)).

Materials and methods

Insects

The wild-type (WT) oriental fruit flies were originally collected from Hainan province, China in 2008 and were reared in the laboratory (27.5 ± 0.5 °C, 75 ± 5% relative humidity, 14-h photoperiod). Larvae and adults were fed with artificial food as previously described (Shen et al., 2013 [↗](#)). Artificial food for adults, consisting of yeast powder, honey, sugar, ascorbic acid and water, was also used as a food source for foraging behavior assays.

Behavioral assays

Virgin females and males, 3-day old, were placed each in groups of 25 flies and reared at 27.5 °C and 60% humidity, and 9-day old flies were subjected to behavioral assays. Food consumption rates of individual flies were measured by the amount Bromophenol Blue dye (BPB) in the fly after the food including 0.1% (BPB) was provided for 1 h for ad libitum feeding, which is long enough time to allow satiation to occur. BPB was measured from decapitated and homogenized bodies in 1 ml PBS buffer with 1% Triton X-100. Absorbance of the supernatant after centrifugation (13000 rpm) for 3 min was measured at 614 nm on a xMarker™ Microplate Spectrophotometer (Bio-Rad). Flies were starved for 12 h prior to the experiments with only water. Foraging behavior assay was performed according to previously published methods with modifications (Li et al., 2022 [↗](#)). In brief, single flies were introduced into chambers (100 mm diameter x 10 mm height) and acclimatized for 20 min. The foraging behavior was recorded using a camera (Sony HDR CX405) for 15 min at 30 fps. Successful foraging was counted when the fly stayed 5 s or longer

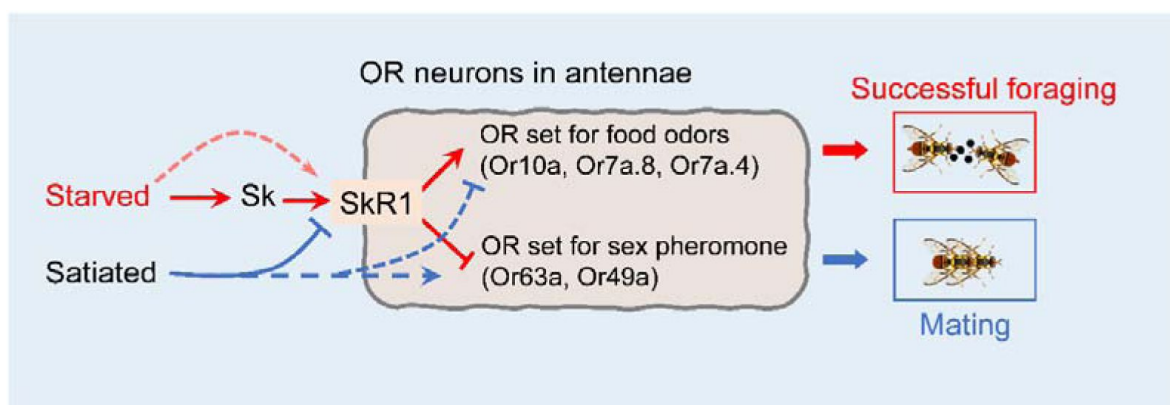


Figure 5.

Schematic representation of peripheral olfactory remodeling in OR neurons of the antenna that arbitrate between mating and foraging behavior in *B. dorsalis*.

Starvation increases the abundance of *SkR1* in the OR neurons (red arrows) and *SkR1* signaling induces the expression of genes encoding ORs that sense food odors, resulting in successful foraging. Satiation suppresses *SkR1* expression and induces the expression of genes encoding ORs that sense opposite-sex pheromones, leading to successful mating (blue arrows). Dashed arrows represent the additional possible pathways that have not been tested in this study, but not excluded in the model. Please see the discussion for details of additional possible factors modulating odorant sensitivity relevant to satiety.

within the 5 mm radius of the center where 10 μ L of artificial food was placed. The experiments consisted of the assays of 3 biological replications for each experimental condition. The behavior was analyzed by EasyFlyTracker (Qu et al., 2022 [DOI](#)) with the default parameters.

