Genetic mechanisms underlying worker caste differentiation in the honeybee

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Düsseldorf, im November 2021 _____

(Vivien Sommer)

CONTENT

CHAPTER I

General introduction

The honeybee *Apis mellifera* is one of the most striking insects when it comes to behavior and how they manage to live as a group of social individuals. When observing a beehive, it seems as if the bees perform work randomly, but taking a closer look, you can see an ingenious division of labor. The functional units of a hive are separated in reproduction and sterile helpers. While reproduction is guaranteed by the queen and drones, every other task from colony maintenance over nursing to foraging is undertaken and coordinated by worker bees (Lindauer, 1952; Rösch, 1930). What is most interesting about this division of labor is that both females, queen and worker, develop from fertilized eggs, while male drones develop from unfertilized eggs. For queen and worker bees, this means that the same genome manifests in different phenotypes and behavior. In addition, worker bees are sterile, which means they live highly altruistic by working for the greater good of the hive. Combined with the fact that worker bees exhibit an enormous repertoire of behavioral traits not found in the queen, raises the question how this advanced social behavior evolved. How and when during caste development is worker or queen-development specified, and which genes are involved? While nutrition has been found as one of the main triggers for queen or worker development, as a prolonged feeding of royal jelly induces queen-development (Haydak, 1970; Kaftanoglu et al., 2010, 2011), the main regulatory pathways of neuronal differentiation and circuit development at a molecular and genetic level are mostly unknown. Group living is quite common in the animal kingdom and can be found in school of fish, a pack of wolves or a herd of elephants, as it increases the probability of survival and reproduction (Hamilton, 1964; Sherman, 1977). The highly social behavior that characterizes honeybee group living is the most complex degree of sociality, challenging our common understanding of the individual will to survive, as worker bees forego reproduction in favor of the common good. This makes the honeybee worker a perfect model organism for studying the genetic basis of advanced social behavior and caste-specific dimorphism associated with a highly variable behavioral repertoire. The unique opportunity in finding and studying a gene that acts as a trigger or even a partly regulator involved in the specification of social behavior in the honeybee is intriguing and the focus of this thesis. Undertaking the challenge of

specific gene manipulation in honeybees followed by behavioral and neuronal analysis, are the experimental setups to unravel aspects of the genetic basis of the worker bee specification.

Advanced social behavior: division of labor in a beehive

In studies dating back to 1609 (Butler), it has already been established that the division of labor in a honeybee hive is based on temporal patterns that determine the tasks to be performed. Unlike primitively living social bumble bee species, where the queen solely founds a colony and at first forages and takes care of the brood herself (Goulson, 2003; Oster & Wilson, 1978), honeybees of the species *A. mellifera* have a clear division of labor in reproduction. Advanced sociality in honeybees is built on the polyphenism of females, where individuals specialize behaviorally and morphologically in reproduction (queen and drones) and sterile helpers (worker bees).

A queen´s (and the drones) responsibility is to provide a continuous flow of offspring, which favored the evolution of body parts that are specialized for reproduction. Since queens mate with several drones only once in a lifetime, they have a spermatheca that can hold up to 5.5 million spermatozoa - enough for her entire life time of up to five years (Gessner & Ruttner, 1977). Further, can a queen lay up to 2000 eggs per day due to the large geminate ovaries, consisting of 200 ovarioles each, of which each in turn is filled with maturing eggs (Franck et al., 2002; Snodgrass & Morse, 1910). A queen´s behavioral repertoire is specialized on laying eggs by displaying a fixed action pattern driven by a consistent stimulus response triggered by an empty cell where an egg can be placed. By laying eggs a queen strolls around the hive and simultaneously signals her presence with the queen´s pheromone (queen mandibular pheromone; QMP). QMP suppresses the maturing worker bee ovaries and thus guarantees social cohesion and the reproductive monopole of the queen (Hoover et al., 2003; Slessor et al., 1988). With the exception of the mating and swarming flight a queen never leaves the hive. The role of drones in reproduction is quite simple and solely consists of the insemination of the queen. For this reason drones developed strong wing muscles needed for the mating flight and pronounced reproductive organs with paired gonads consisting of testioles arranged to testes, which can inject six to 12 million spermatozoa in a queens oviduct (Woyke, 1960).

Collectively said, evolution favored traits easing reproduction for queens and drones, and in case of the queen, also to be efficient for generating offspring for up to five years.

Worker bees on the other hand are sterile, which is even more astonishing in context of the role they have within in a beehive. The survival of the colony depends on the task performance of the worker bees, as they take care of the brood, food storage and foraging. To do so worker bees display a striking number of task specializations leading to a second, age-dependent division of labor among this caste that is accompanied by behaviorally and physiological specializations, also known as temporal polytheism (Robinson, 1992). This age-dependent task division starts from a young age, where worker bees perform tasks in the brood area, then move towards

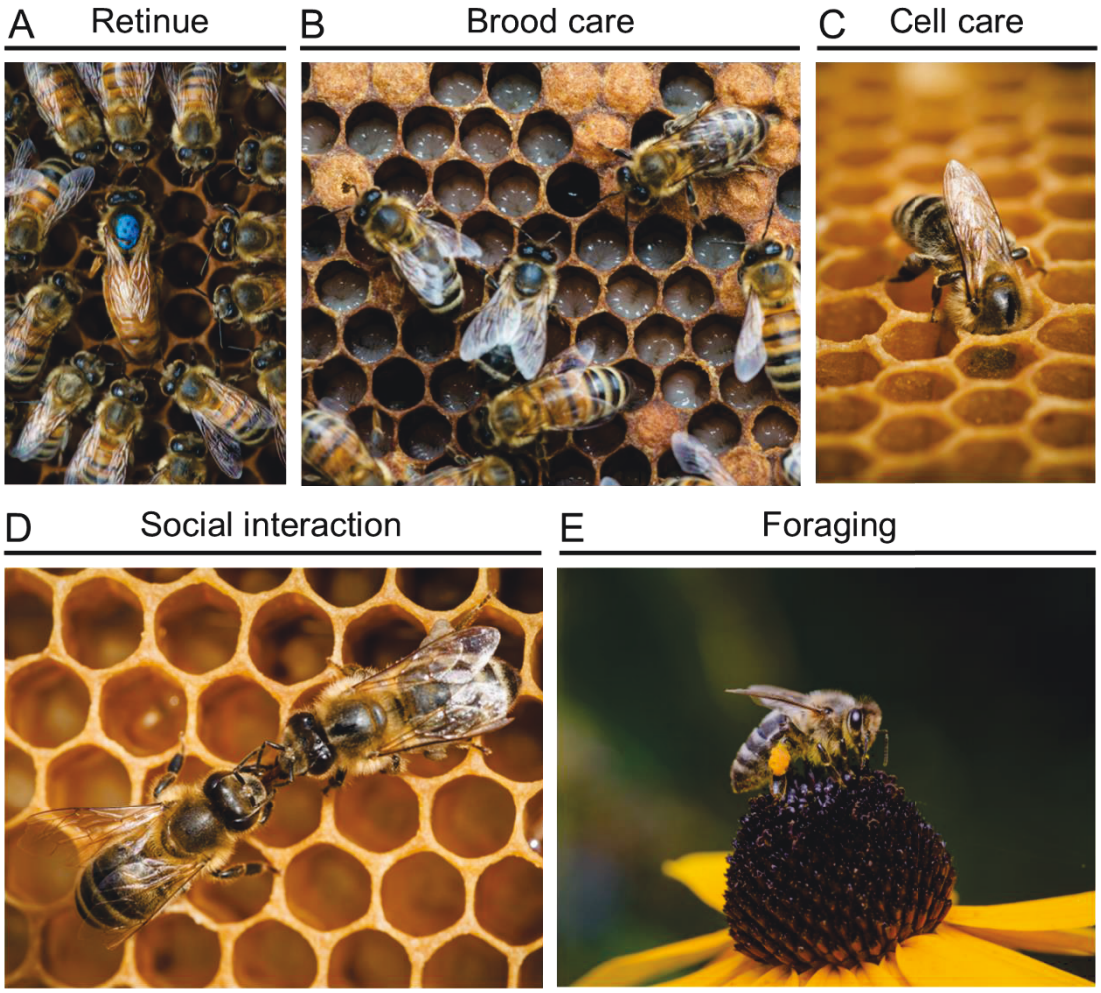

Figure 1. Examples of tasks performed by worker bees. Worker bees perform age-dependently different tasks. Nurse bees from the age 4 to 12 days display for example queen retinue behavior (**A**) or take care of the brood by inspecting cells (**B, C**). Middle-aged bees from the age of 12 to 21 days perform cell maintenance and nectar work throughout the hive, which also includes inspecting cells (**C**). Worker bees of all ages display social interactions, with trophallaxis (**D**) being important for the food exchange between nestmates and therefore a crucial part of social group living. Worker bees reaching the age of 21 days leave the hive and start foraging (**E**). The pollen baskets with orange lumps of pollen are visible in **E**.

the periphery of the hive while they age and at last start foraging outside. Newly hatched bees are unable to sting or fly and spent the first few days acquiring these abilities. These bees further contribute to cell cleaning, but are mostly inactive or show grooming for the rest of the time (Seeley, 1982). The following nursing caste at the age of four to 12 days is crucial for the hive survival as their main task is to take care of the brood (Fig. 1b, c; Ribbands, 1953; Seeley, 1982). Brood care includes continuously inspecting cells and processing sensory information about the cell content, state and age of brood which manifest in the execution of flexible tasks adjusted to the stimulus perceived (Siefert et al., 2021). Nurse bees therefore need to process a multitude of information to be able to perform the correct task. Their age-dependent task repertoire is specialized to the brood area, where they mostly take care of larvae by feeding Royal jelly to future queens and a little lesser to worker larva. Royal jelly is produced by the hypopharyngeal glands (HPG), a paired exocrine gland in the front of the head capsule of worker bees (Deseyn & Billen, 2005). The HPG is producing jelly age-dependently and reaches its peak volume and secretion activity about six to eight days of worker bee age, which is exactly in the nurse stage of worker bees (Deseyn & Billen, 2005; Snodgrass & Morse, 1910). With increasing age of worker bees, the volume and secretion decrease and a different mixture of proteins and enzymes, for example invertase which is used by middle-aged bees to ripen nectar into honey is secreted. With the onset of foraging the secretion of the HPG however stops completely (Robinson, 1992; Ueno et al., 2015). Next to brood care nurse bees also feed other adult nestmates (Fig. 1d; Crailsheim, 1991, 1992; Johnson, 2008a). With the mouth-to mouth food transfer trophallaxis nurse bees take care of other worker bees, drones and the queen by providing a protein rich nutrition (Crailsheim, 1992). The queen is mainly fed by nurse bees displaying retinue behavior (Fig. 1a; Allen, 1955). During contact with the queen, retinue nurse bees take in QMP and thus act as messengers for the queen’s presence by spreading QMP, assisting the queen in keeping her reproductive monopole (Seeley, 1979; Velthuis, 1972). Worker bees from the age of 12 to 21 days form the middle-aged caste and display a task repertoire which is observed throughout the colony and less in the brood area (Fig. 1c; Johnson, 2008a). They build combs, work on honey cells, or work with propolis in all comb areas and move long distances to perform these tasks. Bees of this caste have been observed receiving and storing nectar and also guarding the nest entrance (Johnson, 2008a;

Seeley, 1982). Once a bee reaches the age of three weeks, she transitions to the outside and becomes a forager. From this point on these bees do no longer engage in within-colony tasks and specialize on collecting nectar, pollen, water or propolis (Calderone and Page, 1988; reviewed in Seeley, 1995). The worker-specific pollen "baskets" on the hindlegs, the corbicula, enables foragers to collect pollen (Fig. 1e). Although the transition from nurse over middle-aged to the forager caste seems predetermined by age, it is very flexible and adjusted to the needs of the colony (Robinson, 2002). By experimentally removing pupal combs form a colony Milojevic (1940) and Haydak (1963) showed, that this colony was able to adapt to the new environmental conditions by producing nurses over several months. Demonstrating that even though the behavioral development and task performance seems to be strictly regulated by age, it can be adapted flexibly to environmental changes.

Collectively, the beehive is a superorganism which relies on the flexible task repertoire of worker bees combined with their highly altruistic behavior. Processing multimodal stimuli that can lead to several behavioral outputs, makes the genetic basis for the programming of such outcome of great interest for behavioral research. For this reason, the worker bee has rapidly risen to be one of the model organisms to study polyphenism and the basis of the specification of complex social behavior. Given the fact that both females of the honeybee, develop from the same initial genetic conditions, gives the chance to determine molecular and genetic mechanisms involved in the manifestation of caste-specific behaviors.

Sex and caste determination in the honeybee

To uncover the mechanisms underlying the specification of such diverse behaviors, the first step is to understand the genetic basis of sexes in the honeybee. In *A. mellifera* we have a unique form of haplodiploid sex-determination that is regulated by complementary sex determination. Females and males are hereby defined by an allele-dependent system of protein-protein interactions (Fig. 2). While females, both queen and worker bee, originate from diploid eggs and are heterozygous at a the sex determining locus (SDL), males are homo- or hemizygous at the SDL and originate from unfertilized eggs (Beye, 2004; Cook, 1993). The SDL is localized on the third chromosome and includes the primary signal for sex determination in the honeybee, the gene *complementary sex determiner* (*csd;* Beye et al., 1999, 2003).

Femaleness is defined by a heterozygous conformation of the *csd* gene, which induces female-specific splicing of *feminizer* (*fem*) pre-mRNA that induces the translation of a functional and female-specific Fem protein. The female Fem protein then maintains its own expression with an autoregulatory feedback loop that governs splicing of the fem pre-mRNA to the female-specific pathway (Gempe et al., 2009; Hasselmann et al., 2008). As Fem, comparable to the paralogous Csd protein, does not encode an RNA recognition motif domain it interacts with the Transfromer2 (*Am*-Tra2) protein as cofactor for mediating the feedback loop splicing (Nissen et al.,

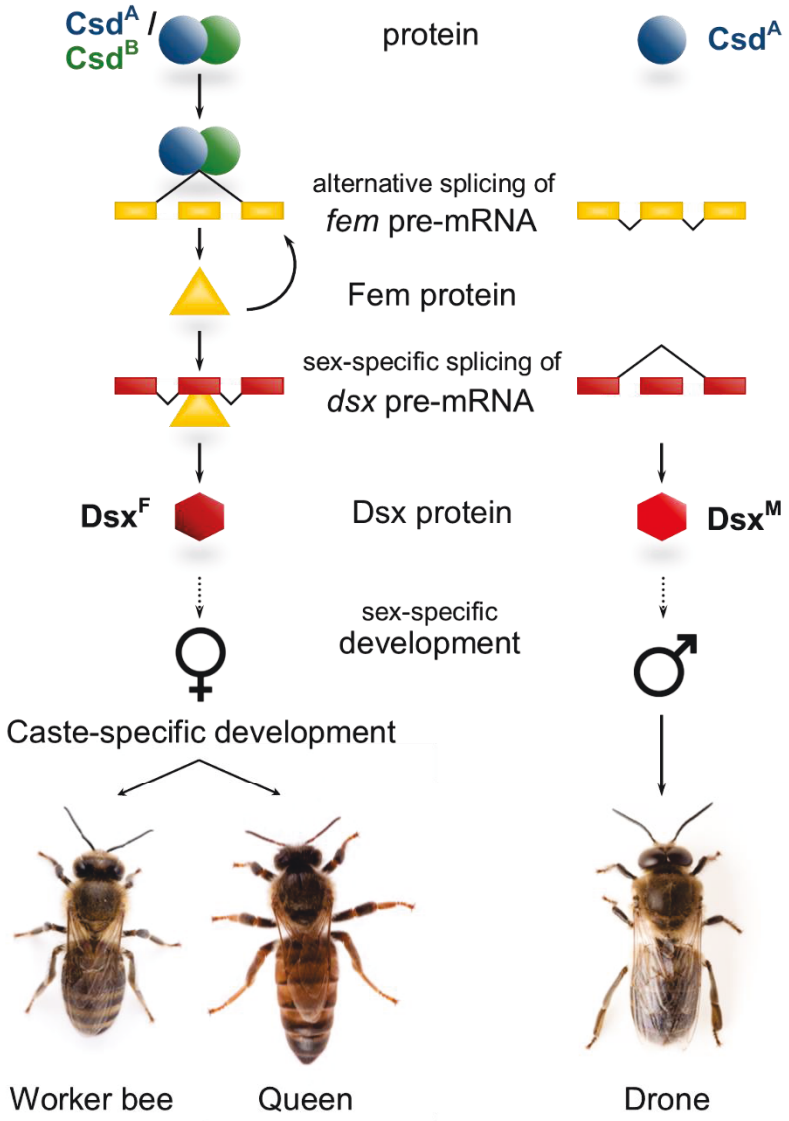

Figure 2: Sex determination in the honeybee *Apis mellifera*. Allelic state of *complementary sex determiner* (*csd*) instructs female or male specification. Sex-specific splicing of *feminizer* (*fem*) induced by heterozygous *csd* conformation. Female Fem protein in conjunction with Transformer2 (Tra2; not displayed) uses autoregulatory feedback splicing loop. Fem splices *dsx* pre-mRNA in females. Due to lack of functional Fem protein in males, no sex-specific splicing in males. Sex specific female (Dsx^F) and male (Dsx^M) Dsx isoforms. Female queen and worker bee origin from identical genetic conditions, hence an additional caste-determining-signal is necessary.

2012). This female-specific *Am*-Tra2-*Fem* complex also regulates the splicing of the downstream target *doublesex* (*Am-dsx*), resulting in a female-specific *Am-dsx*^{F} isoform. Sex-specific splicing is further mediated by the female-specific Am-Tra2 protein in conjunction with the Csd protein by transmitting the allelic state of the csd gene, to successfully induce sex-specific splicing. In males on the contrary, the fem pre-mRNA is not spliced due to the hemi zygotic allelic state of the *csd* gene, leading to an alternative stop codon and resulting in a male-specific and non-functional Fem protein (Hasselmann et al., 2008). With the lack of a functional Fem protein the default splicing of *Am-dsx* pre-mRNA is induced, resulting in a male-specific *Am*-Dsx^{M} protein (Biewer et al., 2015; Burtis & Baker, 1989; Hasselmann et al., 2010). Dsx is thereby the last gene in the initial sex determination cascade acting as bottom master switch for sex-specific development.

In terms of the described sex determination cascade, honeybee embryos are female or male, however females further develop either as worker or queen. Therefore, additionally to the sex differentiation signal a signal for caste differentiation needs to be integrated. While this signal has been found to be triggered by caste-specific nutrition provided during larval development (Haydak, 1970; Weaver, 1955), the genetic underpinnings of the caste-specific regulations and development have yet to be uncovered. Remarkably, until the age of three days (L3) female larvae are bipotent and can develop either into a queen or a worker bee (Shuel & Dixon, 1960; Weaver, 1957). While worker-destined larvae receive royal jelly only about three days and then are fed with restricted amounts of worker jelly (Kaftanoglu et al., 2011; Lindauer, 1952; Rembold et al., 1980), queen-destined larvae receive abundant feeding of royal jelly, resulting in continuously increased sugar levels (Haydak, 1970; Kaftanoglu et al., 2010, 2011). These nutritional differences manifest in an increased level of juvenile hormone (JH) in queens when compared to worker bees (Hartfelder & Engels, 1998). The following caste-specific development has been proposed to be due to several differential expressed genes in response to the different amount of royal jelly and JH (Barchuk et al., 2007; Maleszka, 2018). The affected genes can be described as “master genes” and “effector genes” (de Paula Junior et al., 2020). Master genes code for the insulin/IGF signaling (IIS), target of rapamycin (TOR) JH-pathways that are involved in general body growth and are associated with elevated levels of JH and are thereby directly affected by the nutritional input (Maleszka, 2018; Patel et al., 2007; Wolschin et al., 2011). Effector genes on the other hand act further

downstream and are directly affected by the activity of the master genes. As mentioned above, queens and worker develop caste-specific morphological adaptions, like the ovaries (Capella & Hartfelder, 1998; Lago et al., 2016), leg combs (Bomtorin et al., 2012) and glands (Crailsheim & Stolberg, 1989; Robinson, 1992), which are most likely caste-specifically mediated by such master or effector genes. It has been shown that female brains develop caste-specific, and that first developmental differences can be detected as early as in the third larval stage (Groh & Rössler, 2008; Moda et al., 2013). However, adult queen and worker bees have brains of the same size, but as the queen is larger, worker bees have a proportional larger brain. Most interestingly further studies found, that the antennal lobe and mushroom bodies, known to be the main sensory integration centers of the honeybee brain, have a higher volume in worker bees compared to queens (Groh & Rössler, 2008). This strongly indicates that a caste-specific developmental pathway also navigates the formation of the female nervous system.

Taken together this confirms that next to sex determination, the female polyphenism found in the honeybee is governed by vital molecular and genetic mechanisms, that are activated by external as well as internal factors. Unfortunately, to this date are researchers unable to assign specific queen or worker traits to a key gene, that acts as potential caste-development regulator. Studies like this would greatly help, uncovering the mysteries about how the female polyphenism is genetically defined and would shed light on the evolution of social behavior.

doublesex – a gene with a central role in sex-specific development

As bottom master switch in the sex determination cascade, the gene *dsx* is known to regulate sex-specific differentiation. Could *dsx* also play a role in the regulation of caste-specific development? The regulation of sex determination trough the *fem* (or *tra*)/ *dsx* complex has been conserved in insects (Diptera and Hymenoptera) for more than 280 million years (Biewer et al., 2015). Moreover are the two functional DNA binding and oligomerization domains of the Dsx protein highly conserved among species and the sex-specific splice variants have been shown to affect sexual development (Biewer et al., 2015; Burtis & Baker, 1989; Cho et al., 2007). The role of the *dsx* gene in sexual differentiation is most intensively studied in the fruit fly *Drosophila melanogaster* (*Dm-dsx*). The influence of *Dm-dsx* activity on sexual

development has been shown on many levels. Examples are the sex-specific pigmentation of the abdomen (Rideout et al., 2010; Williams et al., 2008), the male-specific sex combs on legs (Devi & Shyamala, 2013; Rice et al., 2019), the correct sexual identity of the fat body (Coschigano & Wensink, 1993) or the gonads (Camara et al., 2019). In other insects the *dsx* gene has been found to regulate similar sex-specific differentiations. In the horned beetle (*Onthophagus taurus*) for example, the development of male-specific enlarged head horns is promoted by male-specific *Ot*-Dsx^M in males and inhibited in females by *Ot*-Dsx^F activity (Kijimoto et al., 2012). Studies in the silk moth *Bombyx mori* demonstrated that a lack of functional Dsx protein leads to the development of degenerated testes and ovaries, that appear similar in both male and female mutants (Xu et al., 2017). Similar intersex reproductive organs and smaller body sizes were found in *Nl-dsx* mutants of the brown planthopper (*Nilaparavata lugens;* Zhuo et al., 2018). Considering the high impact of the Dsx protein activity on sexual differentiation, it is not surprising that further studies confirmed that not only morphological development but also the capacity to perform specific behaviors is instructed by Dsx activity. More precisely, in *D. melanogaster* is the capacity to perform sex-specific behavior instructed by a sex-specific Dsx- and Fru-positive neuronal cluster. This cluster is critical for regulating several dimorphic behavioral traits, such as correct courtship song production in males (Kimura et al., 2008; Rideout et al., 2007) or sexual receptivity of females (Rideout et al., 2010; C. Zhou et al., 2014). For the plant hopper *N. lugens* similar effects were found, as males with a lack of Dsx protein had difficulties producing a normal courtship signal (Zhuo et al., 2018).