Mating behavior assay was conducted in the courtship chambers (diameter: 3.5 cm; height: 1.5 cm per layer). The virgin females and males were cold anesthetized at -20°C for 1 min and introduced into the courtship chamber separated by a removable transparent film. After 1 h acclimation, the film was removed to allow the paired flies to get in contact, and mating behavior was recorded by a video camera for 15 min. We measured the level of courtship in mutant males and receptivity in mutant females respectively. For courtship assay, a mutant male ($sk^{-/-}$ or $skr^{-/-}$) and a WT virgin female were introduced into the chambers. Courtship rate is the percentage of the mutant males performs attempted copulation, which was used to measure mutant courtship level. For female receptivity assay, a mutant female ($sk^{-/-}$ or $skr^{-/-}$) and a WT virgin male were introduced into the chambers. Copulation rate is the percentage of the mutant females had engaged in copulation, which is used to measure the mutant female receptivity. The number of flies that had performed attempted copulation or engaged in copulation by the end of each 1 min interval were summed within 15 min and plotted as a percentage of total flies for each time point.

Electroantennograms

Electroantennograms were used to record the stimulus-dependent changes in the electrical potential summed over the whole antenna. To test volatiles were food odor or body extract of each sex on the flies conditioned for satiated and starved. Different genotypes of the flies were wild-type, *Sk* mutant, and *SkR1* mutants. Female body extracts were tested by male antennae and male body extracts by female antennae (Table S2). Body extracts were prepared by washing 15 9-day old virgin flies of each sex in 1 ml of hexane for 4 hours (Chen et al., 2023 [DOI](#); Dweck et al., 2015 [DOI](#); Ebrahim et al., 2023 [DOI](#)) at room temperature with gentle shaking.

For electroantennograms, similar to previous study (Li et al., 2022 [DOI](#)), the head (including the antennae) of fly was excised and a glass capillary filled with 0.9% NaCl was inserted into the cut end as the reference electrode. The tip of an antenna was placed into another glass capillary filled with 0.9% NaCl as the recording electrode. The reference electrode was connected to a grounding electrode, while the recording electrode was connected to a $10\times$ high-impedance DC amplifier (Syntech; The Netherlands). The signal was then sent to an analog/digital converter (IDAC-4, USB, Syntech) and transferred to a computer. Finally, the data were analyzed and saved by using GC-EAD software (Syntech). A filter paper strip (3×0.75 cm), loaded with 10 μ L of the odor solution or body extract, was placed into a pipette and delivered a stimulus into a constant, humidified airstream flowing at 100 ml/min by a stimulus controller (Syntech, Hilversum, Netherlands). Two stimuli were made for 0.5 s duration with a 30 s interval. Average EAG response, as shown by mV changes, was used for the calculation. EAG for sex pheromones and body surface extracts were measured from 21:00–23:00, and food odor was measured from 9:00–11:00.

Quantitative real-time reverse transcription PCR

Antennal tissues from flies of different genotypes with different treatments were dissected and immediately stored at -80°C . Total RNA was extracted with Trizol Reagent (Invitrogen) following the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) and specific primers designed using the online software, Primer 3 (<http://primer3.ut.ee/> [DOI](#), Table S1). Quantitative real-time reverse transcription PCR (qRT-PCR) was performed using a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) with NovoStart SYBR PCR SuperMix kit (Novoprotein, Shanghai, China). Two reference genes (Shen et al., 2010 [DOI](#)), α -tubulin (GenBank: GU269902) and RPS3 (GenBank: XM_011212815), were used selected to normalize the expression levels for the calculation in qBASE (Hellemans et al., 2007 [DOI](#)). Each experiment comprised 3 independent biological replicates and two technical replicates.

CRISPR/Cas9-mediated mutants

In vitro synthesis of single guide RNA was performed as previously described (Li et al., 2022 [\[4\]](#)). In brief, the suitable target sites of *Sk* and *SkR1* genes were searched by CasOT (Xiao et al., 2014 [\[5\]](#)), and the corresponding single guide RNAs (sgRNAs) were synthesized *in vitro* and purified using the GeneArt Precision gRNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). We designed and synthesized one sgRNA for the *Sk* gene (Fig. S4) and two single guide RNAs for the *SkR1* gene (Fig. S5).