In terms of the effects a loss of function of Dsx protein causes, the question arises how does this gene affect and regulate these specifications of sexual dimorphic behavioral and morphological traits? The answer is encoded in a domain, enabling the Dsx protein to interact with DNA and act as transcription factor in a sex-specific manner. The DM (Doublesex/MAB-3) domain, first discovered in Dsx proteins in *D. melanogaster* and *C. elegans*, binds its target DNA as a dimer, using an oligomerization domain (OD1) to form most likely homo- or heterodimeric complexes (Zhu et al., 2000). DNA is hereby targeted using a unique zinc finger motif consisting of two intertwined CCHC and HCCC Zn^{2+}- binding sites (Zhu et al., 2000). Early on studies demonstrated that mutations within the zinc-binding site lead to intersex phenotypes (Erdman et al., 1996; Hildreth, 1965). The DM domain is identical in both

female and male, indicating that the Dsx^M as well as Dsx^F protein bind to identical DNA targets (Clough et al., 2014; Erdman & Burtis, 1993; Erdman et al., 1996; Zhu et al., 2000). Due to this fact, the sex-specific regulatory activity found in Dsx proteins, is thought to be regulated by a second oligomerization domain (OD2) localized at the c-terminal end of Dsx (Erdman et al., 1996). The OD2 is spliced sex-specifically causing the female and male *dsx* transcripts to have different lengths. The female OD2 is with 27 aa shorter than the male OD2 with 110 aa. This in turn means, that the proteins Dsx^F and Dsx^M can target the same genes, but have different regulatory effects on them (Arbeitman et al., 2004, 2016; Bayrer et al., 2005; Lebo et al., 2009). In *D. melanogaster*, the Dsx protein interacts with genes involved in the specification of sexual dimorphisms, for example the egg *yolk protein 1* (Burtis et al., 1991; Coschigano & Wensink, 1993), *bric-abrac* (Williams et al., 2008) and *flavin-containing monooxygenase-2* (Luo & Baker, 2015) and while expression of these genes is upregulated in females, it is downregulated in males. In *Bombyx mori* it has been shown that female-specific *Bm*-Dsx^F induces the expression of *vitellogenin* (*vg;* Suzuki et al., 2003, 2005), a precursor of egg yolk, confirming the findings in *D. melanogaster*, that the *vg* gene is a direct target of the Dsx protein. A recent study in *D. melanogaster*, showed that for correct development of female-specific brain morphology, the female Dsx isoform needs to interact with the Hox gene *abdominal-B* (*abd-B;* Ghosh et al., 2019). As the protein complex of Dsx and AbdB interacts on sex-dimorphic apoptotic enhancers, Ghosh et al. (2019) identified a novel interaction of these transcription factors, that has a high potential also acting on other genes. What makes this study of particular interest is, that it provides evidence that the Dsx protein could generally use homeodomain containing TFs, such as AbdB, as cofactor to regulate almost any kind of tissue and sex-specific development, extremely expanding the regulatory potential of the Dsx protein.

In the honeybee studies using RNA interference to induce a knockout of the *fem* gene generated a complete switch from female to male-development (Gempe et al., 2009). Further analysis in respect of the *dsx* transcript showed, that only the male-splice variant can be detected in these individuals, indicating that femaleness is bound to the activity of a functional female-specific Fem protein and that female-specific splicing of the *dsx* gene is directly affected by that (Gempe et al., 2009). Unfortunately, further studies focusing on the regulatory impact the gene *dsx* has on the sex- and also caste-specific development are missing in the honeybee but are

critical to understand the specification of femaleness in this species. In regard of the female polyphenism observed in the honeybee hive, *dsx* might play an essential role in worker-differentiation as it has the potential of being co-opted to also regulate caste-specific development next to the ancestral role of regulating sex-specific development.

Outline

As early as 1866 a “element” was discovered, that suggested that heritable information of pea plants, such as flower color, are located within the cell (Mendel 1866). It was Wilhelm Johannsen who then named this element a gene (1909), and now 155 years later, we are able to identify genes in about 85.000 species, allowing us to study gene-specific effects on almost any form of developmental outcome. Being able to directly connect the activity of a single gene to a specific outcome in morphology or behavior has been a challenge many researchers have taken up on. Due to the honeybee reproduction system, generating genome-edited bees is especially challenging, as it requires to generate a lot of genetically manipulated queens or even more genetically manipulated worker bees. With the establishment of CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR associated (Cas) systems (CRISPR/Cas), that induces double strand breaks at the desired target sequence we are now in the unique position to target specific genes (Jinek et al., 2012a). Caste-differentiation in the honeybee underlies differential nutrition and still largely unknown genetic and molecular mechanisms that can now be studied using CRISPR/Cas directly and non-invasively targeting genes and analyzing the direct effect of gene manipulation. Caste differentiation in honeybees is not only interesting with respect to the origin of the female dimorphism, for most however also likely plays a key role in the emergence of social traits and division of labor. The gene *dsx* has been identified as one of the key regulators for sexual development in *D. melanogaster* (Rideout et al., 2010; H. Zhou et al., 2021). The highly conserved domains of *dsx* among insect species, suggest similar regulatory mechanisms for the honeybee sexual differentiation. Or that for example Fem or Tra2, that directly target the gene *dsx*, may have similar effects on sex-specific development in honeybees. The overall question that comes up when trying to understand female honeybee development is: where and when is caste differentiation specified?

To answer this question, the first part of my thesis will focus on whether nutrition is the only factor responsible for the morphological development of queen and worker bees or if morphological specification is mediated by genetic regulation from the sex determination cascade. Caste is first differentiated by nutrition (Asencot & Lensky, 1988; Haydak, 1970) and a possible mechanism to sense the nutritional stimuli could be governed by the IIS/TOR pathways, which influence growth during development (Colombani et al., 2003; Ikeya et al., 2002). However, it is unclear whether this pathway affects caste-differentiation or if the IIS-/TOR-pathway is activated due to a queen- or worker-developmental program. In the horned beetle it was shown that the male horn size is directly affected by nutrition, indicating a link to genes with sex-specific activity (Kijimoto et al., 2012). I will use CRISPR/Cas9 to manipulate genes of the sex determination cascade in female embryos and following will rear them in-vitro on different nutrition regimes. Following I will analyze the morphological development in detail to uncover the role of sex-specific genes in caste differentiation and how nutrition is involved.

In the second part of my thesis, I try to unravel the enigma of how worker behavior is genetically specified in the worker honeybee. The behavior displayed features a wide range of very flexible and altruistic tasks exclusively done by worker bees (Johnson, 2008a). Characteristics unique for the worker bee must be somehow genetically programmed during development in form of caste-specific morphological and neuronal differentiation. I will combine two powerful tools, CRISPR/Cas9 and computer-based bee behavior tracking (Blut et al., 2017) to analyze the behavior of genetically manipulated worker bees of the nurse stage. I choose to target the gene *dsx* for this part of my thesis, as its role in regulating sexual differentiation on the behavioral level has been demonstrated in other species (Rideout et al., 2010; Zhuo et al., 2018). Mutated worker bees monitored on an individual level in a hive-like environment will bring substantial insights into how this gene affects the specification of worker bee behavior. This will shed light on the hypothesis whether sex-determination genes have been co-opted for caste determination or if other genetic cascades or nutritional triggered machineries induce caste specification. Histological analyses of the mutant worker brains will further determine the role of the gene *dsx* during neuronal development of the honeybee.

CHAPTER II

Manuscripts

Manuscript I

A genetic switch for worker nutrition-mediated traits in honeybees

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Abstract

Highly social insects are characterized by caste dimorphism, with distinct size differences of reproductive organs between fertile queens and the more or less sterile workers. An abundance of nutrition or instruction via diet-specific compounds has been proposed as explanations for the nutrition-driven queen and worker polyphenism. Here, we further explored these models in the honeybee (*Apis mellifera*) using worker nutrition rearing and a novel mutational screening approach using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) method. The worker nutrition-driven size reduction of reproductive organs was restricted to the female sex, suggesting input from the sex determination pathway. Genetic screens on the sex determination genes in genetic females for size polyphenism revealed that *doublesex* (*dsx*) mutants display size reduced reproductive organs irrespective of the sexual morphology of the organ tissue. In contrast, *feminizer* (*fem*) mutants lost the response to worker nutrition-driven size control. The first morphological worker mutants in honeybees demonstrate that the response to nutrition relies on a genetic program that is switched “ON” by the *fem* gene. Thus, the genetic instruction provided by the *fem* gene provides an entry point to genetically dissect the underlying processes that implement the size polyphenism.

Author summary

In honeybees, nutrition drives dimorphic size development of reproductive organs in fertile queens and sterile workers. The first induced morphological mutants in honeybees demonstrate that this developmental plasticity requires a genetic program that is switched “ON” by the *feminizer* (*fem*) gene.

Introduction

Highly social insects are characterized by caste dimorphism, with morphologically and physiologically distinct reproductive queens and more or less sterile workers (Evans & Wheeler, 2000; Simpson et al., 2011; Trible & Kronauer, 2017). In honeybees, the development of two distinct phenotypes is controlled by different nutrition, and it is a prominent example of developmental plasticity and polyphenism (Maleszka, 2018; West-Eberhard, 2003). One major concern for the study of caste development involves explaining how a usually sterile worker and a queen that lays up to 2,000 eggs per day develop from different diet and feeding regimens (Buttstedt et al., 2016; Corona et al., 2016; Maleszka, 2018). Worker-destined larvae receive restricted amounts of a reduced sugar content diet (worker jelly [WJ]), while queen-destined larvae receive large quantities of a sugar-rich diet (royal jelly [RJ]; Asencot & Lensky, 1976, 1988; Haydak, 1970; Leimar et al., 2012). WJ and RJ drive the development of female larvae in two distinct morphs. Workers have a five-day longer developmental time, lower body mass, two small ovaries containing few ovarioles, and mid- and hind-leg structures adapted for pollen collection and transport. Queens have a five-day shorter developmental time, larger body mass, and two large ovaries that contain many more ovarioles, and they lack the pollen collection structures on the legs. Two types of models have been proposed to explain how diets and feeding regimens mediate worker/queen development. The Nutrition/Growth model suggests that queen/worker development is driven by the amount of food and balance of nutrition (Buttstedt et al., 2016; Leimar et al., 2012; Rembold & Lackner, 1981), which modulate a developmental program. Queen-destined larvae have abundant nutrition, and organ growth is only limited by the intrinsic program. Worker-destined larvae have a shortage of nutrition that restricts growth and influences metabolic parameters accordingly. In contrast, the Instruction model proposes that the RJ has a compound (or compounds) that instruct the development of queens (Kamakura, 2011; Rembold et al., 1974; Von Rhein, 1933). In support of the Instruction model, research over the past decades has attempted to identify a single compound from RJ (Rembold et al., 1974; Rembold & Lackner, 1981; Von Rhein, 1933) that can determine queen development. A recent study provided evidence that the protein royalactin has queen-determining activity (Kamakura, 2011). However, follow-up experiments in another laboratory were unable to repeat these results (Buttstedt et al., 2016), questioning the existence of a single determinant for queen

development [4]. Gradually increasing the sugar levels of WJ and altering the composition of RJ-containing diets produced workers, intercastes, and eventually queens (Asencot & Lensky, 1976, 1988; Kaftanoglu et al., 2011; Leimar et al., 2012), but it failed to rear only queens. The more continuous caste characteristics resulting from different feeding regimes (Nijhout, 2003) have been proposed in support of the Nutrition/Growth model. The RJ and the WJ produce different reaction norms of the general developmental program that determines the caste polyphenism. An alternative explanation is that the essential higher sugar levels for queen-destined larvae are a secondary effect and reflect the higher energy requirements for the faster and larger-growing queen organs of an otherwise instructed queen program. The rearing of larvae at day 5 in queen-less colonies yielded bees with ovariole numbers that were discontinuous (either more worker or queen-like distributed), while other queen and worker traits were either absent or present in a noncorrelated fashion (Dedej et al., 1998), suggesting two distinct states of the developmental program and the possible existence of regulatory switches (Gempe et al., 2009). One possible mechanism by which nutrients are sensed by bee larvae is the insulin/IGF signaling (IIS) and target of rapamycin (TOR) pathways, which link the abundance of nutrition with worker and queen differential gene expression (Patel et al., 2007; Wang et al., 2013; Wheeler et al., 2006; Wolschin et al., 2011). Indeed, nutritional input can also influence growth and metabolic programs via the IIS and TOR pathways in mammals and other insects (Colombani et al., 2003; Ikeya et al., 2002; Slaidina et al., 2009). However, whether regulation of the IIS and TOR pathways drives caste differentiation or whether the regulation is a response to the activation of a queen develop-mental program is currently unknown. Consistent with the faster and larger growth of queens, gene expression studies have revealed the upregulation of physio metabolic genes in queens, reflecting their higher metabolic rate (Barchuk et al., 2007; Cameron et al., 2013). Chromatin modifications and DNA methylation analyses have indicated distinct epigenetic states in worker- and queen-destined larvae, suggesting another level of regulatory control associated with caste-specific gene expression (Foret et al., 2012; Kucharski et al., 2008; Wojciechowski et al., 2018). Here, we explored whether nutrition is the only factor directing size polyphenism and whether further genetic instruction from the sex determination pathway is required. To do so, we introduced a method to screen mutations directly

in worker bees using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technique.

Results

Worker nutrition is not a general driver for the reduced size of reproductive organs

According to the Nutrition/Growth model, nutrition is the only driver of reduced reproductive organ size, the most prominent trait in caste development. Males, like queens, receive high amounts of sugar during larval development (Mandla & Kumar, 2016) and develop large reproductive organs unlike sterile worker bees. Gradually increasing the sugar levels of WJ produces intercaste development (Asencot & Lensky, 1976, 1988; Kaftanoglu et al., 2011). Hence, if a shortage of nutrition in the worker diet (and reduced sugar levels) is the only driving component, we would expect that this diet would also mediate the size reduction of reproductive organs in males.

We manually reared genetic females and males on worker nutrition (Kaftanoglu et al., 2010, 2011) and compared their phenotypes with those of workers and genetic males reared in the colony (Fig. 1, Table S1 and S2). The reproductive organs of genetic female bees raised on worker nutrition either inside the colony (n = 14) or manually outside (n = 15) were equivalent in size (Fisher's exact test, df = 1, P = 1). In both laboratory- and colony-reared genetic females, there were few ovarioles, and the size of each ovary was small compared with the size of the heads (Fig. 1 and Table S1). This contrasts with the large ovaries of the female larvae fed a queen diet in the hive (queens alone cannot be consistently reared under laboratory conditions

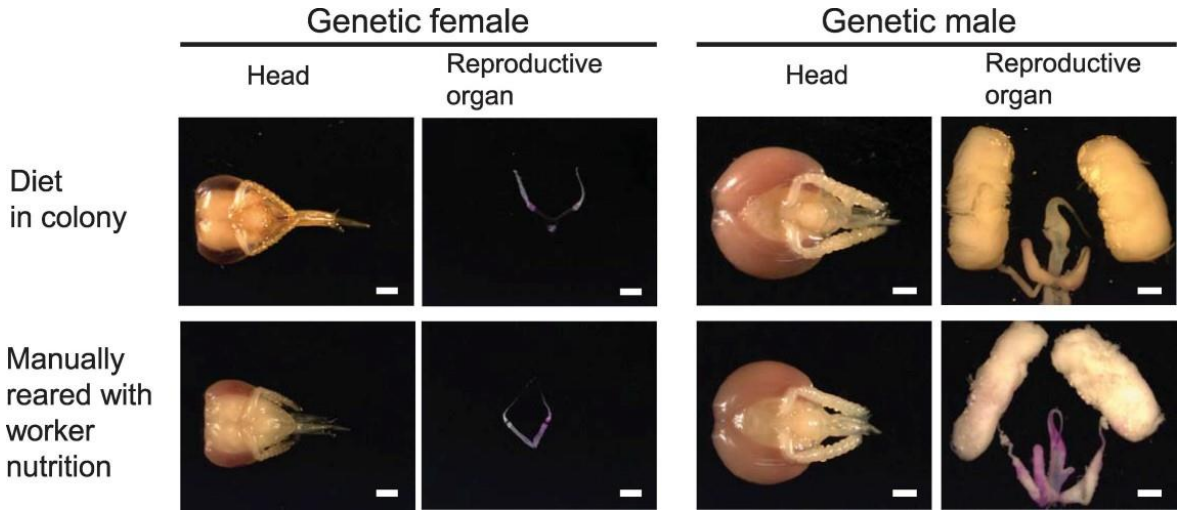


Figure 1. Reproductive organ and head phenotypes of females and males reared on worker nutrition in the laboratory and in the colony. Scale bar = 1 mm.

(Buttstedt et al., 2016); see Fig. 4A and 4B as an example of a queen phenotype). This result indicates that our manual feeding regime mirrors the effect of a worker diet in the hive (Kaftanoglu et al., 2010, 2011). To examine whether only the balance and amount of nutrition (low amount of sugar) determine small reproductive organs, we reared genetic male larvae on worker nutrition in the laboratory and compared these with males that received high amounts of sugar in the colony (Mandla & Kumar, 2016). Genetic males that were reared on the worker nutrition diet had large male reproductive organs (Fig. 1 and Table S2). They were equivalent in size (n = 20) to the males obtained from the colony (n = 8) that were reared on drone nutrition (Fisher's exact test, df = 1, P = 1). These results indicate that worker nutrition (and a shortage of sugar) is not the only requirement for the size polyphenism, suggesting input from the sex determination pathway.

Somatic and mutational screening in reared bees

We next established a method that enables the mutational screening of sex-determining genes directly in worker bees using the CRISPR/Cas9 method (Jinek et al., 2012a; Kohno et al., 2016; Wiedenheft et al., 2012). Following traditional mutant approaches, we would need to produce mutant queens and drones that need to be crossed to generate double-mutant worker bees. If we could mutate all nuclei in the embryo, we would be able to directly rear mutated worker bees without maintaining colonies and performing crossings. To examine whether we could mutate worker bees entirely using the CRISPR/Cas9 method, we tested different embryonic injection conditions. To determine the robustness of this approach, we studied at least two sites for three genes, the *doublesex* (*dsx*), *fruitless* (*fru*), and loc552773 genes (Fig. S1). Only the *dsx* gene was used later on for phenotyping. We injected into the anterior embryos of very young female embryos (0 to 1.5 hours after egg deposition; Schulte et al., 2014). We tested a set of single guide RNAs (sgRNAs; Table S3) at different concentrations and observed that we repeatedly mutated each injected embryo.

The fragment length (FL) and sequence analyses of the amplicons in larval stage 1 larvae revealed that up to 100 % of the *fru* and *dsx* and 60 % of the loc552773 target embryos were mutated (Table 1, S4, S5 and Fig. 2). The wildtype (WT) allele was consistently not detected in 30 of the 39 mutated larvae (77 %), suggesting that all nuclei (to the level of detection) and both alleles in the larvae were mutated (generating double mutants). More than two mutated sequence variants were detected in a single larva (3 %), while singly mutated sequences together with the WT allele were detected in 8 larvae (20 %; Table S4 and S5). Indels occurred most frequently between the 5 bp to 1 bp range, with 44 % of mutations being deletions and 20% resulting in insertions (Table S5 and S6). All mutations occurred at the designated target site. Therefore, our results on the adjustments demonstrate that nearly 80 % of the injected embryos had mutations on both alleles (double mutants) affecting the bee entirely (absence of mosaicism). This high proportion enabled us to screen for mutant effects of the sex-determining genes directly in the injected bees.

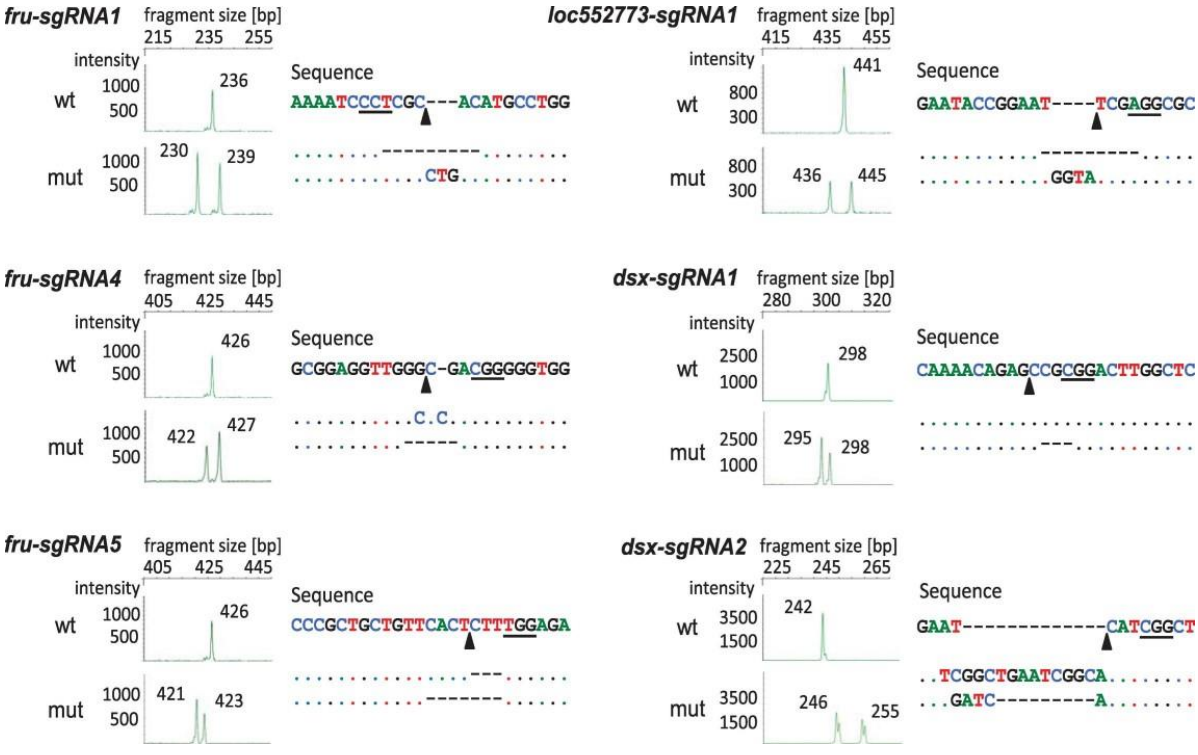


Figure 2. Examples of FL and nucleotide sequence analyses of the targeted genomic sites of single bees using the efficient CRISPR/Cas9 method. FL analysis is presented on the left, and the nucleotide sequences are presented on the right for single bees. Examples of WT alleles and mutated sequences are shown. The cleavage site of the Cas9 protein is indicated with arrows. The PAM site (the essential targeting component for CRISPR/Cas9) is underlined in the nucleotide sequence. Dashes indicate deletions. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; FL, fragment length; mut, mutated sequences; PAM, Protospacer adjacent motif; WT, wildtype.

Table 1. Frequency of the mutated honeybee larvae based on FL analyses at single base-pair resolution of the amplicons.

Treatment	pg of Cas9 mRNA per embryo	pg of sgRNA per embryo	No. of surviving embryos 24 after injection	No. (%) of hatched L1 larvae	No. geno-typed larvae	No. of larvae with length variant[1]	Effi-ciency of mutage-nesis[2]
***fru*-sgRNA1**	800	29.2	105	10 (10 %)	8	2	20 %
***fru*-sgRNA2**	400	14.6	467	72 (15 %)	7	6	86 %
***fru*-sgRNA1**	240	8.8	78	2 (3 %)	2	2	100 %
***fru*-sgRNA4**	400	14.6	125	3 (2 %)	3	3	100 %
***fru*-sgRNA5**	400	14.6	98	10 (10 %)	10	10	100 %
loc-sgRNA1[3]	400	14.6	93	7 (8 %)	5	3	60 %
loc-sgRNA2	400	14.6	102	31 (31 %)	28	1	4 %
***dsx*-sgRNA1**	400	5.5	52	1 (2 %)	1	1	100 %
***dsx*-sgRNA1**	400	3.7	93	5 (5 %)	4	1	25 %
***dsx*-sgRNA2**	400	5.5	178	2 (1 %)	2	2	100 %
***dsx*-sgRNA2**	400	3.7	89	5 (6 %)	5	5	100 %
***dsx*-sgRNA2**	400	0.7	52	21 (26 %)	19	3	16 %
H_2O	-		48	37 (56 %)	11	0	0 %
Non-injected	-		65	55 (85 %)	19	0	0 %

[1]Fragments differed in length compared with fragments isolated from 7 nontreated (WT) larvae.
[2]Realtive ratio of the number of mutant larvae to the number of all larvae.
[3]Targeted the gene loc552773.
Abbreviations: Cas9, CRISPR-associated protein 9; FL fragment length; pg, picogram; sgRNA, single guide RNA; WT, wildtype.