The sgRNA and cas9 protein (Invitrogen) were gently pipetted and mixed in nuclease-free water to form a mixture of 400 ng/μL sgRNA and 300 ng/μL Cas9 protein. Eggs collected within 20 min of oviposition were injected within 2 h with the mixture of sgRNA and Cas9 protein using an IM300 Electric Microinjector (Narishige, Tokyo Japan). The injected eggs were incubated at 27.5 ± 0.5 °C and 75 ± 5% relative humidity for hatching.

Mutant screening and off-target analysis

Heterozygous G1 offspring were obtained using 9-day-old virgin adults (G0) crossed to wild-type opposite sex. Suitable mutant G1 (*sk*^{+/-} and *skr1*^{+/-}) were backcrossed to WT flies for at least eight generations, then homozygous mutant flies (*sk*^{-/-} and *skr1*^{-/-}) were obtained by self-crossing. Genomic DNA was extracted from individual flies (G0) or a single hind leg (G1 and later generations) by incubating tissue samples in 30 μL InstaGene Matrix (Bio-Rad, Hercules, CA, USA) at 56 °C for 30 min and inactivating the enzyme at 100 °C for 10 min. Fragments of 300–500 bp spanning the sgRNA target sites were amplified using the primers (Table S1). The fragments were genotyped using the QIAxcel Advanced System (Qiagen, Hilden Germany), and potential edited products were directly sequenced or cloned into vector pGEM-T Easy (Promega, Wisconsin, Madison, USA) for Sanger sequencing to identify the mutation.

To assess off-target effects, CasOT was used to search potential off-target sites for the *Sk* and *SkR1* genes. All loci with one potential mismatch allowed in the seed region and up to three potential mismatches in the non-seed region were searched on the genome sequence (<https://db.cngb.org/search/project/CNP0003192/> [\[6\]](#)). Two potential off-target sites were found for *Sk* gene and three for *SkR1* gene. PCR amplification and sequencing of fragments including potential off-target sites were performed to check for mutations. In examination of the potential off-target sites with > 4 bp mismatches to the sgRNA sequences, no mutations were found in the off-sites (Fig. S10).

Immunohistochemistry

Rabbit anti-BdSkR1 antibody was generated by immunization with the peptide N'-RQGLRKCRDQWP-C'. Mouse anti-BdOrco antibody was raised by immunization with two peptides, N'-GTNPNGLTRKQE-C' and N'-HWYDGSEEAFTF-C'. Peptide were synthesized and conjugated with keyhole limpet hemocyanin through additional cysteine residues at their N termini by Zoonbio Biotechnology (Nanjing, China). Rabbit anti-BdSk antibody was a gift from Dr. Chuan Zhou (Wu et al., 2020 [\[7\]](#)). The antisera used to recognize Sk peptide were raised in New Zealand white rabbits using the synthetic peptide N'-GGDDQFDDYGHMRFG-C'.

Immunohistochemistry was performed as previously described (Li et al., 2022 [\[4\]](#)). In brief, whole brains of 9-day-old flies were dissected in phosphate-buffered saline (PBS), fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4 °C, and washed (3 × 15 min) in PBST (0.5% Triton X-100 in PBS). The tissues were blocked in 5% normal goat serum for 20 min at room temperature, then incubated in primary antibody for 48 h at 4 °C. After three washes in PBST (3 × 15 min), the tissues were then incubated in secondary antibody overnight at 4 °C. After washing in PBST again, the tissues were mounted on a slide with 100% glycerol for imaging. Adult antennae were dissected and embedded in Tissue-Tek O.C.T. Compound (Sakura, Tokyo, Japan). Sections (18 μm) were

prepared using a Thermo HM525 NX microtome at -22°C and then fixed in 4% PFA in PBS at room temperature for 2 h. The subsequent procedures were as described above for the brain tissues. Primary antibody used: rabbit anti-Sk (1:1000), rabbit anti-SkR1 (1:500), mouse anti-Orco (1:200). Secondary antibodies used: goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000, Cell Signaling Technology), goat anti-mouse IgG conjugated to Alex Fluor 647 (1:1000, Cell Signaling Technology). Samples were imaged using an LSM780 confocal microscope (Carl Zeiss, Jena, Germany) and processed with Image J software (Schneider et al., 2012 [DOI](#)).