The *feminizer* gene is required for small size polyphenism

To examine whether the *feminizer* (*fem*) gene is required for small size polyphenism, we mutated the gene in genetic females and reared them with worker nutrition. The *fem* gene instructs female development and maintains the female signal during development, as revealed from *fem* interference RNA (RNAi) knockdown and mosaic studies using a non–worker- specific diet for bee rearing (Gempe et al., 2009; Hasselmann et al., 2008). The *Fem* protein is encoded by female-specific spliced *fem* transcripts but not the male spliced variant, which harbors an early stop codon (Schulte *et al.*, 2014; Fig. 3A). The female splicing of *fem* is directed by the *complementary sex determiner* (*csd*) gene when the genotype is heterozygous (Beye *et al.*, 2003; Fig. 3A). If the *fem* gene is required for small size polyphenism, we would expect that worker nutrition cannot drive size reduction when *fem* is

inactive. If the *fem* gene is dispensable, worker nutrition would drive size reduction even when the *fem* gene is inactive.

We induced mutations at two target sites in the first half of the female open reading frame (ORF) of the *fem* gene with *fem*-sgRNA1 and *fem*-sgRNA2 (Fig. S1 and S2) and reared genetic females with worker nutrition to larval stage 5. Fifteen percent of the reared and injected genetic females (heterozygous for the *csd* gene; Table S7) were double mutants for nonsense mutations as revealed from the sequenced amplicons (Table S8 and Fig. S2). These double mutants (n = 4) had large gonads (Fig. 3B and 3D) compared with the small gonads of WT genetic females reared on worker nutrition (n = 38, Fisher's exact test, df = 1, $P < 0.001$, S9 Table). The large gonads in the mutants were of the male type. They consisted of packed layers of multiple testioles of the same size as those of the males reared on worker nutrition (Fig. 3B) and those of the males in the colony (Fig. 1). The female *fem* mutants lost the female *dsx* transcript and only displayed the male *dsx* transcript (Fig. 3C), demonstrating that the mutant bees entirely switched in their development from female to male identity. These results indicate that *fem* is required for size polyphenism or that size polyphenism relies on the intrinsic program of the female differentiating tissue induced by *fem*.

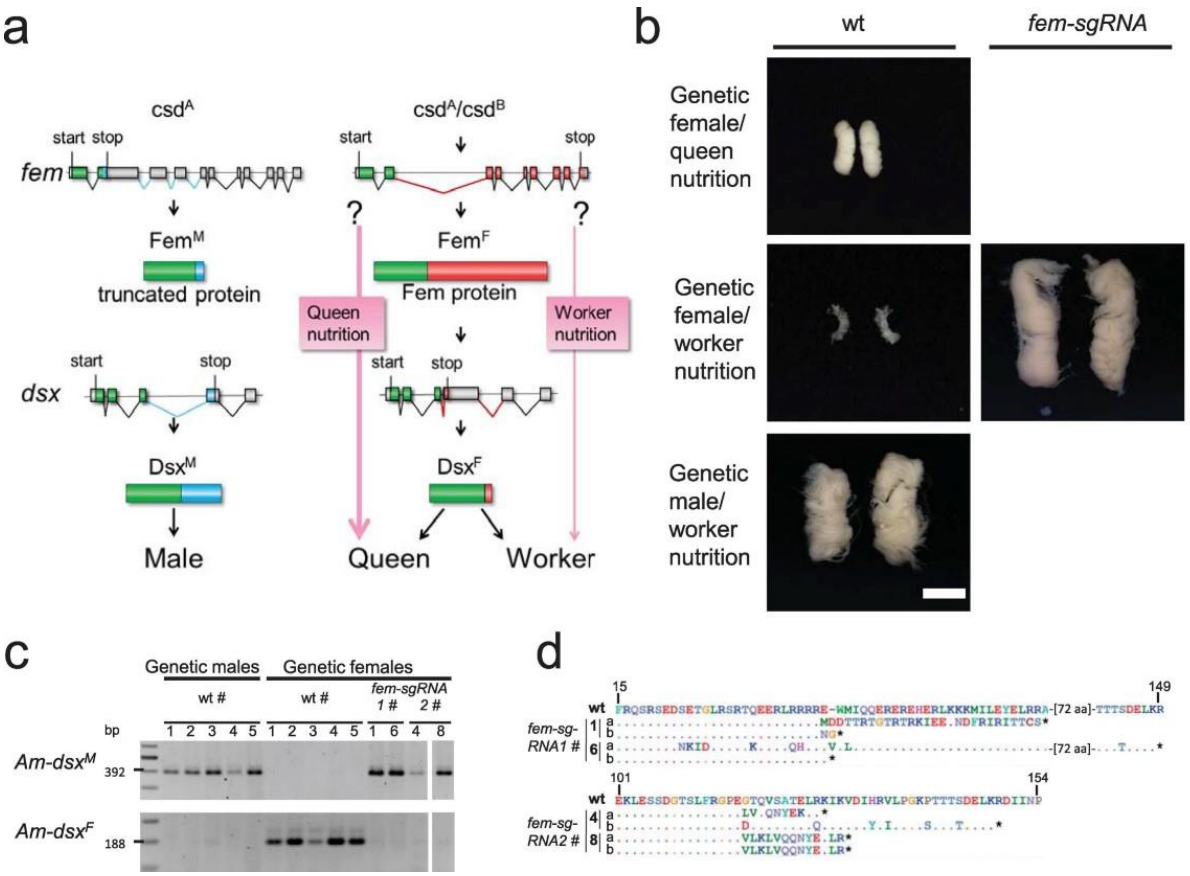


Figure 3. Size polyphenism of gonads in genetic females at larval stage 5 that were double mutants for the *fem* gene. (a) Model of the known components of the sex- determining pathway in honeybees with nutritional differences in females. (b) Gonad development at larval stage 5. (Right) A pair of large gonads (male type) from *fem* sgRNA2-treated genetic females reared on worker nutrition. The gonads display densely packed layers of folded testioles, similar to those observed in haploid males (WT males). (Left) Pairs of small gonads (female type) from WT workers and genetic female bees reared on worker nutrition. AWT large queen ovary from a queen reared in a colony on queen nutrition. A large WT testis of a haploid male manually reared on worker nutrition. (c) Male *dsx* (*dsx*^{M}) and female *dsx* (*dsx*^{F}) transcripts in mutated genetic females with male phenotypes (*fem*-sgRNA1 or *fem*-sgRNA2). Male and female transcripts were separately amplified by RT-PCR, and the male and female fragments of each single bee were resolved via agarose gel electrophoresis. Numbers indicate different control and mutated bees. (d) Deduced amino acid sequences from sequenced amplicons of the *fem* gene at the designated CRISPR/Cas9 cleavage sites for the four worker-nutrition-reared genetic female larvae with large gonads of the male type. Stars indicate premature translation stop codons. Numbers indicate different mutated bees. Scale bars, 1 mm. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; *dsx*^{F}, female *dsx*; *dsx*^{M}, male *dsx*; RT-PCR, reverse transcription PCR; sgRNA, single guide RNA; WT, wildtype.

dsx is dispensable for small size polyphenism

To examine the role of female *dsx* on size polyphenism of the reproductive organ, we mutated the *dsx* gene in genetic females and reared them on worker nutrition. If *dsx* is dispensable, we would expect small size polyphenism even when *dsx* activity is compromised. In Drosophila melanogaster, the *dsx* gene essentially controls, beside the reproductive organs, all aspects of somatic sexual differentiation (Cline and et al., 1996; Williams & Carroll, 2009), and it controls at least reproductive organ development in other insects that belong to different insect orders, including hymenopteran insects (Hediger et al., 2004; Mine et al., 2017; Shukla & Palli, 2012; Suzuki et al., 2005). The *dsx* transcripts in honeybees are sex-specifically spliced by the presence of the *Fem* protein in females and the absence of the *Fem* protein in males (Gempe *et al.*, 2009; Fig. 3A). The sexual splice variants encode a transcription factor with an intertwined zinc-containing DNA binding (DM) domain and male- and female-specific termini at the carboxyl end (Cristino et al., 2006; Dearden et al., 2006; Matson & Zarkower, 2012; Retschnig et al., 2014; Zhu et al., 2000). We mutated the *dsx* gene at two target sites in the non–sex-specific expressed N-terminal portion. *dsx*-sgRNA2 targeted the DM domain, whereas *dsx*-sgRNA6 targeted a downstream region in exon 3 (Fig. S1). The treated genetic females were reared on worker nutrition and were examined for morphological changes of the reproductive organ and head. Genotyping of the mutated bees with morphological changes via next-generation sequencing (NGS) of the amplicons revealed that they were regularly double mutants with an approximate ratio of 1:1, suggesting that the mutations belong to the two chromosomes of the diploid set. If we detected more than two sequence variants per bee, we excluded these bees from further phenotype analysis as they were genetic mosaics (e.g., a mosaic of differently mutated cells). Eleven (17 %) of the adult or pupal bees had intersex morphology in the reproductive organs compared with the WT genetic females (Table S10). No effect was observed for the heads. The following mutations were the most common ones in the genetic females: (i) different nonsense mutations that introduced new stop codons at various positions in exons 2 and 3, (ii) deletions of amino acids in the DM domain mainly the histidine codon at amino acid position 68 (ΔH68), and (iii) deletion of the alanine codon (ΔA191) at amino acid position 191 (Fig. 4 with the deduced amino acid sequences and Fig. S3 with the detected

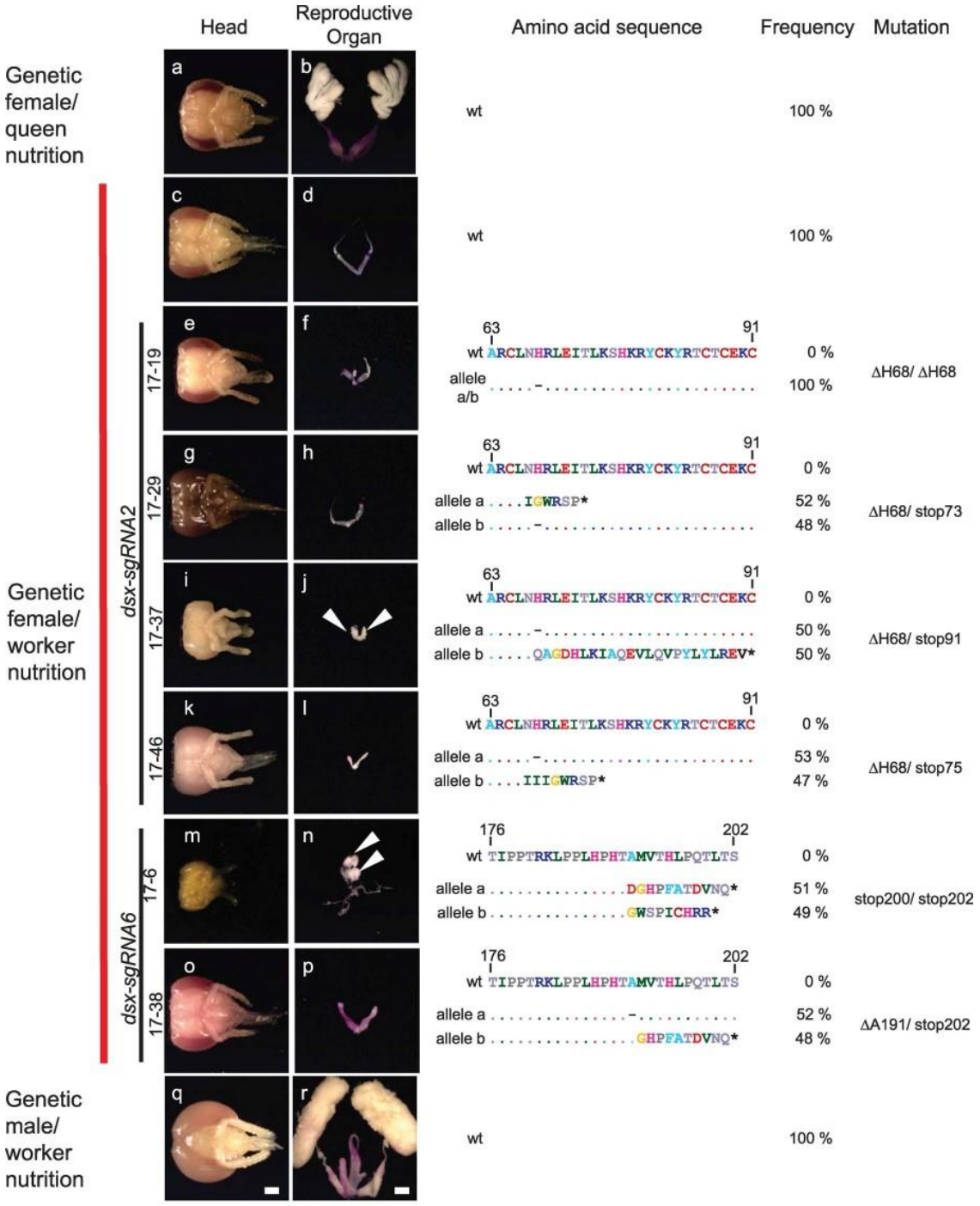


Figure 4. Size polyphenism of the reproductive organs in genetic female double mutants for the *dsx* gene. Pictures of the head and internal reproductive organs of mutated and WT control bees are shown on the left, while the genotypes at the *dsx* locus with the deduced amino acid sequences are displayed on the right. Mutated and control genetic females and males were reared on worker nutrition. Queens were reared on the queen diet in a colony (we cannot mimic queen rearing in the laboratory). The WT amino acid sequence is shown above the detected alleles for comparison. (a, b) WT genetic female reared on queen nutrition (RJ) in the colony. (c, d) WT genetic females manually reared on worker nutrition. (e-l) Genetic females reared on worker nutrition that were double mutants for *dsx* via the *dsx*-sgRNA6 (note that a small part of the worker bee heads 17–39 [i] is missing due to the dissection process). (m-p) Genetic females reared on worker nutrition that were double mutants for *dsx* via the *dsx*-sgRNA2. (q, r) Genetic males manually reared on worker nutrition. Organs were stained with aceto-orcein (reddish coloring) to facilitate the dissection process. Testis tissues are marked with arrows. Scale bar, 1 mm. Dashes in the sequence indicate deletions, and stars illustrate early translational stop codons. RJ, royal jelly; WT, wildtype.

nucleotide sequences). The ΔH68 mutation removes a histidine of the DM domain that is essential for the zinc binding and DM domain functions (Murphy et al., 2015; Zhu et al., 2000) and that is conserved between vertebrates and invertebrates (Fig. S4). The intersex reproductive organs were all of the same small size (*n* = 11) as the worker reproductive organs in WT genetic females that were manually reared on worker nutrition (*n* = 17, Table 2, Fisher's exact test, df = 1, *P* = 1). The small intersex reproductive organs displayed either male gonads with poorly or non–sex-specifically differentiated duct systems (*n* = 4), as observed in stop200/stop202 and ΔH68/stop91 genetic females (arrows in Fig. 4 and S5).

The potentially earlier developmental stage of some of these mutant bees cannot explain why these male-like gonads are so small because the distinct size differences of male and worker gonads are also present at earlier pupal stages (Fig. S6). In other cases, the reproductive organs were underdeveloped (*n* = 7), and the oviducts were consistently misshaped while the ovarioles were repeatedly missing, as identified in ΔH68/ΔH68, ΔH68/stop73, ΔH68/stop75, and ΔA191/stop202 genetic females (Fig. 4 and S5). The heads of the mutant genetic females with intersex reproductive organs were all of worker type (*n* = 11, Fig. 4 and Table S10), suggesting that *dsx* is not required for sexual development of the head. The results of the consistently small, intersex reproductive organs with varying degrees of masculinization suggest that *dsx* is not required for size polyphenism.

Table 2. The size of the intersex reproductive organs in genetic females double mutant for *dsx* and reared on worker nutrition.

Sex	Nutrition	Genotype	Reproductive organ	Numbers	Size of reproductive organ[a]	
					< 2.5 mm; < 0.7 times the size of the head width	> 6 mm; > 1.2 times the size of the head width
Genetic female	Manually reared on worker nutrition	*dsx* double-mutants	Intersex	11	11 (100 %)	0 (0 %)
	Queen diet in colony	Wildtype	Worker	17	17 (100 %)	0 (0%)
Genetic male	Manually reared on worker nutrition	Wildtype	Male	16	0 (0 %)	16 (100 %)

[a] Length between the fused left and right part of the reproductive organ to its end in the sagittal plane.

Discussion

Caste polyphenism in honeybees is determined by different nutrition with the size of the reproductive organ as an important trait. Most studies suggest that the balance and amount of nutrition (Nutrition/Growth model) drive the size polyphenism between queens and workers. Our genetic and rearing results now suggest that the response to nutrition relies on a genetic program that is switched on by the *fem* gene. The genetic females with a mutant *fem* gene show large size reproductive organ (large polyphenism), while WT genetic females (Fig. 5A) reared on the same worker nutrition have only small reproductive organs (small polyphenism). Genetic females that have a mutated *dsx* gene (operating downstream of *fem*) do show small reproductive organs (small size polyphenism; Fig. 5A). *dsx* mutants produce intersex reproductive organs and male-like gonads that are all of small size, demonstrating that small size does not rely on female development of the tissue. The small size polyphenism also did not result from *dsx* malfunction because (i) small phenotypes were consistently observed irrespective of the different degrees of *dsx* malfunctions we introduced by missense and nonsense mutations (Fig. 4) and (ii) *dsx* mutations in other insects did not influence the size of the reproductive organs (Hediger et al., 2004; Hildreth, 1965; Xu et al., 2017). Thus, the results together suggest that the *fem* gene is required for the small size polyphenism. We conclude that the *fem* gene must be switched “ON” so that size polyphenism can be executed (Fig. 5B). The essential role of the *fem* gene in small size polyphenism assigns a further key function to the *fem* gene. Previous studies demonstrated that the *fem* gene is also required to (i) induce entire female development in response to the primary signal *csd* (Gempe et al., 2009; Hasselmann et al., 2008) and to (ii) maintain the female signal during development via a positive regulatory feedback loop (Gempe et al., 2009). Whether *fem* also instructs the large size polyphenism of queens needs further functional testing once a queen-only rearing protocol has been developed for the laboratory (Buttstedt et al., 2016).

The genetic instruction via the *fem* gene provides an entry point to dissect nutrition-mediated control. Our results suggest that the *fem* gene switches “ON” the machinery that is required for sensing the worker nutrition and for implementing the size polyphenism. Because the *fem* gene encodes a serine arginine rich (SR)-type protein, the direct targets of the *fem* gene involved in size polyphenism may also be activated by sexual splicing. The *fem*-controlled candidate genes can be functionally

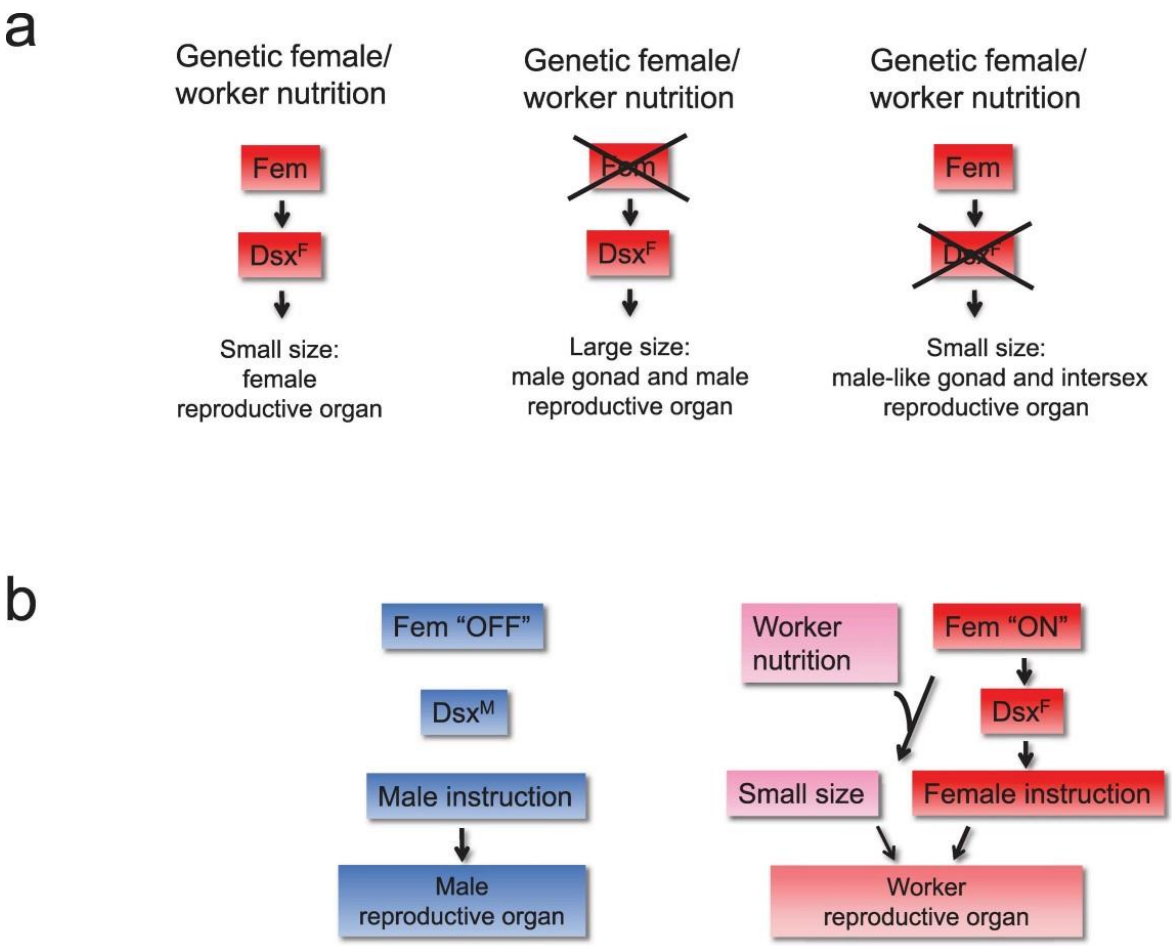


Figure 5. The role of the sex-determining genes *fem* and *dsx* in size polyphenism. (a) Schematic presentation of the mutant effects of *fem* and *dsx* gene on size polyphenism. Genetic female bees reared on worker nutrition produce only small reproductive organs. Genetic females with a mutant *fem* gene show no small size polyphenism of reproductive organs. Genetic females that have a mutated *dsx* (operating downstream of *fem*) do show size polyphenism of the intersex reproductive organ and male-like gonads. Thus, we conclude that the *fem* gene is required for the small size polyphenism. Crosses mark the genes that we compromised using CRISPR/Cas9-induced mutations. (b) The role of the *fem* gene for caste development. The gene products of the sex determination pathway (Fem, Dsx^F, Dsx^M) are shown in red(female) and blue (male) boxes. The nutrition-mediated process is shown in pink. Arrows indicate regulatory relationships. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9.

tested by determining whether they affect the size polyphenism. The function will be directly tested in mutated genetic females as demonstrated in this study.

Our mutant analysis further demonstrates that *dsx* controls female differentiation of the reproductive organs. The mutant honeybee phenotypes of the reproductive organs in honey- bees yielded similar phenotypes as in female D. melanogaster. Female *dsx*-mutant fruit flies have reproductive organs of varying intersex phenotypes. The organs are often underdeveloped with occasionally developed ovaries but are frequently of the "male type" (Bownes et al., 1983; Hildreth, 1965; Schüpbach, 1982). The internal duct system can develop into a mixture of

female/male or single poorly differentiated ducts (Hildreth, 1965). RNAi-mediated knockdown studies on the beetle *Tribolium molitor*, housefly *Musca domestica*, and sawfly *Athalia rosae*, as well as conditional expression and CRISPR/Cas9 experiments on the silkworm *Bombyx mori*, have revealed sex-related effects on internal reproductive organ development (Hediger et al., 2004; Matson & Zarkower, 2012; Mine et al., 2017; Shukla & Palli, 2012; Suzuki et al., 2005; Xu et al., 2017). Our results support a conserved role for *dsx* in the sexual development of the reproductive organ. However, in honeybees there is a nutrition-driven size control of reproductive organ development that operates upstream of or in parallel with *dsx*-regulated sexual development.