RNA-seq analysis

Total RNA was extracted from the head epidermal tissues, including the antennae, of fed WT flies, starved WT flies, and starved *sk^{-/-}* flies using TRIzol reagent (Invitrogen). Three replicates were performed. Library construction and sequencing was performed by Novogene with Illumina HiSeq2000 platform (Novogene Bioinformatics Technology Co.Ltd, Beijing, China). The raw data were analyzed after filtering the low-quality sequences by fastp with the default parameters (Chen et al., 2018 [DOI](#)). Sequences were aligned to the *B. dorsalis* genome (<https://db.cngb.org/search/project/CNP0003192/>) using Hisat2 (version 2.2.1) (Kim et al., 2015 [DOI](#); Yang et al., 2023 [DOI](#)). Samtools (version 1.7) was used to convert sam files to bam files in the process (Li et al., 2009 [DOI](#)). Finally, to calculate the count, we use featureCounts (version 2.0.1) (Liao et al., 2014 [DOI](#)). The expression level of genes from the RNA sequencing was normalized by the FPKM method. Differential expression analysis was performed using the DESeq2 R package (1.16.1) (Anders and Huber, 2010 [DOI](#)).

Functional heterologous expression of ORs and calcium imaging

Heterologous expressions of ORs were made in Human Embryonic Kidney-293 (HEK-293) cells, which were cultured in Dulbecco's modified Eagle medium (DMEM, 11995065, Gibco, Thermo Fisher Scientific, USA) containing 10% Fatal Bovine Serum (FBS, 10091148, Gibco) and 1% penicillin-streptomycin-Amphotericin B solution (C0224-100 ml, Beyotime) with 5% CO_2 at 37°C . Cells for transfection were seeded onto a 96-well black well plate (165305, Thermo Fisher Scientific, USA). Approximately 50%–70% confluent HEK-293-cells were transfected by $2\text{ }\mu\text{g}$ pcDNA3.1 plasmid containing respective olfactory receptors with BdOrco of *B. dorsalis* using TransIT[®]-LT1 Transfection Reagent (MIR 2300, Mirusbio) for 24 h.

For calcium imaging experiment, the culture media was replaced to $100\text{ }\mu\text{L}$ of $2.5\text{ }\mu\text{M}$ Fluo-4 AM (F14201, Invitrogen) and incubated at 37°C with 5% CO_2 for 1 h. Subsequently, after another 30 min of incubation at room temperature, the culture media containing Fluo-4 was removed, and the cells were washed three times with Hanks' balanced salt solution (HBSS; 14065056, Gibco, Thermo Fisher Scientific, USA) in order to fully remove the Fluo-4 residue. Then, $99\text{ }\mu\text{L}$ of HBSS was added to each well and plate was placed in the dark at RT to be tested. Finally, $1\text{ }\mu\text{L}$ of test odor was added to each well and fluorescence immediately was measured for at least 80 s. Finally, images were captured by a Zeiss Observer 7 inverted microscope (Carl Zeiss, Jena, Germany). Test odors were dissolved in dimethylsulfoxide (DMSO, ST038-100ml, Beyotime) and prepared five final concentrations: 10^{-7} , 10^{-6} , 10^{-5} ,

10^{-4}-M and 10^{-3}-M . the fluorescent ratio ($\Delta F/F$) was calculated as peak intensity divided by basal intensity (intensity before ligand perfusion). For all experiment, the response to DMSO was used as negative control.

Statistical analysis

For qRT-PCR result analysis, relative expression levels were calculated using qBase (Helleman et al., 2007 [DOI](#)) based on the expression of the reference genes. Statistical analysis is performed using GraphPad Prism and indicated in each figure legend. Data were first verified for normal distribution using the Shapiro-Wilk normality test. One-way ANOVA, followed by Tukey's multiple

comparisons (if normal distribution) and Kruskal-Wallis and post-hoc Mann-Whitney U-tests (if non-normal distribution), was used for comparisons among multiple groups. Values are reported as means $\pm$ standard errors of the mean (SEM).