The first CRISPR/Cas9-induced morphological mutants in honeybees introduced a new genetic screening method for worker bees. We efficiently induced mutations in injected embryos using the CRISPR/Cas9 method (Jinek et al., 2012a; Wiedenheft et al., 2012) and directly screened for somatic mutations in the reared honeybees (somatic mutation approach). Up to 100 % of the embryos were mutated, and mosaicism among the mutated embryos was rare (<10 %). The previous studies in honeybees using CRISPR/Cas9-induced mutations report on 1 out of 2 queens with only 12 % and 2 out of 4 queens with only 5 % and 10 % mutant drone offspring, suggesting that the previously published method has a substantial lower rate and produced strong mosaicism in the queens (Kohno et al., 2016; Kohno & Kubo, 2018). These previous studies generated no worker bees that would require further crossing experiments. With very early embryonic injections (Schulte et al., 2014) and a selection step to identify the most efficient sgRNAs and Cas9 concentrations, we generated mutation rates of up to 100 % and no mosaicism in worker bees directly. The rearing of the mutated embryos to worker bees was performed under controlled conditions in the laboratory (Kaftanoglu et al., 2010, 2011). This required no rearing of queens and drones and crossing experiments. The procedure was demonstrated for mutations at two target sites for two genes and their morphological changes (Fig. 3 and 4). The absence of mosaicism and completeness of mutagenesis of this procedure were shown by the results that most mutated bees lost the WT allele (they were double mutants; Fig. 2, 3D and 4) and that double *fem* nonsense mutations produced an entire female to male switch, including *dsx* splice products (Fig. 3C). This somatic mutation approach does not require further crossing experiments and laborious maintenance of hundreds of colonies and therefore offers the prospect of

larger genetic screens in honeybees. In other insects in which somatic mutation approaches have been applied (Mazo-Vargas et al., 2017; Zhang & Reed, 2016), the adults were genetic mosaics in which parts of the butterfly wing were WT while other parts were mutated. Enhancing the efficiency of mutagenesis can thus provide an opportunity for somatically testing gene functions in insects that are not yet genetically trackable.

Methods

sgRNA and mRNA syntheses

Cas9 mRNA was synthesized from the Cas9 gene (Hwang et al., 2013; Vector MLM3613, ID #42251, Addgene, Cambridge, MA) using a linearized plasmid via the T7 promoter and the mMES-SAGE mMACHINE Kit (Ambion, Darmstadt, Germany). mRNAs were polyadenylated using the Poly(A) Tailing Kit (Ambion). Target sites for the sgRNAs were identified via Optimal Tar-get Finder software (http://tools.flycrispr.molbio.wisc.edu/targetFinder/). sgRNAs were 20 nt long with a G nucleotide at the 5´-end. sgRNAs with no off-target effects or with at least three nucleotide mismatches to alternative target sites were selected. sgRNAs were generated via PCR without a template using two overlapping oligonucleotide sequences containing the sequence of the T7 RNA polymerase transcription start site, the gene-specific target site and the Cas9 protein-binding site. sgRNAs were synthesized using a RiboMax Kit (Promega, Madison, WI) according to the manufacturer's instructions. RNAs were purified using the MEGAclear Kit (Ambion).

Microinjections and rearing

Embryos were microinjected 0 to 1.5 hours after egg deposition (Beye et al., 2002; Gempe et al., 2009; Schulte et al., 2014) using 53 mm injection pipettes (Hilgenberg, Malsfeld, Germany). Cas9 mRNA or protein (New England Biolabs, Ipswich, MA) was applied at 400 to 2,000 ng/µl and mixed with sgRNAs using a molar ratio of 1:2 to 1:0.75. The number of injected embryos that hatch can vary greatly between experiments and sgRNAs (5 % to 40 %). Rearing was performed using a mass rearing technique for the worker bees (Kaftanoglu et al., 2010, 2011). Freshly hatched larvae were provisioned only once with the worker larval diet (50 %- 53 % RJ, 4 % glucose, 8 % fructose, 1 % yeast extract, and 30 %- 34 % water), approximately 120 to 170 mg of which was consumed (Kaftanoglu et al., 2010, 2011). The larvae were incubated at 34 ˚C and 90 % humidity until the larval stage 5 or to adults. For pupal rearing we also used a slightly different diet for larvae at stage 5 (50 mg diet 2 [50 % RJ, 12 % fructose, 6 % glucose, 2 % yeast extract, and 30 % water]).

DNA preparation, RNA isolation, and cDNA synthesis

For genotyping, genomic DNA was isolated from freshly hatched L1 or L5 larvae (Hunt & Page Jr, 1995) using the peqGOLD Tissue DNA Mini Kit (VWR, Darmstadt, Germany). RNA was isolated using the TRIZOL method (Thermo Scientific, Braunschweig, Germany), and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Second-strand. cDNA synthesis was performed by adding 10 µl of 10× DNA Polymerase Buffer, 40 U DNA Polymerase I, 0.8 U Ribonuclease H, and 65.68 µl of dH2O to 20 µl of the cDNA first-strand synthesis product. Double-stranded cDNA was purified using the EZNA Cycle Pure kit (Omega Bio-Tek Inc., Norcross, GA).

PCR, sequencing, and FL analysis

All mutant bees were genotyped by sequencing the amplicons of the targeted site. PCR amplifications were performed using standard conditions (Hasselmann & Beye, 2004) and GoTaq polymerase (Promega). Oligonucleotide sequences were synthesized at Eurofins (Ebersberg, Germany). Amplicons were either cloned and sequenced (Sanger sequencing [Eurofins]) or sequenced via NGS. NGS index PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA), and purification of the Index PCR products was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). NGS was performed on an Illumina MiSeq system using the MiSeq Reagent Kit version 2 (500 cycles; Illumina), generating 800,000 paired-end reads with a read length of 2 × 250 bp, resulting in approximately 15,000 paired-end reads per sample. We removed contamination by removing sequences that were less frequent than 5 %. The FLs of hexachlorofluorescein (HEX)-labeled amplicons were determined using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and Peak Scanner software (Thermo Scientific). For the *fem* mutants, we conducted fragment and sequence analysis on the amplicons of the cDNAs to ensure that the many *fem*-related sequences observed at the genomic *fem* locus (derived from duplication events; Koch *et al.*, 2014) were not amplified.

Supporting information

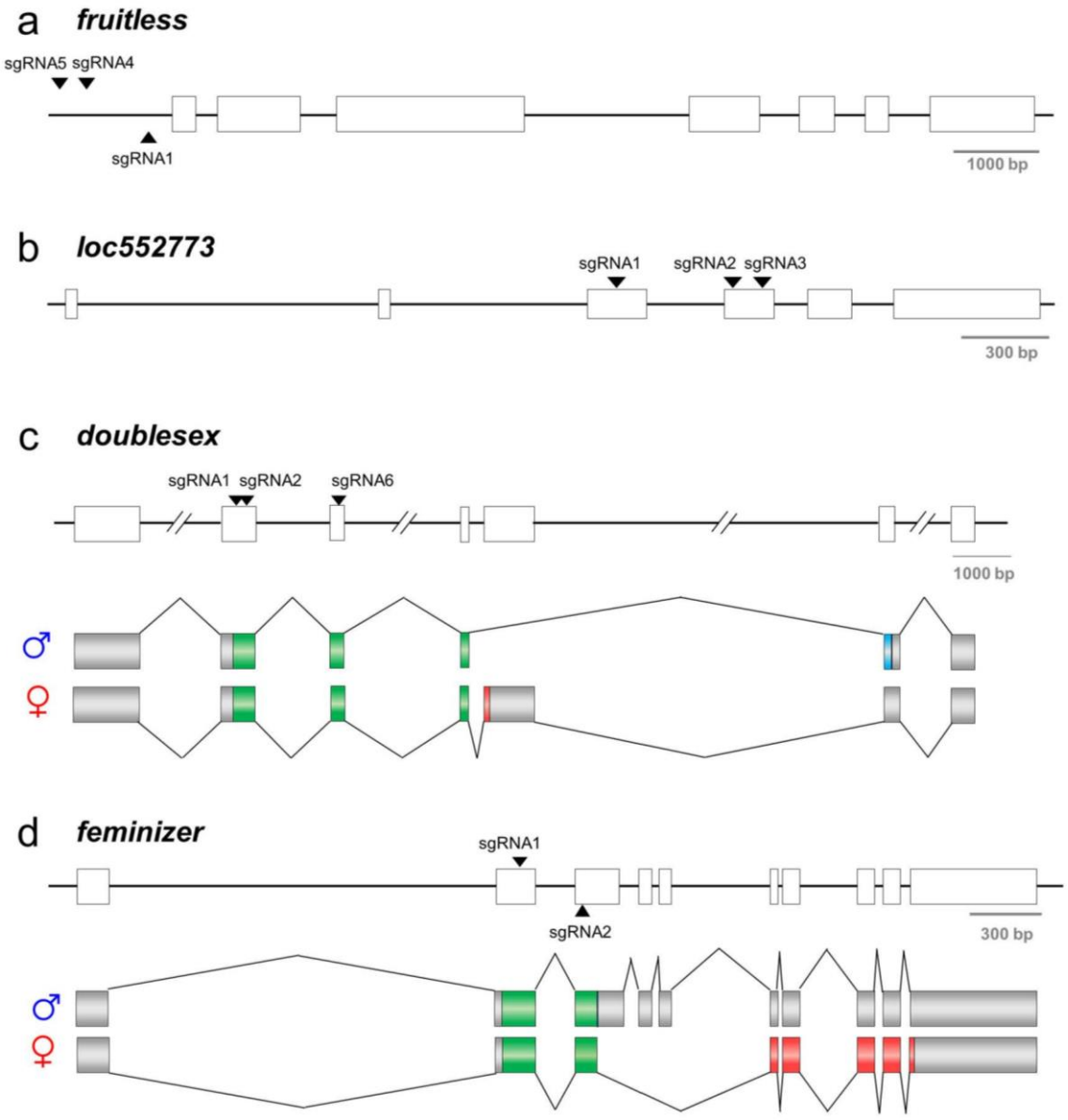
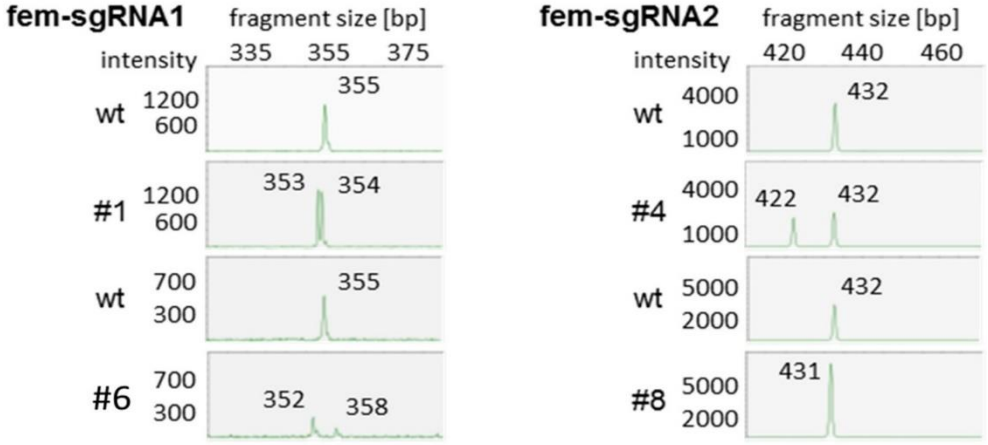


Figure S1: Genes and targeted genomic sites. Genomic organization of the genes *fru* (a), loc552773 (b), *dsx* (c), and *fem* (d) with the designated sgRNA target sites (black arrows). Boxes indicate exons. If genes transcribe sexual splice variants, they are presented. Green boxes indicate common, red the female-specific, and blue the male specific ORF of the sexual transcripts. *dsx*, doublesex; *fem*, *feminizer*; *fru*, *fruitless*; ORF, open reading frame; sgRNA, single guide RNA.

a



b

Injected sgRNA	Larva	Alignment of *fem* sequences
fem-sgRNA1	1	Wildtype AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT Allele a AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT Allele b AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT ACGACGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAA ACGACGTAGACG--AATGGATGATACAACAAGAACGGGAACGAGAACACGAA ACGACGTAGACG-GAATGGATGATACAACAAGAACGGGAACGAGAACACGAA
fem-sgRNA1	6	Wildtype TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG Allele a TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG Allele b TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG ACAATCACGCAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAA ACAATCACGCAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAA ACAATCACGCAGTGAAGATAACAAAATTGATCTGCGTTCAAGAACAAAAGAAGAA CGATTACGACGTAGACGCGAA---TGGATGATACAACAAGAACGGGAACGAGAAC CGATTACGACGTAGACGCGAA---TAGAT---ACAACAAGAACGGGAACGAAAAT CGATTACAACATAGACGCGAAGTGTGGTTGATACAACAAGAACGGGAACGAGAAC ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGT GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGT GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAAT AATGCATCAAACACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAG AATGCATCAAACATATCTAAAACATTTATATTATCCGAAAAATTAGAATCTTCAG AATGCATCAAATATATCTAAAACATTTATATTATCCGAAAAATTAGAATCTTCAG ATGGTACATCTTTATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACT ATGGTACATCTTTATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACT ATGGTACATTTTTATTTAGAGGACCAGAAGATACTCAAGTTAGTGCAACAGAATT ACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAACAACAACA ACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCTAGGAAAACCAACAACAACA ACAAAAAATTAAAGTAGATATTTATAGGATTTTGCCAGGAAAATCAACAACAACA TCTGATGAACTTAAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG TCTGATGAACTTAAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG ACTGATGAACTTAAATGAGATATTATCAATCCTGAAGATGTGATGCTCAAAAG

fem-sgRNA2	4	Wildtype sequence for comparison TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAGACAATCACG Sequence a (size: -10; 9/38 sequences, 24 %) TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAGACAATCACG Sequence b (size: 0; 24/38 sequences, 63 %) TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAGACAATCACG Sequence c (wildtype; 2/38 sequences, 5 %) TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAGACAATCACG Sequence d (size: 0; 3/38 sequences, 8 %) TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAGACAATCACG CAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATTACGA CAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATTACGA CAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATTACGA CAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATTACGA CAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATTACGA CGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAAAGATTGA CGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAAAGATTGA CGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAAAGATTGA CGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAAAGATTGA CGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAAAGATTGA AGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGAGAAAAAATTATC AGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGAGAAAAAATTATC AGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGAGAAAAAATTATC AGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGAGAAAAAATTATC AGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGAGAAAAAATTATC GAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGTAATGCATCAAAC GAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGTAATGCATCAAAC GAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGTAATGCATCAAAC GAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGTAATGCATCAAAC GAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGTAATGCATCAAAC ACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAGATGGTACATCTT ACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAGATGGTACATCTT ACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAGATGGTACATCTT ACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAGATGGTACATCTT ACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAGATGATATATCTT TATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACTACGAAAAATTAA TATTTAGAGGACCAGA----------GTTAGTGCAACAGAACTACGAAAAATTAA TATTTAGAGGACCAGAAGATACTCAAGTTAGTGCAACAGAATTACAAAAAATTAA TATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACTACGAAAAATTAA TATTTAGAGGATTAAAAGGTACTCAAGTTAGTGCAACAGAACTACGAAAAATTAA GGTAGATATTCATAGAGTTTTGCCAGGAAAACCAACAACAACATCTGATGAACTT GGTAGATATTCATAGAGTTTTGCCAGGAAAACCAACAACAACATCTGATGAACTT AGTAGATATTTATAGGATTTTGCCAGGAAAATCAACAACAACAACTGATGAACTT GGTAGATATTCATAGAGTTTTGCCAGGAAAGCCAACAACAATATCTGATGAACTT GGTAGATATTCATAGAGTTTTGCTAGGAAAACCAACAACAACATCTGATGAACTT AAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG AAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG AAATGAGATATTATCAATCCTGAAGATGTGATGCTCAAAAG AAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG AAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG
fem-sgRNA2	8	Wildtype GGTACATCTTTATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAA Allele a GGTACATCTTTATTTAGAGGACCAGAAG-TACTCAAGTTAGTGCAA Allele b GGTACATCTTTATTTAGAGGACCAGAAG-TACTCAAGTTAGTGCAA CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC

Figure S2: The nucleotide sequences of the *fem*-mutated genetic females that were reared on worker nutrition and that have large-sized gonads of the male type. (a) Diagrams of the FL analysis for each of the 4 individuals and WT worker bee examples. (b) The nucleotide sequences. We conducted fragment and sequence analysis on amplicons of cDNA to ensure that the many *fem*-related sequences observed at the *fem* locus (derived from duplication events) [63] were not amplified. The designated binding sites of the sgRNAs are underlined. Sequence b in larvae #4 resulted from fusion of exon 3 with exon 5. The sequences in larvae #4 resulted from fusion between exon 3 and other

fem-related sequences (Koch et al., 2014). The WT sequences were obtained from a sample of 5 WT worker larvae (5 clones each). cDNA, complementary DNA; FL, fragment length; WT, wildtype.

Injected sgRNA	Larva No.	Alignment of *dsx* sequences
dsx-sgRNA2	17-19	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele a/b ....---..... Allele a ....---..... Allele b ....---.....
dsx-sgRNA2	17-29	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele a ....----..... Allele b ....---.....
dsx-sgRNA2	17-37	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele a ....---..... Allele b ....--.....
dsx-sgRNA2	17-46	Wildtype CGATGTCTGA----------ATCATCGGCTG Allele a ....----------..... Allele b .........TCATGATCCTGC.....
dsx-sgRNA6	17-6	Wildtype GCATCCTCACACTGCGATGGTCACCCATTTG Allele a ....--..... Allele b ....-.....
dsx-sgRNA6	17-38	Wildtype GCATCCTCACACTGCGATGGTCACCCATTTG Allele a ....---..... Allele b ....-----.....

Figure S3: Genotypes of *dsx*-mutated females of Fig. 4 as obtained from NGS analyses. The *dsx* WT nucleotide sequences are represented as a reference sequence. NGS, next-generation sequencing; WT, wildtype.

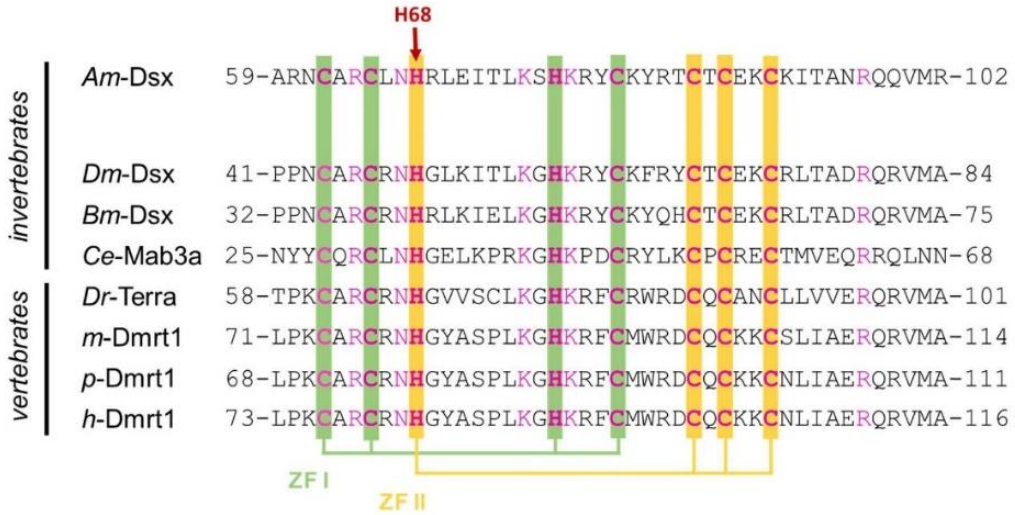


Figure S4: Alignment of the amino acid sequence harboring the zinc finger motifs (ZF I and ZF II) of the DM domain. The deleted conserved histidine at position 68 of the honeybee sequence (*Am*) is highlighted with an arrow.

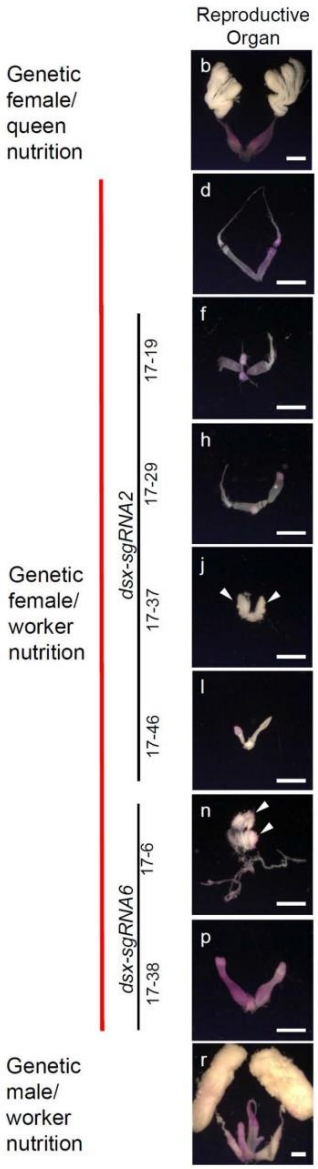


Figure S5: The intersex reproductive organs of Fig. 4 at higher magnification. Scale bar, 1 mm. The genetic females were double mutant for *dsx* and reared on worker nutrition. For further details, see legend of Fig 4 in the main text.

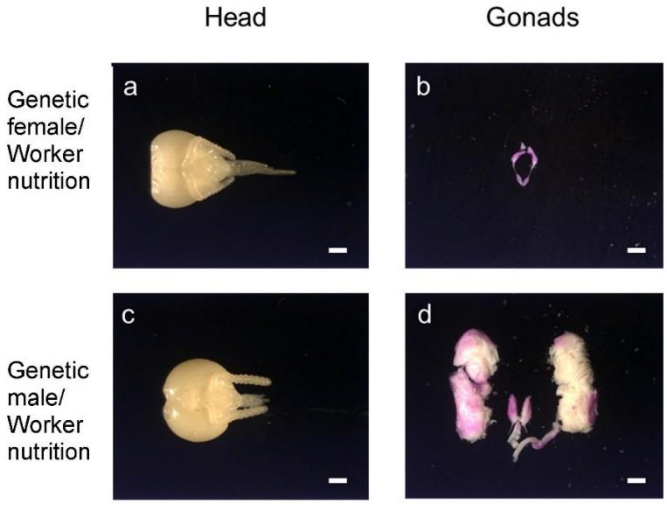


Figure S6: The phenotypes of worker nutrition-reared genetic females and genetic males at an early pupal stage. These females have the typical reduced reproductive organ of workers and the fully developed reproductive organs of males. Head and (a) and (c) and reproductive organ (b) and (d). Gonads were stained with aceto-orcein (reddish coloring) to facilitate the dissection process. Scale bar = 1 mm.

Table S1: The worker bees reared in the colony and the genetic female bees reared manually on worker nutrition.

			Worker phenotypes		
		Number	Head[1)] (Triangular shaped; upper part straight between compound eyes)	Size of female reproductive organ[2)] (Length < 2.5 mm; < 0.7 times the size of the head width)	Ovariole number[3)] (<25)
Genetic female	**Worker diet in colony**	14	14 (100 %)	14 (100 %)	14 (100 %)
	Manually reared on worker nutrition	15	15 (100 %)	15 (100 %)	15 (100 %)

[1)] Frontal view of head. In contrast to workers, queens have a roundish shaped head; the upper part is curved between compound eyes (see Fig. 4a and b in the main text).
[2)] Length between the fused left and right part of the reproductive organ to its end in the sagittal plane. The length in queens is > 6 mm and > 1.2 times the size of the head width.
[3)] Ovariole number in queens is > 100 (Leimar et al., 2012).

Table S2: The genetic male bees reared in colony and manually on worker nutrition.

		Male phenotypes	
	Number	Head[1)] (Round head; complex eyes nearly meet in the upper part)	Size of male reproductive organ[2)] (Length > 6 mm; < 1.2 times the size of the head width)
Male diet colony	8	8 (100 %)	8 (100 %)
Manually reared on worker nutrition	20	20 (100 %)	20 (100 %)

[1)] Frontal view of head.
[2)] Length between the fused left and right part of the reproductive organ to its end (sagittal plane). The length in worker is < 2.5 mm and < 0.7 times the size of the head width.

Table S3: Nucleotide sequences of the sgRNAs. Sequences complementary to the designated genomic target site are shown in bold letters. sgRNA, single guide RNA.