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Additional files

Supplemental figures and tables [↗](#)

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Joint Public Review:

Summary:

The behavioral switch between foraging and mating is important for resource allocation in insects. This study characterizes the role of sulfakinin and the sulfakinin receptor 1 in changes in olfactory responses associated with foraging versus mating behavior in the oriental fruit fly (*Bactrocera dorsalis*), a significant agricultural pest. This pathway regulates food consumption and mating receptivity in other species; here the authors use genetic disruption of sulfakinin and sulfakinin receptor 1 to provide strong evidence that changes in sulfakinin signaling modulate antennal responses to food versus pheromonal cues and alter the expression of ORs that detect relevant stimuli.

Strengths:

The authors utilize multiple complementary approaches including CRISPR/Cas9 mutagenesis, behavioral characterization, electroantennograms, RNA sequencing and heterologous expression to convincingly demonstrate the involvement of the sulfakinin pathway in the switch between foraging and mating behaviors. The use of both sulfakinin peptide and receptor mutants is a strength of the study and implicates specific signaling actors.

Weaknesses:

The authors demonstrate that SKR is expressed in olfactory neurons, however there are additional potential sites of action that may contribute to these results.

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Author response:

The following is the authors' response to the original reviews.

Joint Public Review:

Summary:

The behavioral switch between foraging and mating is important for resource allocation in insects. This study investigated the role of the neuropeptide, sulfakinin, and of its receptor, the sulfakinin receptor 1 (SkR1), in mediating this switch in the oriental fruit fly, Bactrocera dorsalis. The authors use genetic disruption of sulfakinin and of SkR1 to provide strong evidence that changes in sulfakinin signaling alter odorant receptor expression profiles and antennal responses and that these changes mediate the behavioral switch. The combination of molecular and physiological data is a strength of the study. Additional work would be needed to determine whether the physiological and molecular changes observed account for the behavioral changes observed.

Strengths:

(1) The authors show that sulfakinin signaling in the olfactory organ mediates the switch between foraging and mating, thereby providing evidence that peripheral sensory inputs contribute to this important change in behavior.

(2) The authors' development of an assay to investigate the behavioral switch and their use of different approaches to demonstrate the role of sulfakinin and SkR1 in this process provides strong support for their hypothesis.

(3) The manuscript is overall well-organized and documented.

Weaknesses:

(1) The authors claim that sulfakinin acts directly on SkR1-positive neurons to modulate the foraging and mating behaviors in *B. dorsalis*. The authors also indicated in the schematic that satiation suppresses SkR1 expression. Additional experiments and more a detailed discussion of the results would help support these claims.

(2) The findings reported could be strengthened with additional experimental details regarding time of day versus duration of starvation effects and additional genetic controls, amongst others.

Recommendations for the authors:

Major issues

(1) As written the introduction is somewhat fragmented and does not lay out a clear rationale for the current study in the species used by the authors. Others, including Guo et al. (2021) and Wang et al. (2022), have previously shown that sulfakinin signaling pathways are important for feeding and receptivity regulation in *D. melanogaster*. Thus, the novelty of this study should be more clearly articulated.

The introduction in the revision is significantly changed to improve the description for the rationale of study (lines 60-66 in the revision).

(2) In addition, the Introduction should provide more specific background information on the pheromonal activity of oriental fruit fly body extract, the odor-preferences, and the sex pheromone of this species compared to that of model insects such as *Drosophila melanogaster*.

The revision contains a paragraph of introduction for chemical ecology of oriental fruit fly that is related to this study (lines 67-75).

(3) It isn't clear what the first image in Figure 1C represents - is this a schematic of the area or does it represent data?

The Fig 1C and the associated figure caption are revised. The figure is more visible by changing the track colors. The figure caption is revised as "Representative foraging trajectories in the 100 mm diameter arenas within a 15-min observation period of flies starved for different durations."

(4) The authors should include examples of the EAG recordings following the stimulation with food volatiles or pheromones, not only the results of their analyses. This could be included in the main figures or even in supporting information.

As suggested, we added the examples of the EAG recordings following the stimulation with food odors and body extracts in the Figure 1 and Figure 3.

(5) The demonstration that removal of the antennae severely impairs mating is dispensable because the antennae are required for other functions in addition to olfaction.

We agree that the roles of the antennae are likely more than the olfactory function. As suggested, we removed the data.

(6) It is currently difficult to understand how the authors measured successful rates of foraging. Please provide more details.

In the revision, we added a sentence describing the method for measuring in detail. See line 269-273.

(7) The expression of sulfakinin does not change significantly in the antennae following starvation (Figure 2A). Do the authors know whether they change in the central nervous system under these conditions? Have the authors (or has anyone else) checked the expression pattern of sulfakinin in the antennae? This information would help determine whether the sulfakinin signal that acts on SkR1 is released from neurons in the central nervous system (Figure S4C) or whether it is also released from the neurons in the olfactory organs. Based on the immunochemistry results shown in Figure S4C, it would also be interesting to determine whether the intensity of anti-sulfakinin immunoreactivity changes before versus after starvation. This could help establish whether sulfakinin is released during starvation.

We added the expression data showing the the mRNA level of Sk in the head that is higher after refeeding in Fig. S3. The change in the expression of Sk is also added in the text (lines 107-110). We were unable to identify the Sk neurons in the antennae suggesting possibility of the direct action of humoral Sk on the antennae.

(8) In Figure 2A, the authors show that the expression levels of some neuropeptides system components change during starvation. However, it would be helpful if the authors could include more detailed information on how the results are shown in the figure legends (e.g., the expression level of each candidate in fed flies was set as 1, etc).

We revised the figure caption to explain the Figure 2 with the expression values in the figure legend.

(9) In Figure 2D, null mutant males of sulfakinin and SkR1 consume more food at all times compared to the wild type. However, the corresponding mutant females consume more food only at night. Is this because the wild-type female flies eat more food during the day? In a related issue, Figure 2D shows differences in food consumption measured at different times of day, however, this is not directly addressed in the text, which instead mentions that "the amount of excess food consumed by the mutants was dependent on the duration of the starvation period in both sexes".

Thank you for the important suggestions. We speculate that the difference of feeding amounts of females occurring only at night is due to the high basal feeding rate of females during the daytime, masking the increase in feeding in the knockout of Sk signaling. As suggested, we have added a relevant description of the difference in food consumption. In

addition, we changed the Y-axis scale in the figure for a justified comparison between males and females. See line 123-128.

(10) It isn't clear how the time of day relates to the duration of starvation. This suggests that mutant females only consume more at 21:00 (presumably at night) whereas males consume more throughout the day. Does this suggest an interaction with the circadian system? What is the duration of starvation in Figure 3A? In a related issue, in Figure 4 it would be useful to know what time of day the EAG analysis was done because the data shown in Figure 2D suggests that the time of day significantly impacts behavioral responses. And does the red versus blue color scheme of the OR subunits represent up/downregulated levels in wild-type animals? Please define this for the reader.

In addition to the response to the point 9, responding to the issue of feeding amount in females. As the reviewer noted, there was indeed a diurnal difference in food amount consumed by *B. dorsalis*. However, whether this is related to circadian rhythms is something we haven't studied for further in-depth. Measuring food intake at these 3 times of day, we all ensured that the duration of starvation was the same 12 h. The duration of starvation in Figure 3A is 12h. We have mentioned this in the manuscript. See line 267-268.

The EAG for sex pheromones and body surface extracts were measured from 21:00-23:00, and food odor was measured from 9:00-11:00. The times of the experiments are described in the revision. See line 309-311.

Accordingly, we made a revision of the figure caption for explaining the colored fonts. Red color represents a set of ORs related with foraging and blue color is for a set of ORs related with mating. Therefore, the ORs with red color were upregulated in starved wild-type animals and the ORs with blue color were downregulated in starved wild-type flies. We have defined this in the revised manuscript. See line 672-673.

(11) The authors convincingly show that SKR1 is present in the antennae and is co-expressed with orco. It would be useful to discuss whether this receptor is also expressed in other tissues where there may be additional sites of action of this pathway.