Molecule	Nucleotide sequence
fru-sgRNA1	**GAAUGCACCAGGCAUGUGCG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
fru-sgRNA4	**GCUGGCGGAGGUUGGGCGAC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
fru-sgRNA5	**GCCCGCUGCUGUUCACUCUU**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
fem-sgRNA1	**GAUUACGACGUAGACGCGAA**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
fem-sgRNA2	**GCACUAACUUGAGUACCUUC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
loc-sgRNA1	**GGCUGGAAUACCGGAAUUCG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
loc-sgRNA2	**GAACGUGGUCUUCACCUUCA**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsx-sgRNA1	**CTTGCTCGTTTTGTCTCGGC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsx-sgRNA2	**CACGTGCTACAGACTTAGTA**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsx-sgRNA6	**CAACGUAGGAGUGUGACGCU**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAA GGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

Table S4: The numbers of mutated larvae and the numbers of length-modified (different to the WT) sequences. WT, wildtype.

		No. of length-modified sequences[1)]			**Sum**
		1	2	3	
No. of larvae	**Without wt allele (%)**	10 (26 %)	20 (51 %)	1 (3 %)	21 (79 %)
	With wt allele (%)	8 (21 %)	0 (0 %)	0 (0 %)	8 (21 %)

[1)] Determined by comparing the sequence length of the treated larvae with a sample of 7 non-treated (wildtype, wt) larvae.

Table S5: Nucleotide sequence changes detected in the mutated larvae at the designated target site. At least 10 clones for each larva were sequenced. These nucleotide changes were consistently not observed in 7 nontreated (WT) larvae. The sequence complementary to the sgRNAs are underlined. sgRNA, single guide RNA; WT, wildtype.

Injected sgRNA	Larva No.	Alignment of the nucleotide sequence at the target sites
fru-sgRNA4	1	Wildtype GAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGCGACGGGGGTGGCG Allele a GAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGC---------- Allele b GAGGGGACGGGTGGAAGCTGGCGGAGG---------- GCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTGG --CGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTGG ----------CTAAAGGGGAAAGGGGGGTGG
fru-sgRNA4	2	Wildtype GGAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGCGACGGGGGTGGC Allele a GGAGGGGACGGGTGGAAGCTG----------ACGGGGGTGGC Allele b GGAGGGGACGG---------- GGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTG GGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTG ---------- GCGGGAGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG GCGGGAGTGGTGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG ----------CGAACATAAAATCCCTCG CACAT CACAT CACAT
fru-sgRNA4	3	Wildtype AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele a AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele b AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA GGTTGGGC-GACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTGGCCCGACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTG-----ACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG
fru-sgRNA5	2	Wildtype GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACTC Allele a GGCTTCAACGCGGCTCGGTTGGGTGGTGACCCGCTGCTATTCCCT- Allele b GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACT- TTTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA
fru-sgRNA5	4	Wildtype GTTGGGTGGTGGCCCGCTGCTGTTCACTCTTTGGAGAGGAAAGGGT Allele a GTTGGGTGGTGGCCCGCTGCTGTTC---CTTTGGAGAGGAAAGGGT Allele b GTTGGGTGGTGGCCCGCTGCTGTTCA--CTTTGGAGAGGAAAGGGT TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAG---GAGGA GGTGGGGGAGAGTGG GGTGGGGGAGAGTGG GGTGGGGGAGAGTGG

fru-sgRNA5	7	Wiltdype GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACTC Allele a GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACT- Allele b GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTT----- TTTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA --TGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA --TGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA
dsx-sgRNA1	12	Wildtype TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele a TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele b TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGC---GGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT
dsx-sgRNA1	16	Wildtype TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele a TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele b TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGT---GGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT
dsx-sgRNA2	9	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAT--- Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGATCGGC Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGATC ----------CATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA TGAATCGGCACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA ---------ACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA
dsx-sgRNA2	11	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGCTG Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGCTG CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA TCGT--GAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA TCGT--GAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA
dsx-sgRNA2	68	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCGT CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA --GCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA
loc552773-sgRNA1	3	Wildtype CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAT- Allele a CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG Allele b CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGCTGG ----------TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC CGCCACCCATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC AATA------TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC

loc552773-sgRNA1	6	Wildtype CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAT- Allele a CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG Allele b CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG ---TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG TATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG TATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG
loc552773-sgRNA1	7	Wildtype CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAT- Allele a CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGG---- Allele b CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG ---TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG -----AAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG TATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG
loc552773-sgRNA2	9	Wildtype GTTCGAGATCTCAAAGCGGATGTCGAGAACGTGGTCTTCACCTTCA Allele a GTTCGAGATCTCAAAGCGGATGTCGAGAACGTGGTCTTCACTTTCA Allele b GTTCGAGATCTCAAAGCGGATGTCGAGAACGTGGTCTTCACC-TCA AGGTGAACTTTGAGAAACTCCATTTCCAAGGGAAGTATCAGATCGACGCGAGGGT AGGTCAACTTTGAGAAACTCCATTTCCAAGGGAAGTATCAGATCGACGCGAGGGT AGGTGAACTTTGAGAAACTCCATTTCCAAGGGAAGTATCAGATCGACGCGAGGGT

Table S6: The detected deletions and insertions mediated by CRISPR/Cas9 method in a sample (*n* = 25) of mutated nucleotide sequences. CRISPR/Cas9, clustered regularly inter-spaced short palindromic repeats/CRISPR-associated protein 9.

Indels (bp)	#	Relative frequency
> -20	2	8 %
-20 to -11	3	12 %
-10 to -6	2	8 %
-5 to -1	11	44 %
+1 to +5	5	20 %
+6 to +10	0	0 %
+11 to +20	2	8 %
> +20	0	0 %

Table S7: The heterozygous, female genotype of the *csd* gene in the *fem* double nonsense mutants.

Injected sgRNA	Larva No.	Alignment of the hypervariable region of the *csd* alleles
fem-sgRNA1	1	*csd* allele 1 GAACCTAAAATAATTTCATCTTTATCGAACAATTACAATTAT *csd* allele 2 GAACCTAAAATAATTTCATCTTTATCGAGCAATTACAATTCT AATAATAATAATTATAATAATTATAATAATTATAATAATTATAATAATAATTATA AACAATTATAATAATTATAGTACTAATTAT------------------------- ATAATAATTATAATAAAAAATTA------TATTACAATATTAATTATATTGAACA --------------AAACAATTACAATATTGTTACAATATTAATTATATTGAACA AATTCCTGTTCCTGTT AATTCCTATTCCTGTT
fem-sgRNA1	6	*csd* allele 3 GAACCTAAAATAATTTCATCTTTATCGAATAAGACAATACAT *csd* allele 4 GAACCTAAAATAACTTCATCTTTATCGAACAATTACAATTCT AATAATAATAATTAT--------------------------AAAAAATTATATT AATAATTATAATAATTATAATAAATATAATTATAATAATTCTAAAAAATTATATT ACAATATTAATTATATTGAACAAATTCCTATTCCTGTT ACAATATTAATTATATTGAACAAATTCCTGTTCCTATT
fem-sgRNA2	4	*csd* allele 5 GAACCTAAAATAATTTCATCTTTATCGAACAAGACAATACAT *csd* allele 6 GAACCTAAAATAATTTCATCTTTATCGAACAATTACAATTAC AATAATAATAAATATAATTATAATAATAATTATAATAATAATTGTAAAAAATTAT AACAATTATAATAATAATTAT-----------------------AAACCATTAT ATTACAATATTAATTATATTGAACAAATTCCTATTCCTGTT ATTACAATATTAATTATATTGAACAAATTCCTGTTCCTGTT
fem-sgRNA2	8	*csd* allele 8 GAACCTAAAATAATTTCATCTTTATCGAATAATACAATACAT *csd* allele 9 GAACCTAAAATAATTTCATCTTTATCGAACAAGACAATACAT AATAATAATTATAAATATAATTATAATAATAATTATAATAA---TTATAAAAAAT AATAATAAT---AAATATAATTATAATAATAATTATAATAATAATTGTAAAAAAT TATATTACAATATTAATTATATTGAACAAATTCCCGTTCCTGTT TATATTACAATATTAATTATATTGAACAAATTCCTATTCCTGTT

Table S8: Large gonads of the male type in genetic female double mutant for *fem*.

Treatment	Number	Number of bees with large gonads (male type) and double nonsense mutations
***fem*-sgRNA** (sgRNA1/sgRNA2)[1)]	27 (11/6)	4 (15 %) (2/2)
Untreated	38	0 (0 %)

[1)] 400 pg *Cas9* mRNA together with 5.5 pg *fem*-sgRNA1 or 14.6 pg *fem*-sgRNA2 were injected per embryo.

Table S9: Reproductive organ size of genetic females at larval stage 5 that were double mutant for *fem* and that were reared on worker nutrition.

			Length at larval stage 5	
		Number	> 2.5 mm, Testes	< 1.5 mm, Ovary
Genetic female / manually reared on worker nutrition	Double missense mutation in *fem*	4	4 (100 %)	0
	Wildtype	38	0	38 (100 %)

Table S10: The reared genetic females with intersex reproductive organ that were double mutant for *dsx*.

Treatment	Experiment	Number of bees at larval stage 1	Number of bees at stage of phenotyping[1)]	Bees with worker head[2)]	Bees with intersex reproductive organ	% of intersexes with *dsx* double mutations
***dsx*-sgRNA2**	1	125	41	41 (100 %)	4 (10 %)	100 %
	2	362	11	11 (100 %)	5 (45 %)	100 %
***dsx*-sgRNA6**	-	39	11	11 (100 %)	2 (18 %)	100 %
Untreated	-	82	34	34 (100 %)	0 %[3)]	0 %

[1)] Genetic mosaics were excluded.
[2)] Frontal view: triangular shaped; upper part straight between compound eyes
[3)] 17 out of 34 were dissected.

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Manuscript II

doublesex specifies initiation and sustainment of distinct worker behavior in the honeybee *Apis mellifera*

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Abstract

Honeybees (*A. mellifera*) display a unique form of division of labor accomplished by caste dimorphism. The female caste origins from the same genome, but manifests in different behavior and phenotypes. While the queen reproduced, every other task from brood care to foraging is taken care of by worker bees. The genetic underpinnings of how this caste dimorphism is specified are still unknown. Here we used CRISPR/Cas9 to mutate the functional domain of *doublesex* (*dsx*), a gene highly conserved in structure and function and known for its effect on sex-specific behavior in *Drosophila*. We observed individual worker bee behaviors on a comb using BBAS (bee behavioral annotation system; Blut et al. (2017)) and found that the *dsx* gene is required for the specification of task-related behaviors specific for worker bees. We find *dsx* essential for the initiation of task engagements as cell inspection (CI; <5 sec) and long work in cell (WIC;>5 sec) frequencies were significantly reduced in *dsx*-mutants. As the average length of WIC was markedly reduced in *dsx*-mutants, we further conclude that the sustainment of task engagement is also instructed by *dsx*. This all in a task-stimuli dependent manner. Further, significant reduction in frequency and length of trophallactic contacts in *dsx*-mutants, while other interactions were not affected, indicates that *dsx* instructs the development of very specific worker traits, that are associated with social group living. Neuronal malformations in 25 % of *dsx*-mutants, suggest *dsx* regulated caste-specific neuronal development. This is supported by Dsx expression found in the division corresponding to the basal ring (Dbr) of the ventral lobe and corresponding neurons. Here we provide evidence that a single gene, the transcription factor *dsx*, instructs the differentiation of specific behavioral traits and neuronal development of worker bees, that are associated with the evolution of social behavior.

Introduction

Animals are born with a set of behaviors that enhance the prospect for survival and reproduction (Tinbergen, 2010). The complexity of such behaviors is remarkable, ranging from purely instinctive driven actions to behaviors that are flexibly adjusted to the environment. Our understanding of how this is genetically encoded and determined during development is rudimentary at best, as classic approaches struggle to assign key factors to a behavioral manifestation. Key components for specification such as transcription factors (TFs), have multiple functions, for example, the neuronal cell diversity with its terminal morphology found in the *Drosophila* brain, is specified by a specific set of TFs and a specific combination of terminal selector genes (Konstantinides et al., 2018).

To understand molecular processes that influence the manifestation of behaviors, different allelic states and CRISPR-Cas9 mediated knockdowns have been very informative. This includes, for example, the *npr-1* neuropeptide receptor gene in *C. elegans* (De Bono & Bargmann, 1998) and the *foraging* cGMP-dependent protein kinase gene in *Drosophila* (Osborne et al., 1997), both impacting social feeding behavior. In ants the *odorant receptor co-receptor* (*orco*) gene mediates odor and pheromone perception, development of antennal lobes and the plasticity of social behavioral traits (Trible et al., 2017; Yan et al., 2017). In honeybees a recent study showed, that reproductive organ size is dependent on the activity of the gene *doublesex* (*dsx*), a conserved zinc-finger TF-protein, which suggests a key role in caste and sex differentiation (Burtis et al., 1991; Cho et al., 2007; Scott E. Erdman & Burtis, 1993; Roth et al., 2019). This corresponds with findings in *Drosophila*, where aspects of sex-specific behaviors and morphology have been identified to be programmed by the genes *dsx* and *fruitless* (*fru*), as male courtship and morphology is dependent on these genes (reviewed in Dauwalder, 2011; Ryner et al., 1996; Villella & Hall, 1996). Disrupting sex-specific *dsx* and *fru* positive neurons in the fruit fly brain, causes a reduction of courtship in males and malfunctional egg laying as well as copulation behavior in females (Rideout et al., 2007, 2010). If we are to understand the programming of such behaviors on the molecular level, examining more sophisticated behaviors is essential. For this, the fact that *dsx* is conserved in its functional domain in the honeybee *Apis mellifera* in combination with the wide variety of both robust and flexible behavior displayed by worker bees and the possibility of precise genetic manipulation (Roth et al., 2019), provides an ideal

model organism to study gene-dependent behavioral development.

The more or less sterile worker bees and the reproductive queen *A. mellifera* originate from an identical genome, however displaying morphological and behavioral differences as a result of developmental programming. Both females develop from fertilized, diploid eggs and the female sex determination is initiated by heterozygosity at the *complementary sex determiner* (*csd*) gene (Beye et al., 1999, 2003). At the bottom of the sex determination cascade is *dsx*, which is sex-specifically spliced dependent on the allelic state of *csd*, which induces sex-specific splicing in the genes *feminizer* (*fem*) and *transfromer2* (*tra2*), genes upstream of *dsx* and both essential for the female-specific development (Gempe et al., 2009; Hasselmann et al., 2008). As the bottom master switch the Dsx protein in the honeybee holds a possible solution for understanding the specification of distinct physiology and behavior (Giurfa & Giurfa, 2003; Menzel, 2012; Seeley, 1995).

Worker bees elaborate a highly social behavioral repertoire with up to 50 different manifestations and very flexible adjustments of their task engagements (Johnson, 2008a). For example, feeding larvae requires workers to check a cell and adjust their response to the nutritional state of the larvae, resulting in highly variable time spent per larvae (manuscript submitted Blut et al. 2021). To exhibit such high plasticity in behavioral initiation responses, worker bees need to integrate activity of nestmates, the state of the hive and food availability to be able to engage with the most vital task (Johnson, 2008b; Seeley, 1982). Task priority is evaluated by information gathered through for example antennation, trophallaxis or the waggle dance performed by nestmates. If necessary, worker bees also actively recruit nestmates by secreting an alarm pheromone to defend the hive for unwelcome intruders (Boch et al., 1962; Slessor et al., 2005). In contrast, are a honeybee queens task engagements driven by consistent stimulus responses specialized for reproduction, such as checking whether or not a cell is empty for egg laying (reviewed in Brutscher, Baer and Niño, 2019). Behavioral procedures are evolutionary adapted for reproductive success, for example a queen does not feed herself but is fed through trophallaxis by worker bees. Further, does she use the constant retinue behavior displayed by young worker bees to spread the queen mandibular pheromone (QMP). QMP, a pheromone of low volatility, constitutes to social cohesion and the reproductive monopole of the queen by suppressing ovary maturation in worker bees (Hoover et al., 2003; Slessor et al., 1988, 2005). These examples highlight the flexible, versatile,

and socially oriented behavior displayed by worker bees, compared with the behavioral repertoire of a queen, which is reproduction oriented and relies on the worker bee. Details on how these differences manifest in the nervous system development are unknown, but analyses of brain-morphology indicate that neuronal wiring is caste-specific as main brain areas for processing information, the mushroom bodies, show differences in size between queens and worker bees (Groh et al., 2006). With the recent finding that the developmental regulator *dsx* integrates the sexual and caste determining signal in the honeybee (Roth et al., 2019), aspects of caste-specific morphological differentiation can be attributed to the activity of a single gene. Making it a candidate gene to affect the programming and specification of worker-specific behaviors in honeybees. We used targeted mutations, CRISPR/Cas9, of the *dsx* gene to compromise its functionality. We postulate that *dsx* activity specifies aspects of worker-specific behavior during development, while sex-specific activity of the *dsx* gene will not affect vital behavioral abilities. However specific worker characteristics, like brood care or worker-worker interactions will be affected. By using a computer based behavioral bee annotation system (BBAS by Blut et al., 2017) worker behavior will be detected and quantified under natural conditions, enabling a detailed detection of individual behavioral traits. Further a setup for honeybee-specific stimuli perception followed by analysis of the worker brain morphology will give insights into the effect of malfunctional *dsx*. This will help to better understand the role of *dsx* in caste-differentiation and further, might be able to unravel parts of the genetic fundament of honeybee behavioral specification.

Results

Activity from the *dsx* gene is required for specifying cell inspection and work in cell behaviors

A key aspect of group living is that worker bees forego reproduction and perform different tasks to collectively maintain the colony while they share information and food. Central to worker bees of the nurse stage is that they engage in different tasks that are devoted to the collective rearing of the brood. Typically, nurse bees inspect cells (IC) on the comb by entering a cell for less than 5 seconds (Johnson, 2008a), where they encounter either food (honey or pollen), a larva, or an empty cell. Cells with the same task opportunities are usually clustered in the same area on the comb. When inspecting, the bees eventually engage in work in the cell (WIC) by feeding brood, cleaning empty cells, processing or taking in food which is indicated by an increase in the time spent in the cell (Johnson, 2008a; supplementary Video V 1-4). In order to determine whether the *dsx* gene specifies these worker-specific tasks, we generated homozygous *dsx* mutant (*dsx*$^{stop/stop}$) worker bees using CRSPR/Cas9 induced mutations (Jinek et al., 2012b; Roth et al., 2019; Fig. 1 a, b) targeting the nucleotide sequence before the DNA binding domain (Znf of the DM domain type (Matson & Zarkower, 2012)). We induced mutations in early embryos and reared entirely mutated *dsx*$^{stop/stop}$ worker bees at high frequency (n = 67; supplementary Table S2, 3) with no mosaicism which we identified using deep sequencing of amplicons (Roth et al., 2019). The proportion of CRISPR/Cas9 treated and wildtype control adult worker bees did not differ during in-vitro rearing ($P > 0.2$, df = 1, Fisher´s exact; supplementary Table S4) suggesting that the treatment has no effect on survival.

We then followed the behaviors of *dsx*$^{stop/stop}$ and wildtype control worker bees in small colonies with about 450 other wildtype and hive-reared worker bees (Fig. 1c). We continuously tracked the behaviors of worker bees at the nurse age when they were seven days old using computer-based tracking (Blut et al., 2017) on combs where cells in the same area were filled with larvae, pollen and sugar solution or were left empty for all replicates (supplementary Fig. S5, Table S6-8). The *dsx*$^{stop/stop}$ worker bees did not display gross abnormalities for the sequence of CI and WIC

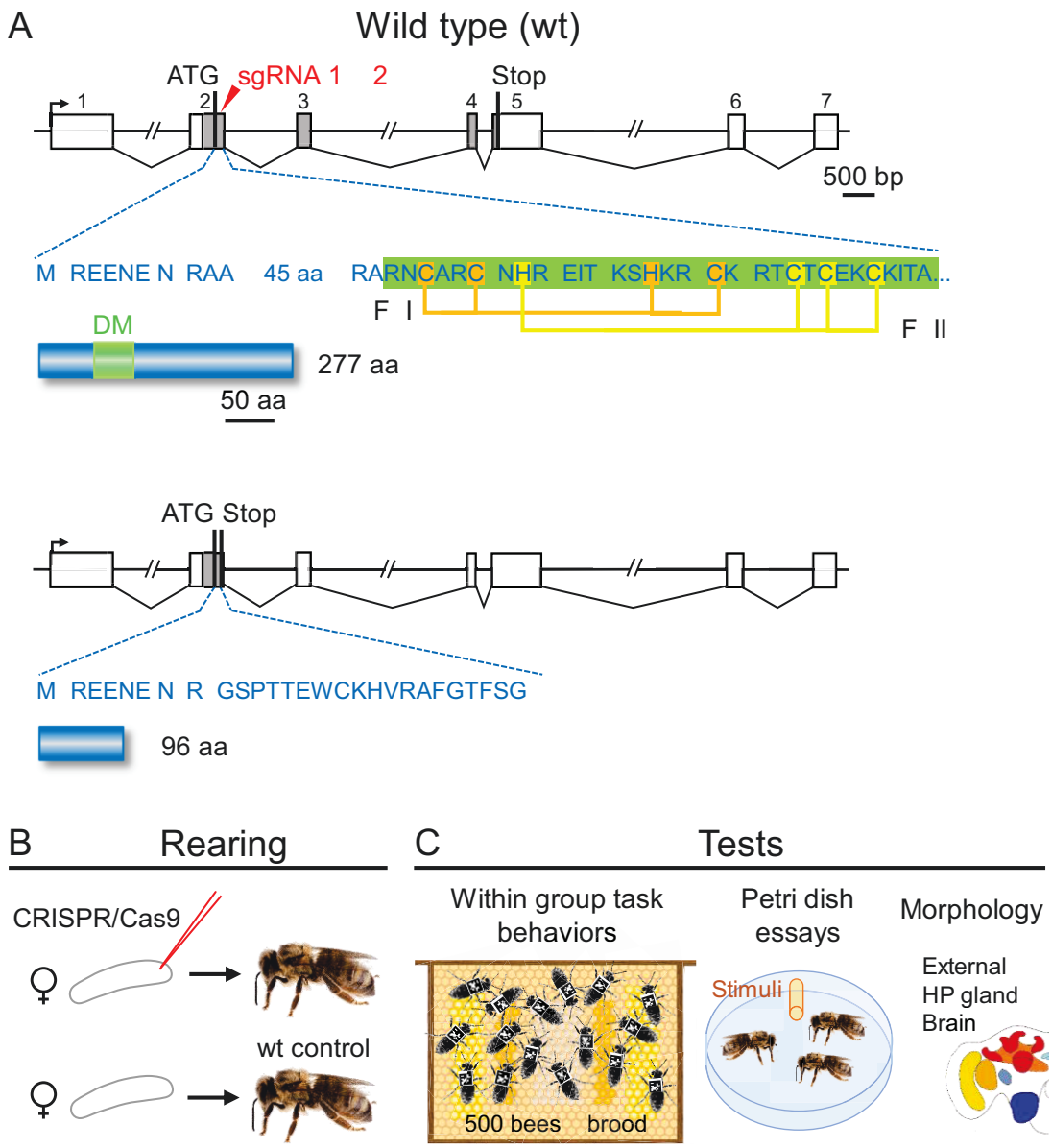


Figure 1: The generation and behavioral testing of *dsx*$^{stop/stop}$ mutant worker bees. A. Scheme of the genomic organization of female-specific spliced *dsx* gene and the targeted mutation. Boxes are exons and interconnected lines indicates the female splice variant (male splice variant is provided in supplementary Fig. S1). Translation start and stop are indicated. Gray boxes indicate the ORF (open reading frame). Arrow (red) indicates target sites of the two sgRNAs 1 and 2. The sequence (blue) displays the amino acids encoded by wildtype and the *dsx*$^{stop/stop}$ mutation. Green, DM domain consisting of two zinc fingers (ZFI and ZF II). Below, schematic presentation of the proteins (blue). As consequence of the stop mutations (asterisk) the *dsx*$^{stop/stop}$ mutants lack a functional DM domain (supplementary Table S23). **B.** The generation of worker bees. Female embryos were either *dsx* mutated using CRISPR/Cas9 method or not (wildtype (wt) controls) and were in-vitro reared to adult stage. Since each bee can carry different mutations, *dsx*$^{stop/stop}$ mutants (without mosaicism) were identified using in-deep sequencing of amplicons that were generated from the sgRNA target sites. **C.** The behavioral and morphological testing of *dsx*$^{stop/stop}$ versus wt control worker bees. Within group task behaviors on brood comb. *dsx*$^{stop/stop}$ and wt control worker bees were individually and computer-based tracked (Blut et al., 2017) on combs in colonies with 450 other hive-reared wildtype worker bees. The combs had same areas with task opportunities in which cells are empty or filled will single larvae, or food (pollen or honey). Petri dish studies were examining responses to light, repellent odor, and alarm pheromone stimuli. Morphological studies were examining external structures, abdomen, head, hypopharyngeal glands (HPG) and the brain.

behaviors (supplementary video material V1-4). Having shown that the *dsx* gene has no active role in specifying the stereotypic components of these task behaviors we next asked whether the *dsx* gene specifies initiation of IC and WIC behaviors. The frequency by which these behaviors are initiated are important as it defines whether a bee engages into a distinct task or not (Johnson, 2008b; Seeley, 1982). The frequency of initiating IC and WIC behaviors were substantially reduced in $dsx^{stop/stop}$ worker bees (IC: Mann-Whitney, z = -2.7, *P* = 0.006; WIC: Mann-Whitney, z = -2.4, *P* = 0.02; Fig. 2a, b; supplementary Table S9). We further quantified initiation of IC behaviors for the different cell types and observed an impairment of IC behaviors for food (Mann-Whitney, z =-2.0, *P* = 0.04) and empty cells (Mann-Whitney, z = -2.0, *P* = 0.04) in $dsx^{stop/stop}$ worker bees. For the WIC behaviors we observed that the median frequency estimates of the $dsx^{stop/stop}$ worker bees were only half of the wildtype controls when focused on brood cells (Mann-Whitney, z = -1.9, *P* = 0.05; Fig. 2b, supplementary Table S9). These impairments suggest that the *dsx* gene is actively involved in specifying the initiation features of IC and WIC behaviors. Another feature that regulates task engagement is how long WIC behavior is sustained. The time spent per WIC was significantly reduced in $dsx^{stop/stop}$ worker bees (Mann-Whitney, z = -2.0, *P* = 0.04; Fig. 2b'; supplementary Table S10). When examining the different cell types, we found that the time spent was specifically and markedly reduced for cells containing food (Mann-Whitney, z = -2.3, *P* = 0.02; Fig. 2b': supplementary Table S10). These results indicate that the *dsx* gene plays an active role in sustaining the work in the cell.

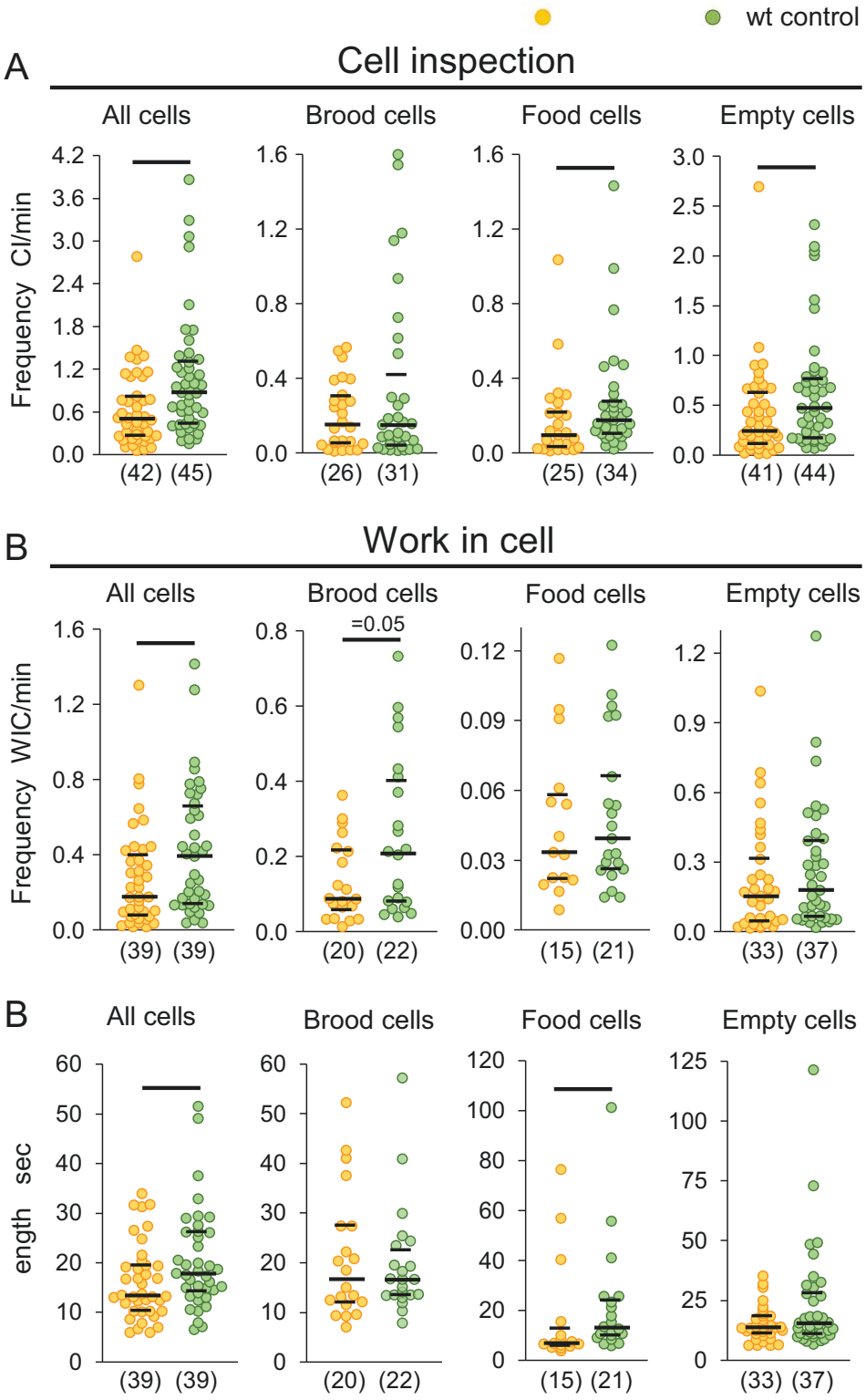


Figure 2: *dsx* gene activity is required for cell inspection and work in cell behavior. Inspecting cell (IC) and work in cell (WIC) behaviors of *dsx*$^{stop/stop}$ and wildtype control worker bees. **A.** Frequency of IC observed for all cell types (P = 0.006, z = -2.7, Mann-Whitney-U-test (MWU)). Frequency of IC for larvae containing cells (P = 0.7, z = -0.7, MWU), food cells (P = 0.04, z = -2.0, MWU) and empty cells (P = 0.04, z = -2.0, MWU). **B**. Frequency of WIC observed for all cell types (P = 0.02, z = -2.3, MWU). Frequency of WIC for larvae containing cells (P = 0.05, z = -1.9, MWU), food cells (P = 0.5, z = -0.7, MWU) and empty cells (P = 0.3, z = -1.1, MWU). **B'** The length of WIC behavior observed for all cell types (P = 0.04, z = -2.0, MWU). The length of WIC for larvae containing cells (P = 0.8, z = -0.2 MWU), food cells (P = 0.02, z = -2.3, MWU) and empty cells (P = 0.2, z = -1.3, MWU). The median (middle line) and quartiles are displayed. *n* values are shown in parentheses. Min: minutes.

dsx activity specifies sustainment of trophallaxis

A central feature of group living is social food sharing or trophallaxis behavior (reviewed in LeBoeuf, 2020). Nurse bees engage into trophallactic behaviors that provide other nurse bees with food to feed the young (Crailsheim, 1998). There is a sequence of behaviors leading to trophallaxis that also provide a path to distribute information about the nutritional state of the colony to other colony members (Farina & Grüter, 2009; Mc Cabe et al., 2006; Schulz et al., 2002). Typically, two bees initiate antennation during an encounter that eventually initiates begging behavior of the recipient and extension of the proboscis (Fig. 3a). This behavior may or may not trigger the extension of the proboscis of the other, donor bee, that possibly results in trophallaxis and sharing of food (Crailsheim, 1998; Farina & Wainselboim, 2001; Free, 1959; supplementary Video V5-7). The specific cues controlling the initiation of these distinct behaviors are still unknown.

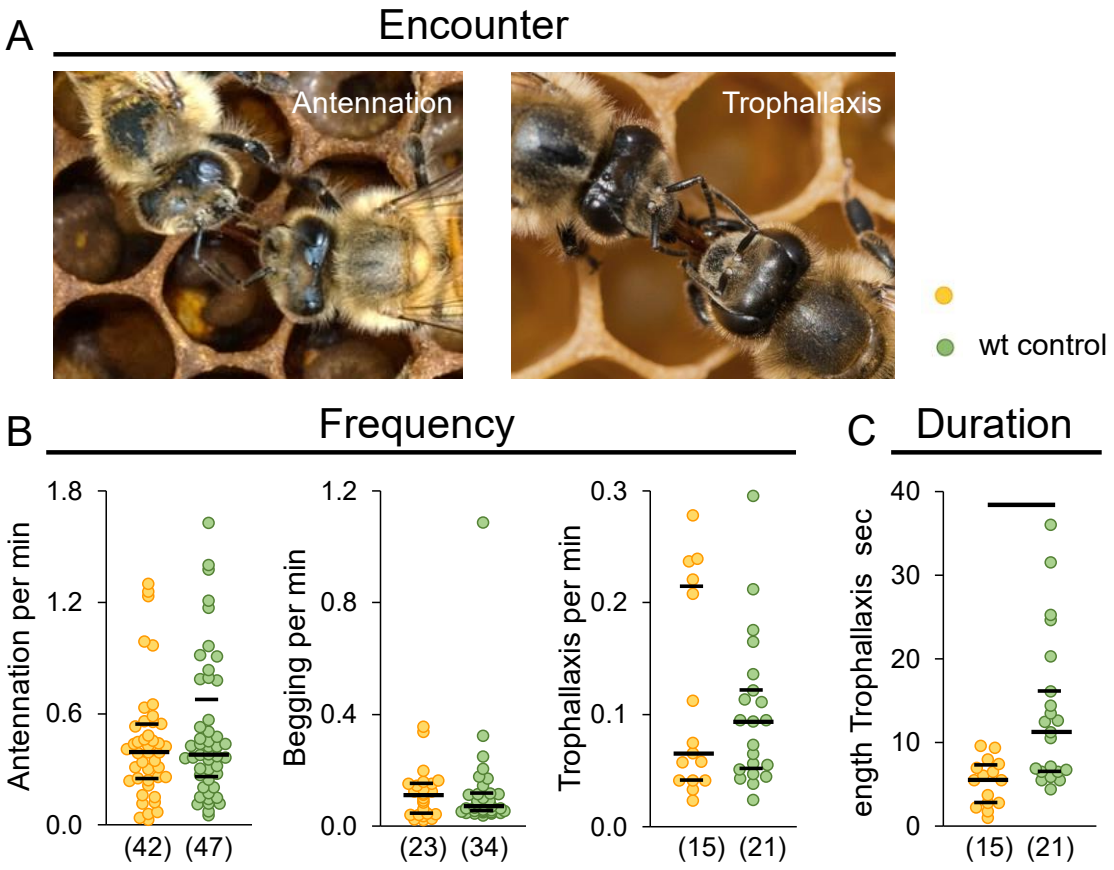


Figure 3: The *dsx* gene activity is essential for the sustainment of trophallaxis behavior. Antennation, begging and trophallaxis behaviors of *dsx*$^{stop/stop}$ and wildtype control worker bees. **A.** Examples of antennation and trophallaxis behavior of two worker bees. **B.** Frequency of antennation (P = 0.7, z = -0.5, Mann-Whitney U-test (MWU)), begging (P = 0.7, z = -0.7, MWU) and trophallaxis (C; P = 1, z = -0.06, MWU). **C.** Duration of trophallaxis behaviors (P = 0.001, z = -3.2, MWU). The median (middle line) and quartiles are presented. n values are shown in parentheses. Min: minutes.

We investigated the effects of the *dsx* gene disruption on these behaviors and observed no anomalies in the sequence and stereotypic performance of antennation, begging and trophallaxis behavior in $dsx^{stop/stop}$ worker bees (supplementary Videos V8-11). In order to determine whether the *dsx* gene specifies the initiation of antennation, begging or trophallaxis behaviors we examined their frequency and found that these initiations were not impaired in $dsx^{stop/stop}$ worker bees (Mann-Whitney, $z < -0.06$, $P > 0.7$; Fig. 3b; supplementary Table S11). An important determinant that regulates the amount of food that is shared between bees is the duration of trophallaxis. The duration of trophallaxis behavior was significantly and markedly decreased from 11.3 to 5.5 seconds median estimates in wildtype controls versus $dsx^{stop/stop}$ worker bees (Mann-Whitney, $z = -3.2$, $P = 0.001$; Fig. 3c; supplementary Table S11). These results on trophallaxis duration suggest that the sustainment of trophallaxis behavior is impaired. Collectively, these results suggest that the *dsx* gene function is specifically required to instruct the sustainment of trophallaxis behavior.

dsx activity is not essential for gross external morphology, maturation, work-related mobility, and sensorimotor functions

The initiation and sustainment impairments described cannot be explained by defects of the work area related moving behavior on the comb. The $dsx^{stop/stop}$ worker bees did perform as well as wildtype control bees in our measures of the time spent in distinct work areas, the visiting behaviors of these distinct work areas harboring the different cell types and mobility behavior (Mann-Whitney, $z < -0.3$, $P > 0.1$; Fig. 4a-c; supplementary Table S12-14) suggesting no impairment. The initiation and sustainment impairments can also not be explained by external morphological defects. Head-, antennae- and body-morphology and body sizes of $dsx^{stop/stop}$ worker bees did not differ from the wildtype control bees (Fig. 4d, e). The $dsx^{stop/stop}$ and wildtype control worker bees had triangular shaped heads measured in head length to width ratio for $dsx^{stop/stop}$ ($n = 29$) and wildtype controls showing no differences ($n = 26$; Mann-Whitney, $z = -0.3$, $P = 0.7$) and wild-typic antennae with 13 segments observed in all $dsx^{stop/stop}$ ($n = 17$) and wildtype control worker bees ($n = 11$; $P = 1$, df = 1, Fisher´s exact; supplementary Table S16, 17). Furthermore, are the initiation

and sustainment defects not due to impairments of the physiological transition to the nurse stage. Bees entering the nurse stage develop secreting hypopharyngeal glands (HPG; Deseyn & Billen, 2005; Richter et al., 2016), which provide components of the diet for larval feeding. All *dsx*$^{stop/stop}$ (*n* = 24) and wildtype control (*n* = 27) worker bees had fully developed HPG (*P* = 1, df = 1, Fisher´s exact), as they were composed of secretory acini that were arranged along collecting ducts (Fig. 4f, supplementary Table S15, 16; Ahmad et al., 2021).

However, the only differences we observed were for the reproductive organ development as previously reported (Roth et al., 2019; supplementary Fig. S18). By further testing essential sensorimotor functions in *dsx*$^{stop/stop}$ worker bees, we demonstrate that *dsx* activity does not affect the response to light (Mann-Whitney, z = -0.6, P = 0.6), repellent odor (Mann-Whitney, z = -0.3 , *P* = 0.8), or the honeybee

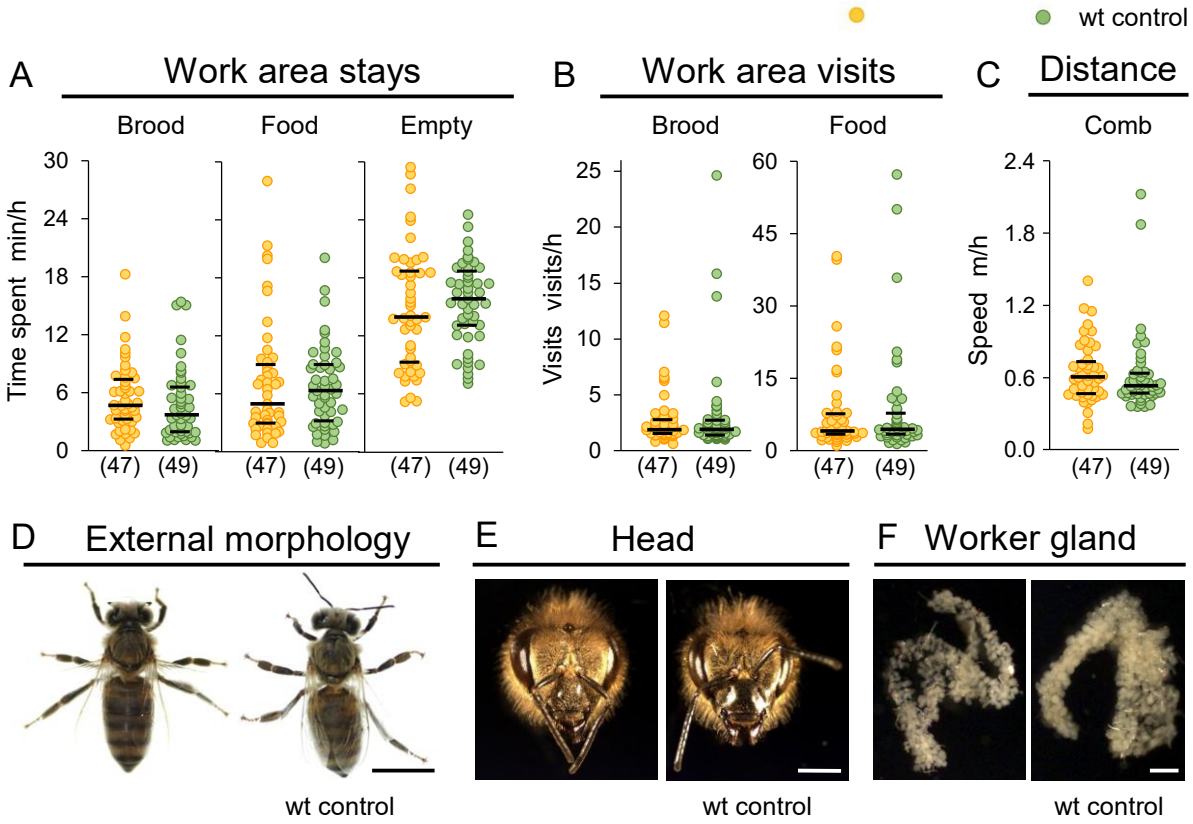


Figure 4: The *dsx* activity does not specify work-related mobility behaviors, gross external morphology, and maturation. (A to C) mobility behaviors of *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees on the comb and among distinct work areas. The median (middle line) and quartiles are presented. *n* values are shown in parentheses. Min: minutes, h: hours. **A.** The length of staying in equivalent work areas in which cells either contain larvae (*P* = 0.1, z = -1.5, Mann-Whitney-U-test (MWU)), food (*P* = 0.7, z = -0.4, MWU) or were empty (*P* = 0.4, z = -0.9, MWU). **B.** Frequency of work area visits. Areas containing either larvae (*P* = 0.8, z = -0.6, MWU) or food (*P* = 0.5, z = -0.3, MWU). **C.** The walking distance on the comb (*P* = 0.2, z = -1.3, MWU). **D.** Entire external morphology. *dsx*$^{stop/stop}$ (*n* = 17) and wt control (*n* = 11) worker bees had the same number of abdominal segments (6 = wild-typic). Scale 5 mm. **E.** Head (observed from front) external morphology. *dsx*$^{stop/stop}$ had triangular shaped worker bee heads. Scale 1 mm.

alarm pheromone component IPA (Mann-Whitney, z = -1.1, *P* = 0.3; Fig. 5; supplementary Fig. S19), indicating that the initiation and/or sustainment impairments in IC and WIC and trophallaxis behaviors are not due to general or gross sensorimotor defects. In fact, both *dsx*$^{stop/stop}$ and wildtype control bees responded to the repellent odor and IPA with ventilation and reduction in locomotion (*P* < 0.01, Wilcoxon rank sum test, comparison of *dsx*$^{stop/stop}$ and wildtype controls against zero (no change)), indicating a wild-typic perception of this stimuli (Free, 1987; Wager & Breed, 2000). Thus, the loss of *dsx* function specifically disrupts the initiation and/or sustainment aspects of CI, WIC and trophallaxis behaviors. Collectively, these results indicate that *dsx* activity is specifically required to specify the initiation and sustainment features of task behaviors in worker bees.

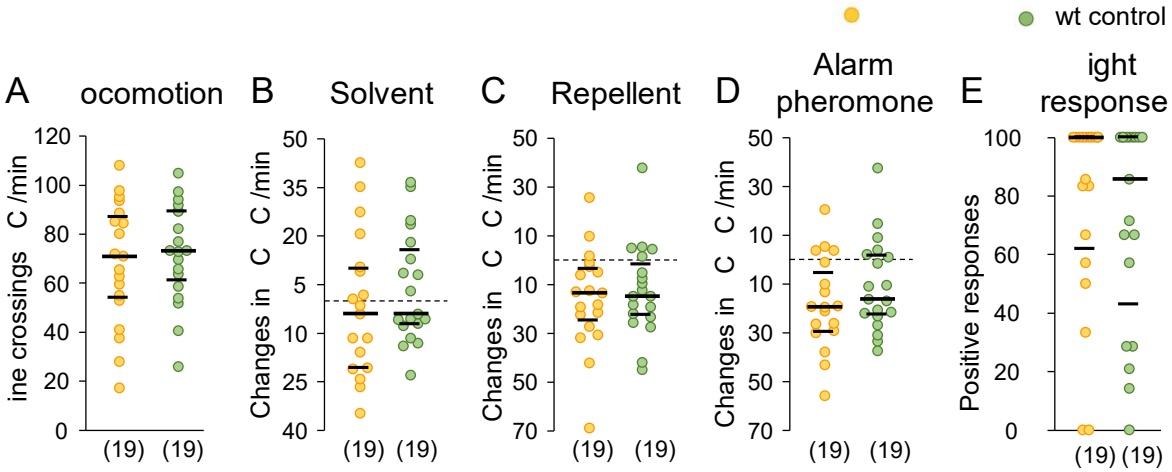


Figure 5: *dsx* activity is not essential for general sensorimotor functions in worker bees. Sensorimotor functions of *dsx*$^{stop/stop}$ and wildtype control worker bees were tested in a petri dish essay. **A.** Locomotion measured in line crossings (LC)/minutes (min). LC/min (*P* = 0.6, z = -0.5, Mann-Whitney U- test (MWU)). **B.** Solvent response measured in LC before and after solvent presentation and calculating the difference. The changes of LC/min after adding the solvent isopropanol were not different (*P* = 0.3, z = -1.1, MWU). Both wildtype control and *dsx*$^{stop/stop}$ worker bees did not respond to the solvent (*P* > 0.4, Wilcoxon rank sum test, comparison of *dsx*$^{stop/stop}$ and wildtype controls against zero (no change)). **C.** Repellent odor response measured as described for solvent and tested by presenting 0.5 µl benzaldehyde. Changes of LC/min were not different after adding the repellent (*P* = 0.8, z = -0.3, MWU). Both wildtype control and *dsx*$^{stop/stop}$ worker bees responded to the repellent by stopping movement and starting ventilation (*P* < 0.01, Wilcoxon rank sum test, comparison of *dsx*$^{stop/stop}$ and wildtype controls against zero (no change)). **D.** Alarm pheromone response measured as described for solvent and tested by presenting 0.5 µl isopentyl acetate (IPA). IPA is a major component of the honeybee alarm pheromone (Free, 1987). Changes of LCs were not different after adding IPA (*P* = 0.3, z = -1.1, MWU). Both bee groups responded to IPA by stopping movement and staring ventilation (*P* < 0.03, Wilcoxon rank sum test, comparison of *dsx*$^{stop/stop}$ and wildtype controls against zero (no change)). **E.** Light response measured by bees walking towards a light pulse. The proportion of positive photo taxis responses was not different (*P* = 0.6, z = -0.6, MWU).

dsx activity is required for the development of central integrative centers

Our behavioral analysis showed that *dsx* activity is necessary for the specification of initiation and sustainment but not the stereotypic sequence of task related behaviors. Since the initiation and sustainment of behaviors are regulated by cues the worker bees encounter, we asked whether chemosensory perception and/or neuronal pathways for the processing sensory information are compromised in $dsx^{stop/stop}$ worker bees. Sensory information processing involves the higher order integration centers, the mushroom bodies, first order neuropils like the antennal lobe for chemosensory information processing and the tracts interconnecting these regions. Examination of the gross midbrain and antennal lobe morphology using f-actin visualization and confocal light microscopy detected among $dsx^{stop/stop}$ worker bees, an individual that lacked one of the four calyx and its associated peduncle of the mushroom bodies (Fig. 6b, c; supplementary z-stack V14, Table S20, 21). Other individuals had extra and distinct, circular f-actin positive signals (reviewed in Groh & Rössler, 2020), which are indicative for a high density of synapses (the microglomeruli; Fig.6d-g), in regions with low f-actin signal within the calyx cup, belonging to the Kenyon cells. Further we identified other f-actin positive structures between the lobula and lateral horn, while the antennal lobes were not affected (Fig. 6h, i; supplementary V13-20). The lack of axon bundles and the extra formations of neuronal tissues which were never identified in wildtype controls, suggest that some neuronal projections were misguided in regions of the midbrain, resulting in varying phenotypes. We then investigated whether *dsx* is expressed in the brain throughout development. Semi-quantitative RT-PCR revealed that the *dsx* gene is expressed during pupation and the formation of the adult brain (Fig. 7a). We conclude from these results that *dsx* activity is essential for projection of distinct neuronal clusters during the development, which involves elements of the higher sensory processing centers of the honeybee midbrain. 25 % of 28 $dsx^{stop/stop}$ worker bees showed these gross morphological defects compared to wildtype controls (n = 29; Fisher´s exact test P = 0.005; Table S21). There may be other subtle changes in the axon projections in other individuals, that we cannot detected on this level of analysis, limited by the staining method and the lack of resolution of neuronal cluster patterns. Because the malformation was restricted to some regions of the midbrain, we next asked whether *dsx* expression and instruction is confined to distinct neuronal cluster

of cells or projections. It has been previously difficult to ascertain the Dsx neuronal clusters. Now, we used CRISPR/Cas9 mediated homologous repair to insert myr-GFP and endopeptidase P2A coding sequence in the place the Dsx translation start codon, so that the transcript would encode both GFP and Dsx^F proteins in the same cell. The worker progeny of such queens never showed a disruption of brain morphology, which we observed in $dsx^{stop/stop}$ worker bees, indicating that the *dsx* activity was not depleted on these $dsx^{myrGFP/+}$ worker bees. To examine the projection of Dsx positive cells we detected the membrane tethered GFP reported using anti-GFP and confocal light microcopy. In brains of 1-day old adult worker bees (*n* = 25) we detected distinct neurons that were labeled by dsx^{myrGFP}. These neurons project from the calyx through the peduncle (Fig. 7b). The distinct area of the ventral lobe (the division corresponding to the basal ring (Dbr); Fig. 7b) these dsx^{myrGFP} positive neurons are projection through, have been reported to provide elements of the chemosensory and visual pathway (Rybak & Menzel, 1993; Zwaka et al., 2016, 2018). Connecting to the Dbr also single dsx^{myrGFP} neurons were found (Fig. 7b). There may be other subtle labeled dsx^{myrGFP} neuronal clusters, that we cannot rigorously detect on this level of analysis, which is limited by the level of dsx^{myrGFP} expression and detection afforded by light microscopy. Nonetheless, these results suggest that *dsx* activity is required to instruct neuronal projections in the mushroom body during development. This involves neuronal projections from the calyx to the peduncle that are elements of the chemosensory and visual pathway.