Indeed, SkR1 is also expressed in the *Drosophila* brain. We added the discussion on the expression and additional sites of action of SKR1 within the central nervous system. See line 200-205.

(12) It isn't clear what the dotted arrows in the model shown in Figure 5 represent.

Dashed arrows represent the additional possible pathways that have not been tested in this study, but not excluded in the model. Please see the discussion for details of additional possible factors modulating odorant sensitivity relevant to satiety. See line 210-229.

(13) In Figure 5, the authors indicate that satiation suppresses SkR1 expression. It would be helpful if the authors tested the expression level of SkR1 in re-fed flies (by feeding the flies after 12h starvation) to see whether levels of expression are rapidly restored to the levels seen in satiated animals. Such a result could further support the claims made by the authors.

Thank for your suggestions. Indeed, refeeding after 12h starvation significantly decreased SkR1. We added the result in supporting information (Fig. S3). See line 713. Results see line 107-110.

(14) The authors show that locomotor activity is unaffected in the mutants but body size comparison would be more useful here since this could also contribute to baseline

differences in meal size.

In the revision, we provided a comparison between WT and *Sk*^{-/-} in the supplementary data. Results showed that mutant flies have the same body size as the WT flies. (Fig. S7) See line 742. Results see line 120-121.

(15) Have the authors tested the behavioral phenotypes of heterozygotes mutant of both Sk and SkR1 flies? This may reveal whether a reduced expression of Sk-SkR1 will also cause significant changes in the foraging and mating behaviors seen during starvation.

We tested the behavioral phenotypes of heterozygous mutant of *Sk* knockout flies. The results showed that foraging and mating behaviors of *Sk* heterozygous mutants were unaffected during starvation, suggesting the mutants are completely recessive. We have added the results in supporting information (Fig. S8). See line 746. Results see line 132-135.

(16) It would be useful to provide information about which SK peptide is detected by the antibody used in Figure S4C. In Figures S4C and S5D, it would be useful to include a counterstain to show that the general morphology is unaffected in the mutants.

As suggested, we added a detailed description for rabbit anti-BdSk antibody. See line 362-363. We have improved the background image to be available to show the general structure. So counter staining would not be essential.

(17) The figure legends for supporting figures need to be improved as they are currently difficult to understand. For example, in S2: what is the meaning of "different removal of antennae"? In S3: it isn't clear how the authors evaluated the responses in EAG experiments; in S4A: there are several DNA sequences that do not appear in the main text of the manuscript; in S4C: the meaning of the boxes and the dots is unclear, as is the figure to the left; in S5D, the authors explain only the suppression of SKR1, yet the figure indicates some images for SKR IHC. These are only a few examples; we ask that the authors revise and improve the legends for supporting figures.

For S2, we removed the data as suggested. For S3, we added a sentence describing the method for measuring in detail. See line 707-709. For S4, the figure in the revision is significantly changed and added a detailed description in the legend (lines 717-724 in the revision). For S5, we have improved our description. See line 731-734. In addition, we have checked all the figure legends of our manuscript and changes were displayed in track version.

Minor issues

(1) It isn't clear what the meaning of "the complexity of sulfakinin pathways" is. Please explain.

We have rewritten the sentence in the revised manuscript by adding the description as "... complexity of *Sk* pathways, spatial and temporal dynamics and multiple ligands and receptors, is...". See line 61-65.

(2) Please double-check the calls to the various figures in the text.

We have double-checked the calls to all the figures in the text to make sure they were correct.

(3) L125: What is the meaning of "olfactory reprogramming"? Please explain.

We rephrased it to "alteration of olfactory sensitivities". See line 145.

| (4) L135: After mentioning qRT-PCR the authors should include a call to a figure that shows these results.

Thank you for your suggestion, the qRT-PCR results are shown in Figure 4B, and we have added it as suggested. See line 154.

| (5) L270: Details are provided for the extraction of the pheromone. However, more details are needed on how the EAG and other functional assays were done.

We have described the assay procedures in detail in the materials and method part. See line 298-311.

| (6) Figure 2B. Please remove the period(".") at the C-terminal end of WT sk.

We are sorry for our mistake. We have corrected it.

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