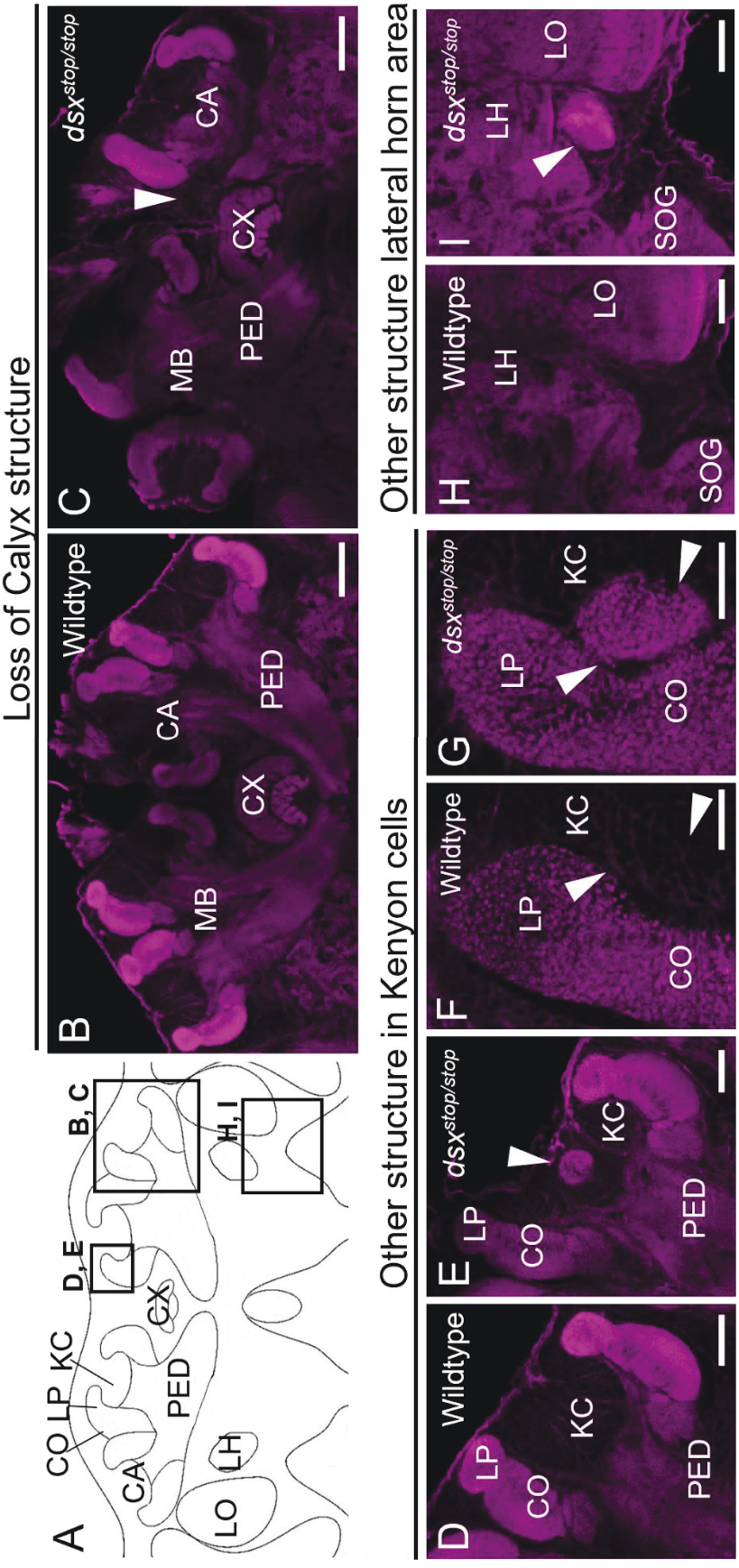


Figure 6: *dsx* activity is required for projections of neuronal clusters in the worker bee midbrain. Phalloidin stained midbrain labeling f-actin. **A.** Schematic drawing of honeybee brain. LO: lobula, LH: lateral horn, CA: calyx, PED: peduncle, CO: collar, LP: lip, KC: Kenyon cells, CX: central complex. Boxes indicate the sections displayed. **B, C.** Section of the mushroom body (MB) CAs in wildtype controls and $dsx^{stop/stop}$ worker bee (#43). Arrowhead marks the loss of one of the four CA and the associated PED in the right hemisphere. **D, E.** Section of the CA in wildtype controls and $dsx^{stop/stop}$ worker bee (#9). Arrowhead marks f-actin positive neuronal structure in the KC. **F, G.** Section of the CA in wildtype controls and $dsx^{stop/stop}$ worker bee (#17). Arrowhead marks neuronal structure in the KC adjunct to the LP and CO. **H, I.** Section of the approximate LH position and LO in wildtype controls and $dsx^{stop/stop}$ worker bee (#17). Arrowhead marks neuronal structure between LH and LO. Scale: B - I 50 µm.

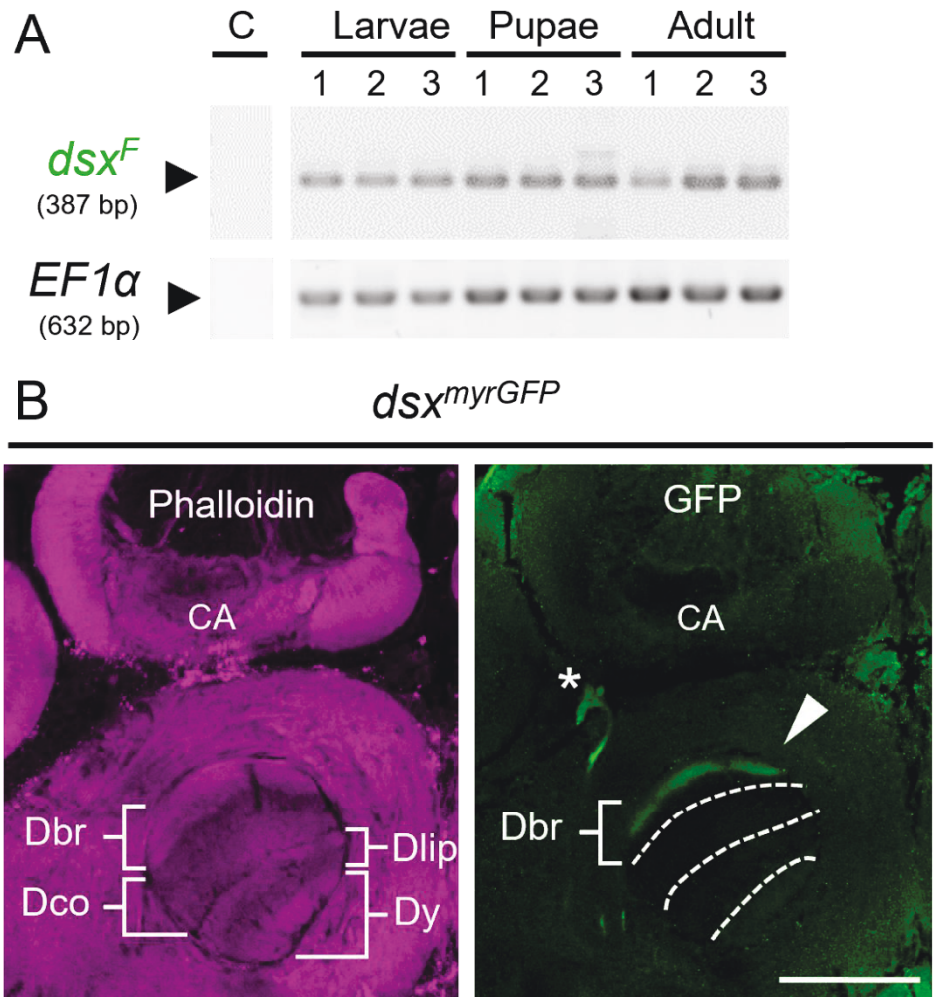


Figure 7: *dsx*^{F} expression in the worker bee CNS. **A.** Female-specific *dsx* transcript (*dsx*^{F}) in brain tissues at different developmental stages. Sex-specific transcripts were amplified vie RT-PCRs from individuals and resolved in agarose gels. PCRs were semi-quantitatively adjusted across individuals using ef-1α gene (EF1α) as control. Pictures are inverted. EF1α: elongation factor 1α, C: null control for RT-PCR. *n* = 3 for each developmental stage. **B.** The *dsx*^{myrGFP} expression in the central nervous system (CNS). Phalloidin (f-actin) and membrane bound GFP staining with a section of the calyx (CA) from the same brain (*n* = 25). The layers of the vertical lobe (division corresponding to the basal ring (Dbr), division corresponding to the collar (Dco)) and the medial lobe (the division corresponding to the lip (Dlip) and the gamma division (Dy)) are indicated in the phalloidin staining. The boundary of these layers (dashed line), the Dbr localization of the *dsx*^{myrGFP} neurons (arrowhead) and their arborizations (asterisk) are indicated. Scale 100 µm.

Discussion

Sophisticated within group behaviors of the honeybee worker, that are involved in the evolution of eusociality are the product of differences in gene activity between castes and sexes. Therefore, to understand how such behavior from non-reproducing worker bees is specified during development requires the identification of those genes and their behavioral role. In this study we show that worker-specific task and trophallaxis behaviors are specified by the genetic switch *dsx*.

We found that inspection of cells, which is vital for most in-hive activities such as brood care and foot maintenance (Johnson, 2008a), is disrupted in nurse stage $dsx^{stop/stop}$ worker bees. For functional group living, it is indispensable that the synergy of initiation and sustainment of tasks is balanced to ensure that important tasks are allocated. Displaying significantly reduced short IC events, the chances for task initiation of $dsx^{stop/stop}$ worker bees are drastically lowered (Fig. 2a, b). The substantially reduced frequency and length of WIC (Fig. 2b'), confirms that WIC initiation depends on IC frequency and additionally implies that $dsx^{stop/stop}$ worker bees contribute less to task sustainment. Including different comb areas, we found engagement probability to be task-stimuli dependent, as WIC behavior in food and brood areas was disrupted in $dsx^{stop/stop}$ worker bees, indicating that dsx activity instructs the formation of distinct behavioral tasks that can be associated with specific stimuli such as honey or pollen (Fig. 2). Further analysis of the mouth-to-mouth food exchange trophallaxis, showed that the *dsx* instructs the formation of another distinct worker behavior, as $dsx^{stop/stop}$ worker bees displayed drastically reduced trophallaxis frequency and length, while other encounters were not affected (Fig. 3). This highly impacts social group living, as trophallaxis is essential for the exchange of information and food (Farina & Grüter, 2009). During these contacts bees exchange nectar samples or pheromones, for example QMP which provides information about the queens presence (LeBoeuf, 2020). Trophallaxis is a repetitive task, especially for nurse bees, as they engage in feeding brood, drones, other worker bees, and even the queen, suggesting that a *dsx* mutation disrupts the functioning of social group life and thus the social stomach that is essential for a colony´s food maintenance (Karsai & Schmickl, 2020; LeBoeuf, 2020).

As individual distance covered, work area visits and stays were not affected, it can be ruled out that individual preferences influenced the performance and probability of these behaviors (Fig. 4a-c). The gene *dsx* has been previously linked to behavioral

specifications. For example, in *D. melanogaster*, *dsx* activity has been shown to be associated with the capacity to perform sex-specific behaviors, such as sexual receptivity in females (Zhou et al., 2014) or correct song production in males (Kimura et al., 2008; Rideout et al., 2007). Similarly, *dsx* mutants of the plant hopper *N. lugens* exhibit disrupted courtship signaling (Zhuo et al., 2018). Strongly indicating that in the honeybee *A. mellifera* the gene *dsx* also directs the capacity to perform distinct behaviors, confirming our results for specific cell inspection behavior of worker bees.

Gross phenotypic defects, such as those found in *D. melanogaster dsx* mutants that lack, for example, the male-specific sex combs on the legs (Devi & Shyamala, 2013) or have differential abdominal pigmentation (Williams et al., 2008), were not identified in this study as all $dsx^{stop/stop}$ worker bees displayed a wildtype phenotype (Fig. 4d-f) and therefore cannot be associated with the disordered behaviors. The reported disruption of reproductive organ development in several insect species (Camara et al., 2019; Xu et al., 2017; Zhuo et al., 2018) which was also recently demonstrated for honeybee pupae (Roth et al., 2019), was also found in the adult $dsx^{stop/stop}$ worker bees of this study (Fig. S18), confirming the conserved and specific role of *dsx* in sexual development.

The behavioral effects could be explained by *dsx*-regulated worker-specific neuronal development. The aforementioned sex-specific behaviors observed in *D. melanogaster* can be associated with a neuronal dimorphism regulated by *dsx* and *fruitless* that manifest in about 60 sex-specific neurons, the P1 and pC1 cluster (reviewed in Yamamoto & Koganezawa, 2013; Zhou et al., 2014). These neurons appear to act as multimodal sensory integrators receiving olfactory, auditory and visual input (reviewed in Auer & Benton, 2016). Interestingly, we found that $dsx^{stop/stop}$ worker bees have other, extra structures in similar higher integration centers of the bee brain, such as the mushroom bodies and the lateral horn (Fig. 6). The *dsx* mutation mostly affected the mushroom bodies, which make with 368.000 intrinsic neurons (the Kenyon cells) up to 40 % of the total number of brain neurons (Mobbs, 1982; Strausfeld, 2002; Witthöft, 1967). Receiving visual and olfactory input, the mushroom body calyces integrate multimodal inputs that are essential for the perception and processing of stimuli and the formation of long-term memory (Durst et al., 1994; Ehmer & Gronenberg, 2002; Gronenberg, 2001; Hourcade et al., 2009), implying that the other structures and the loss of structures in the mushroom bodies

severely disrupt these processes giving a possible explanation for the disrupted behavior observed in *dsx*$^{stop/stop}$ worker bees. Input from the antennal lobe and lobular tract cross in lateral horn region (Brandt et al., 2005; Ehmer & Gronenberg, 2002; Kirschner et al., 2006), suggesting that the extra structures we found in the lateral horn area have a high potential of specifically disrupting chemosensory and visual stimuli transmission (Fig. 6h, i). This is strongly supported by the fact that Dsx expression is limited to the division corresponding to the basal ring of the ventral lobe and further seems to overlap with the A3 neurons connecting the Kenyon cells, calyces, and ventral lobe (Fig. 7c). A3 neurons are GABA-ergic feedback neurons that have been identified to adapt the response in context-specific forms of learning, indicating that they regulate mechanisms that are involved in attention responses to specific stimuli (Filla & Menzel, 2015; Zwaka et al., 2018). Studies in *D. melanogaster* provide evidence that gross structural loss or malformation of the MBs are most likely caused by deficits in axon-guidance pathways (Bates et al., 2010), indicating a possible role of *dsx* in general axon forming processes during development, essential for the development of neuronal tracts such as the A3 neurons or the antennal lobe and lobular tract. A recent study identified a novel interaction between *dsx* and the Hox gene *abdominal-B* (*abdB*) that acts on sexually dimorphic enhancers, drastically increasing the regulatory effect of *dsx* on yet unknown genes that could be involved in axonal guidance or sex-specific neuronal organization regulated by *dsx* (Ghosh et al., 2019).

Although the neuronal deficits of the *dsx* mutants are obvious, they were observed in only 25 % of the mutants examined, implying that the behavioral defects could also be caused by disrupted function of other, non-neuronal tissues. In the honeybee, the fat body has been shown to directly affect behavior through differential gene expression of the egg yolk precursor *vitellogenin* (*vg*). The *vg* gene is thought to be co-opted in worker bees to potentially regulate aspects of task division (Münch & Amdam, 2010). Interestingly, *vg* is a direct target of Dsx in *D. melanogaster* (Burtis et al., 1991; Coschigano & Wensink, 1993), implying that *vg* levels in the honeybee fat body may depend on Dsx activity, strongly suggesting that the *dsx*$^{stop/stop}$ worker bees in this study have disrupted *vg* expression or activity (Engels & Fahrenhorst, 1974). Since *vg* affects immunity, stress resistance, brood care (*vg* expression in HPG) and reproduction (reviewed in Münch & Amdam, 2010) a combination of disrupted *vg* levels and neuronal deformation due to the lack of *dsx*

gene could be the cause of impaired task and trophallaxis performance in *dsx*$^{stop/stop}$ worker bees.

Task engagement is genetically specified

Worker bees of advanced social insects are born with behaviors that are remarkable for their complexity. The worker bees perform sophisticated within group behaviors that collectively produce evolutionary new but species-specific features. Colony-level outputs involve nest building and collective brood rearing, which are linked to the task repertoire, communication and learning abilities of worker bees (Hölldobler & Wilson, 2009). Development hereby endows the capability of these behaviors that are stereotypic and characteristic for the honeybee. Given the complexity of worker bee behaviors, one might expect that the genetic logic underlying the developmental specification of these complex behaviors is fundamentally different from that uncovered for sex-specific behaviors in *D. melanogaster* (reviewed in Auer & Benton, 2016). At the other extreme, it could be argued, that such behavioral complexity requires the combined action of a large number of genes organizing the "hardwiring" of the nervous system (Greenspan, 1995), implying that no single gene has the capacity to specify major aspects of worker bee behavior on its own. For example, a disruption of the *forkhead* box TF FOXP2 in mice brains affects distinct social approach behavior toward interaction partners, while the gross cortical morphology and other behaviors are not affected (Medvedeva et al., 2019).

How sophisticated group behavior of advanced social insects is regulated has long been a central interest of behavioral biology and physiology. In honeybees, a key aspect of group behavior is that worker bees engage in more than 50 behavioral tasks in an age-dependent manner (Johnson, 2008a). Although the set of task behaviors a worker bee engages with gradually change with age, a worker bee performs multiple distinct tasks during a day. By moving on the combs, bees encounter various tasks and task-related stimuli that ultimately trigger behavioral engagement (Johnson, 2008a, b; Seeley, 1982). A worker bee at nurse stage, for example, priorities its engagement into food uptake, feeding the larvae, cleaning the cells and food exchange tasks while other tasks are performed at lower rate. Hence, a key question is how the capability to control task behavior is programmed during development and organized in the nervous system. This question is particularly

challenging since it is not sufficiently resolved how genes program and prioritize choice among multiple tasks during development. Although we focused only on a subset of nurse bee task behaviors, our results show that task initiation and sustainment are programmable features. This is evidenced by the requirement of dsx^F activity for initiation frequency (IC) and sustainment length (WIC) in distinct task behaviors. Thus, it appears that the initiation feature regulates engagement probability with task-related stimuli, whereas the sustainment feature regulates the length of engagement. We suggest that this length control may require sensory input to maintain behavior trough positive feedback. Our findings thus support the general view that specific task engagement is genetically specified (Hunt et al., 1998; Hunt & Page Jr, 1995), as we provide with *dsx* a main effector gene for the developmental programming of initiation and sustainment features. When task engagement is genetically determined, the question of how selection among different tasks is regulated arises. Studies have shown that worker bees engage in different tasks with different priorities (Johnson, 2008a, b; Seeley, 1982), but how a task is ultimately prioritized remains unclear. Our data strongly suggests that the initiation process is related to the task, as $dsx^{stop/stop}$ mutants show quantitative and qualitative impairments that depend on the task the bees encounter (Fig. 2). This implies that the genetic regulation of task initiation, priority and sustainment determines preference for engagement in a particular task, ultimately favoring one task over another. As for group living, the many individually allocated tasks in turn also influence the number of worker bees that initiate a specific task. This has been demonstrated previously as the collective output of a honeybee colony can be regulated by quantitative genetic differences that affect pollen hoarding and stinging behavior in worker bees (Hunt et al., 1995, 1998). In the case of pollen hoarding, higher levels of engagement lead to larger pollen stores in the colony, demonstrating that genetically predetermined task preferences influence group-level outputs (Beshers & Fewell, 2001; Fewell & Page, 1993; Page & Erber, 2002). Hence, our results suggest that initiation, priority, and sustainment are developmentally programmable innate features that impact the characteristic output at the colony level.

How does *dsx* act so specify task engagements?

The developmental programming of behaviorally distinct queens and worker bees from the same genome has been shown to require the input from a caste-determining signal instructed by differential nutrition (Asencot & Lensky, 1988; Haydak, 1970; Leimar et al., 2012). Here, we show that a female-specific transcription factor, the dsx^F gene, is required to directly specify task engagements of worker bees. Importantly, the gene dsx^F does not affect this behavior as it happens, but rather instructs the capacity for specific behaviors in adults during development. This is supported by our finding that disrupting *dsx* activity in female embryos leads to other, extra structures and structural losses in adult worker bees (Fig. 6). Since the worker bees were no more than 13 days old when analyzed, the neuronal differences can be attributed to the absence of Dsx during development and not to dendritic outgrowth in adult worker bees associated with the onset of foraging (Farris et al., 2001). These results suggests that programming of worker traits is a result of the combination of sex- and caste-specific inputs during development. Although worker bees forgo reproduction, sex-specific instruction appears to be an important regulatory factor that also controls worker-specific behaviors. As the capacity to perform behavior resides in the neuronal structure and organization of the brain, uncovering the neuronal basis of task behavior is an important step toward further understanding. At the gross morphological level, mushroom bodies are larger in worker bees than in queens, which appears to be a substrate for the control of more complex behaviors (Groh & Rössler, 2008). Here we provide evidence that aspects of neuronal and synaptic organization in the MB are a consequence of dsx^F activity. Neuronal malformation in the higher integration center of the $dsx^{stop/stop}$ worker bee brains combined with the behavioral impairment in task engagements, suggests that Dsx-positive neurons play a role in integration sensory information to control the initiation and sustainment of tasks. This is strongly supported by our finding that Dsx expression is limited to stimuli processing neurons, further implying that the *dsx* gene instructs worker-specific neuronal wiring. Future studies should aim to demonstrate and characterize the role of *dsx*-expressing brain cells in assessing sensory information and selecting, prioritizing, and modifying behaviors for distinct tasks. This will refine our understanding of how sophisticated group behavior is organized in the nervous system.

It is important to emphasize that *dsx* provides a substantial target for evolutionary change. A recent study in ants indicates that a high degree of social organization may be associated with the evolutionary pattern of *dsx*. While species that have retained the c-terminal OD2 domain of *dsx* in its ancestral form display a less pronounced form of division of labor (DOL), species that have lost the OD2 domain display a higher form DOL (Jia et al., 2018). This suggests strong positive selection on *dsx*, which favors the evolution of advanced living ant species. For DOL, plasticity of task behavior is essential for adaptation to social and environmental change, which we have shown is genetically programmed by the features of initiation, priorities, and sustainment. Constant diversification of task behavior through constant acquisition of new links to sensory inputs creates an evolutionary path that is the hallmark of advances social insects. Our study demonstrates that a single transcription factor, the *dsx* gene, is required for the specification of such advanced group behavior and specifically directs the initiation and sustainment of behaviors that play a key role in the task organization of insect societies.

Methods

Bee handling

The honeybees we used in this study derived from feral *Apis mellifera carnica* colonies from our aviary in the botanical garden of the Heinrich-Heine University, Düsseldorf, Germany. Female embryos (which are diploid) were collected from eggs laid from naturally mated queens, caged in Jenter egg collecting cages (Jenter Queen rearing Kit, Karl Jenter GmbH, Frickenhausen, Germany) which were placed in “Ablegerbeuten” (Holtermann, Germany). Eggs were collected every 1.5 hours (Schulte et al., 2014) from the Jenter egg collecting boxes. Queens were caged for 3 days, and 4 days freed, to maintain a healthy colony. For the tracking we collected newly emerged bees (0-24 hours old), from a sealed comb incubated at 34 °C.

Gene targeting, sgRNA synthesis and microinjection

sgRNA synthesis and microinjections have been previously described (Roth et al., 2019). Target sites for the sgRNAs (1 = GAACGAGCAAAACAGAGCCG, 2 = GTGC ACGATGTCTGAATCAT) were identified using Benchling [Biology Software] (2017), retrieved from *https://benchling.com* and as shown in Roth et al. (2019). Target site of sgRNA1 was 31 bp after start codon and target site of sgRNA2 201 bp after start codon, inducing a deletion of 170 bp (supplementary Table S23). 375 ng/µl Cas9 protein (EnGen Cas9 NLS, *S. pyogenes*, #M0646, New England Biolabs) was used in a molar ratio of Cas9 to sgRNA1 to sgRNA2 of 1:1:1. Embryos were injected each with 400 pl sgRNA/Cas9 mixture at the age of 0.5 - 1.5 hours after oviposition (Gempe et al., 2009) using handcrafted pipettes (53 mm, Hildenberg, Malsfeld, Germany).

In-vitro rearing

Injected and control embryos were kept in humid conditions with 16 % sulfuric acid to prevent mold. On the day of hatching sulfuric acid was replaced with water. Freshly hatched larvae were grafted into nicot pots with 170 µl worker nutrition from Kaftanoglu et al. (2010; diet 7: 53 % royal jelly, 4 % glucose, 8 % fructose, 1 % yeast extract and 34 % autoclaved water) and kept at 90 % relative humidity (generated using saturated solution of K_2SO_4 adapted from Schmehl et al. (2016)) and 34 °C. Before defecation, larvae were transferred onto absorbent Kimwipes (Delicate Task

Wipers, #066664, Kimberly Clark), kept in petri dishes for two days and kept at 70 % rel. humidity (saturated solution of $NaCl_2$ adapted from Schmehl et al. (2016)) and 34 °C from this point on. After defecation, prepupae were separated in 24-well plates covered with filter paper (15 mm, grade 413; VWR, International GmbH, Darmstadt, Germany). Adults were observed closely upon eclosion and introduced to freshly hatched wildtype hive-reared worker bees as soon as they started walking. *In-vitro* reared individuals and hive-reared worker bees were then tagged with 2D barcodes to enable individual tracking (Blut et al., 2017). To create an artificial hive, as many hive-reared wildtype worker bees were added until a colony size of 500 bees was reached (supplementary Table S24). All bees and the queen in the artificial colony were tagged with individual a 2D barcode.

Automatic bee tracking

Computer-based automatic bee tracking was done using the setup described by Blut et al. (2017). We tracked individual worker bee behavior in an artificial hive of 500 bees (see above) in five replicates. Bees were tracked at nurse age, for this reason the artificial hive is kept on a comb with *ad libitum* honey and pollen for 6 days (which equals a bee age of 6 days after emergence) in the dark at room temperature. On the 6^{th} day, bees were cooled down to be transferred onto a standardized comb providing the same amount of honey, pollen and brood for each replica established by Blut et al. (manuscript submitted). To generate the standardized comb, we used templates to position the areas in similar positions on the comb (supplementary Fig. S5). In the pollen area we distributed 30 grams of pollen (“Echter Deutscher Spezial Blütenpollen”, Werner-Seip-Biozentrum GmbH & Co. KG, Butzbach, Germany) equally distributed among the cells. On top of each pollen cell 25 µl of sugar syrup was added (“Ambrosia Futtersirup”, Nordzucker AG, Braunschweig, Germany). The honey area consisted of 550 cells with respectively 200 µl sugar syrup per cell. The brood area consisted of a piece of comb containing 151 3^{rd}-4^{th}-instar stage honeybee larvae that was positioned in the center of the comb (supplementary Table S6). Once the artificial hive was transferred onto the standardized comb, it stayed in an incubator at 34 °C overnight. On day 7 (equaling bee age of 7 days) the tracking was started at room temperature and in the dark for 48 h using the computer-based Bee Behavioral Annotation System (BBAS; Blut et al., 2017). In-vitro reared $dsx^{stop/stop}$ and control as well as hive-reared wildtype worker bees all reached 7 days of age

when the tracking stared, ensuring they reached the nurse stage. After tracking, all *dsx*$^{stop/stop}$ mutant, all control, and some hive-reared wildtype bees were carefully collected and kept in wooden boxes with *ad libitum* pollen paste and water at 34 °C (until sensorimotor tests). Then the detection rate and the brood maintenance efficiency were recorded by counting pupae-staged individuals in the brood area of the standardized comb (supplementary Table S7, 8). For each bee, positional (pollen, honey, brood or empty cell area) and orientation information is obtained with a frame rate of 0.25 seconds. Occupation time (minutes spent in area/hour), number of visits (visits/hour) and speed (meter/hour) were measured using C^{++} and Java script (Blut et al., 2017). Further we calculated occupation time per visit (minutes/visit/hour) with the data given. Parameters were obtained hourly. As a bee's trajectories were not continuous, for individuals that showed a detection rate lower than 10 % per hour (= 6 minutes), this hour was excluded. Additionally, individuals needed to be tracked for at least 12 hours within a 24-hour window (= 50 %) to be included into the evaluation. We then calculated average data according to the thresholds for each individual. For example, after the thresholds were applied one obtained 18 hours (maximum 24 hours) of speed data for an individual, the average speed was calculated by dividing by 18. We evaluated all *dsx*$^{stop/stop}$ worker bees that fulfilled the threshold requirements, resulting in n = 47 for *dsx*$^{stop/stop}$ worker bees. Number of control worker bees was adjusted according to the number of *dsx*$^{stop/stop}$ worker bees evaluated for each replicate but was never lower than n = 8 per replicate, resulting in n = 49 for in-vitro control worker bees. In-vitro reared controls for evaluation were chosen blind and randomly (see supplementary Table S24).

Automatic and manual behavior classification

Using the tracking data, we generated per-frame features using the program JAABA (Kabra et al., 2013) to obtain information about the bees characteristics relative to their nearest nestmate in each frame. Behavior was analyzed for the same bees as described above, however as in the chosen video material some bees were detected and some not, the numbers changed slightly (see supplementary Table S24).

We used the `encounter classifier` from Blut et al. (2017) in 60 minutes of tracking and video material to predict trajectories (video sequences) with encounter behaviors and proceeded with manual evaluation of these trajectories. An average of 21 minutes of trajectories were evaluated for each bee. Note that most encounter

trajectories are only a few frames long which equals seconds in video material. With the help from the automized detection we proceeded to manually evaluate trajectories that were predicted to contain encounter behavior. Manually analysis was necessary as potential behavioral differences exhibited by mutant bees needed to be accounted for. Manual analysis was done using the free video tool VirtualDub (VirtualDub-1.9.11, virtualdub.org) with an software addition that enabled the display of the individual bee ID numbers in the video material (Mersch et al., 2013). Individuals were manually analyzed blinded. We manually analyzed antennation, begging and trophallaxis behavior. Antennation is the palpation of another bees' antennae. During antennation bees can be positioned face-to-face or in angle to each other (supplementary videos V5, 6). A bee performing begging behavior orientates its antennae towards the contact bee, tilts the head up, in some cases extends the proboscis, and reaches the other bee with the front legs (Free, 1959; Korst & Velthuis, 1982; supplementary videos V9, 10). Trophallaxis behavior is observed when two bees are in contact with their antennae and their proboscises are outstretched towards the other bee. Trophallaxis contacts mostly end in food transition (Korst & Velthuis, 1982; supplementary videos V7, 8). Frequency was calculated for antennation, begging and trophallaxis behavior and average length of trophallaxis contacts was measured.

To produce the 'classifier cell inspection', we labelled examples of cell inspection and non-cell inspection behavior in 280 minutes of video material using the graphical interface of JAABA (Kabra et al., 2013) in a group of 250 bees (for more detail Blut et al. 2017). Only cell inspection and non-cell inspection behaviors with high confidence for classification were labeled. We used the information from the per-frame features to train the ´classifier cell inspection´ using machine learning implemented in JAABA. Accuracy of the classifier was determined using cross-validation method in a group of 250 bees. For this we used the default setup of JAABA for cross-validation, we did 10 cross-validations. We generated 91.8 % positive behavior and 87.3 positive non-behavior detections. 8.2 % behaviors were detected false positively and 12.7 false negatively. Following we used the cell inspection classifier to predict trajectories with cell inspection behavior in 150 minutes tracking and video material and subsequently manually evaluated these trajectories, to ensure potential behavioral differences are accounted. Manual evaluation was done as described above. An average of 58 minutes of predicted

trajectories was evaluated for each bee. Note that cell inspection behavior can last several minutes per inspection. Cell inspection events were manually categorized according to the length per cell inspection event. Events < 5 seconds were assigned as inspecting cells (IC) and events ≥ 5 seconds as work in cell (WIC; see supplementary videos V1-4) behaviors. Additionally, the area on the comb (honey, pollen, brood or empty cells) where the cell inspection was performed was noted. Frequency was calculated for IC and WIC behavior and average length of WIC events was measured for respectively each cell area and all cell areas combined.

Data analysis and statistics

Statistical analysis was performed using Systatt and IBM SPSS Statistics 27 software. Mann Whitney U-test was used for pairwise comparison. To test against zero (no change) the one sample t-test was used for parametric data and one sample Wilcoxon for non-parametric data.

Morphological analysis

For morphological analysis bees were anesthetized on ice. Images were taken using a binocular (S8 APO, Leica) with an attached camera (UI-1240LE-C-HQ) and the software uEye Cockpit (IDS). For head images, bees were decapitated and positioned on black glass slides. Head size was measured by dividing the maximum head length from nearest ocelli to bottom by the maximum head width. Head width was measured at the level of antennal basis and in an 90° angle to the head length measurement. Images were analyzed using FIJI (ImageJ 1.53c; Wayne Rasband, National Institutes of Health, USA).

Sensorimotor tests

Individuals were tested in the dark under red light conditions under a laboratory hood. Bees were between 10 and 13 days of age during sensorimotor tests and were conducted subsequent the tracking on the comb. We used a polystyrene Petri dish (14 cm diameter) where we introduced 5 mm openings at the top and the side for ventilation and placed it on a paper grid (1.5 cm^2 pattern; adapted from Humphries et al. (2005); supplementary Fig. S19). The essays for respectively control and $dsx^{stop/stop}$ worker bees were run in presence of two wildtype worker bees reared in

the hive. Bees were placed in the middle of the arena and left 10 to 12 minutes before the essays started. For locomotion, grid mark crosses of the bee were counted for 2 minutes (line crossings/minute). Grids were counted when a line was fully crossed by the head of the bee and repeated crossings which resulted from an immediate change of direction where not counted twice (Humphries et al., 2005). For odor perception we used strip of filter papers (75 mm, grade 413; VWR, International GmbH, Darmstadt, Germany) to present odors through a hole on the side of the Petri dish. Response to solvent was tested by first introducing an empty strip of filter paper for 1 minute, followed by filter paper with 0.5 µl of isopropanol to the arena and measuring line crossing during presentation. For respectively the repellent and pheromone testing we applied 0.5 µl of benzaldehyde/ isopentyl acetate (IPA) to a strip of filter paper, left it for 1 minute before introducing it for 1 minute to the test arena. We measured line crossings for both tests. Positive phototaxis was examined by the number of responses in walking towards and reaching a light pulse. Four LED light sources (220 lumen, 2700 K) evenly distributed around the arena were used for light pulses. Light was given for 10 seconds followed by 10 seconds lights off for respectively six times. During each 10 seconds light on, the light source furthest from the bee was illuminated, then switched off again (Scheiner et al., 2013). For the next 10 seconds light on, the furthest light source from the bee's new position was switched on, and so on. Arenas and grid base were exchanged after each bee tested. Line crossings and light responses were counted from video recordings (60 fps, Full HD, 44100 Hz; Casio Exilim Pro EX-F1) using VSDC *Free Video Editor* (Multilab LLC).

Immunohistology and image processing

For brain dissection, bees were anesthetized on ice, decapitated and the head fixed in wax coated petri dishes. Covered in ice-cold honeybee saline (130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 15 mM Hepes, 25 mM glucose, 150 mM sucrose, pH 7.2) a window between the eyes was cut, the brain tissue dissected and immediately fixed in 4 % ice-cold formaldehyde (Roth, Karlsruhe, Germany) in phosphate-buffered saline (PBS, pH 7.2) at 4 °C for minimum 24 hours. All following washing and incubation steps were performed on a shaker and at room temperature, if not mentioned otherwise. Brains were then washed 3 x 10 minutes in PBS, 10 minutes in PBS with Triton-X 100 (2 % PBS-T) and 2 x 10 minutes in 0.2 % PBS-T,

followed by 1 - 2 hours incubation in 0.2 % PBS-T with 2 % NGS; all steps at room temperature on shaker. Following steps were adapted for the application for *f-actin* or GFP labeling.

For *f-actin* localization brain tissue of 11-to-14-day old bees (dissected after sensorimotor tests) was then incubated in 0.2 % PBS-T with 2 % NGS and 0.2 units of Alexa Flour 568 phalloidin (Molecular Probes, A-12380, Eugene, USA) for 2 days at 4 °C on shaker. Next the brain tissues were washed 4 x 5 minutes in PBS, subsequently dehydrated in an isopropanol series (10, 30, 50, 70, 90, 100 and 100 % isopropanol in PBS, 5 minutes each step), cleared in methylsalycylate (MS; Sigma Aldrich, Steinheim, Germany) and mounted in fresh methylsalycylate.

For GFP labeling brain tissues of 1-day old worker bees were incubated with 1:1000 chicken-anti-GFP (Rockland Immunochemicals, Inc., Limerick, PA, USA) in 0.2 % PBS-T with 2 % NGS for 4 days at 4 °C. Next the brain tissues were washed 3 x 5 minutes in PBS and incubated with the secondary antibody goat-anti-chicken (1:250; Fisher Scientific, Schwerte, Germany) and Flour 568 phalloidin (1:250) in in 0.2 % PBS-T with 2 % NGS for 2 days at 4 °C. The brain tissue was then washed 3 x 5 minutes in PBS, subsequently dehydrated in an isopropanol series as described above, cleared and mounted in fresh MS.

Isopropanol was used to enable a phalloidin labeling of whole mount preparations. Stored at 4 °C in dark until imaging. If samples were stored longer, phalloidin (1:500) was added in the last isopropanol step (100 %). Brain samples from FISH and immunohistochemical staining were imaged with a confocal laser scanning microscope (Leica TSC SP8 STED 3X, Leica Microsystems, Wetzlar, Germany) equipped with a white light laser. Image z-stacks with an optical thickness of 3.0 - 6.0 µm of entire brain samples were imaged using a 20x objective (multi/ NA 0.75) generating tile scans which were merged using the processing tool Mosaic Merge of LAS X (Leica Application Suite X 3.0.0, Leica Microsystems CMS, Wetzlar, Germany). Magnifications were imaged using a 40x objective (water/ NA 1.10). Images were processed using FIJI (ImageJ 1.53c; Wayne Rasband, National Institutes of Health, USA) and LAS X. If necessary, brightness and contrast were adjusted.

CRISPR/Cas9 mediated homology-directed repair

We used CRISPR/Cas9 to insert a DNA fragment via homology-directed repair (HDR) in the gene *dsx* (Wagner and Seiler, in submission). For the N-terminus of GFP we used N-myristoylation (myr) to target proteins in the cell membrane as it has been shown to improve the signal strength (Pfeiffer et al., 2010). The myr sequence encodes the first 85 amino acids of the *Drosophila melanogaster src oncogene* at 64B (Src64B), binding the GFP protein to the membrane (Pfeiffer et al., 2010; Resh, 1999). The myrGFP fragment used was synthesized as a standard gene (Eurofins, Ebersberg, Germany) and delivered inserted into the vector pEX-A258 (standard vector; Eurofins, Ebersberg, Germany). For the design of the 1544 bp long myrGFP DNA fragment, we followed the construct design of Wagner et al. (in submission), which has already been successfully integrated into the honeybee genome via CRISPR/Cas9 mediated HDR (see supplementary Table 20 for nucleotide sequence). We used the same homologous sequences of exon 2 of the *dsx* gene (NCBI; gene ID; 725126; Reference Sequence: NC_037642.) as shown in Wagner et al. (in submission). At the 5´ end of the insert, two nucleotides were inserted for an additional glycine to remain within the open reading frame of *dsx* (supplementary Table S19). The 3´end of the insert consists of a Gly-Ser-Gly (GSG) linker and a 2A peptide (P2A) sequence (Szymczak-Workman et al., 2012). Peptide 2A has a self-cleaving mechanism that separates the GFP protein from the Dsx protein. Sequences for the insert were codon-optimized for a nucleotide distribution typical for the *A. mellifera* genome without generating changes in the amino acid sequence. For injection we amplified the myrGFP DNA fragment by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Braunschweig, Germany) and oligonucleotide primers (5´- GTTGCAGAACGAGGAATCGGGGGAAAG-3´; 3´- TGA TCTTACACTTCTCGCAGGTACAAGTACG-5´; Eurofins, Ebersberg, Germany). The PCR profile was as follows: 160 seconds at 94 °C, 35 amplification cycles of 30 seconds at 94 °C, 40 seconds at 56 °C, 140 seconds at 72 °C and 5 minutes extension at 72 °C in a thermal cycler. The injections were performed as described above using 18.5 pg sgRNA1, 200 pg Cas9-protein and 20 pg of the myrGFP donor DNA for the injection mixture.

Queen rearing and insemination

Eggs were handled as described above and reared to queens (adapted from Schulte et al., 2014). For this freshly hatched larva (~72 hours) were carefully grafted in queen cell cups with gelee royal which were then placed in a queen rearing frame (Holtermann, Germany) and introduced to a queen-less colony. After 6 days in the colony, the queen-frames were placed in an incubator with 34 °C. The emerged queens were placed in small, wooded boxes with freshly hatched worker bees with *ad libitum* water and pollen paste. The queen's genotype was determined using PCR amplification (see DNA preparation). In the following only queens carrying at least one dsx^{myrGFP} allele were used. Between the age of 12 to 19 days the myrGFP queens were treated with CO_2 and inseminated with wild type drones on the following day in collaboration with the lab of Prof. Dr. Bernd Grünewald using standard insemination techniques (Cobey et al., 2013; Collins, 2000). Inseminated queens were kept in "Kieler Begattungskasten" (KBK; Holtermann, Germany) in a free flying arena. Caped brood frames of dsx^{myrGFP} queen colonies were placed in an incubator with 34 °C. Freshly emerged worker bee offspring with one dsx^{myrGFP} allele were dissected at the age of 1 day and used for immunohistological GFP visualization.

DNA preparation

To identify CRISPR/Cas9 mediated mutations and HDR in the *dsx* gene we amplified the sequence area of the sgRNA target sites. DNA was extracted from two opposing legs (one leg for the myrGFP queens) using the innuPrep Mini Kit (Analytic Jena, Jena, Germany).

In order to identify the sequence of mutant bees (injection of sgRNA 1 and 2), DNA was amplified at the deletion site using PCR (5`-ACAACGATAGAGGGACAAACAA CCG-3´, 3`-CACTGCCAATCGGCAGCAAGTG-5`, Eurofins, Ebersberg, Germany). Amplicons were deep sequenced using Illumina MiSeq (MiSeq Reagent Kits, paired-end-reads) by generating on average 89000 reads per amplicon and individual. For read mapping and identification of stop mutants that affected all reads we uploaded the sequencing data to the galaxy web platform and used the public server at usegalaxy.org for analysis (Afgan et al., 2016). Unrelated sequences that made up to 6 % of the variants were removed prior to further analysis.

To identify the CRISPR/Cas9 mediated HDR induced in the *dsx* gene we used PCR standard setup (Hasselmann & Beye, 2004) using Phusion High-Fidelity DNA Polymerase. We designed three PCR (1-3) setups to demonstrate the successful integration of the myrGFP fragment. PCR 1 was used to amplify the upstream sequence of the insertion site (5`-GATTCGTAATAATTCCTGTGC-3´, 3`-CACATAT CCTTCTGGCATCGCAG-5`; Eurofins, Ebersberg, Germany). PCR 2 was used to amplify the downstream sequence of the insertion site (5`-CTGCGATGCCAGAAGG ATATGTG-3´; 3´-CTTCCGCTACTCTTACTTTGAC-5´; Eurofins, Ebersberg, Germany). PCR 3 was used to determine whether the myrGFP fragment was inserted on both are alleles or not (5´- GATTCGTAATAATTCCTGTGC-3´; 3´-CTTC CGCTACTCTTACTTTGAC-5´; Eurofins, Ebersberg, Germany).

RNA and cDNA preparation

We isolated total RNA using a Trizol based protocol (adapted from Vleurinck et al. (2016)). RNA was isolated from single brains of respectively 5th instar larvae, redeye pupa and 1-day old adult worker bees. Each brain sample was homogenized in 250 µl Trizol reagent, vortexed for 30 seconds and following incubated for 5 minutes at room temperature. After adding 50 µl chloroform to the samples, they were vortexed for 30 seconds and incubated for 5 minutes at room temperature. Next the samples were centrifuged for 15 minutes at 4 °C and 13,000 rpm. After centrifugation the upper phase was combined with equal volume of isopropanol, incubated for 10 minutes at room temperature, followed by 10 minutes centrifugation at 4 °C and 13,000 rpm. The total RNA pellet was washed with 250 µl of 70 % ethanol and subsequently dried. The RNA pellet was resolved in 23 µl nuclease free water. For first strand cDNA, mRNA was obtained using reverse transcription with 100 pmol Oligo(dT)$_{18}$ primer and 200 units RevertAid Reverse Transcriptase following the instructions of the supplier (Thermo Fisher Scientific) using 11 µl of total RNA. cDNA was stored at 4 °C.

RT-PCR

RT-PCR reactions were done using Taq polymerase (isolated from our laboratory-derived strain of *Escherichia coli*). The PCR profile was as follows: 2 minutes at 94 °C, 35 amplification cycles of 30 seconds at 94 °C, 40 seconds at 63 °C, 30 seconds

at 72 °C and 5 minutes extension at 72 °C in a thermal cycler. The housekeeping gene *elongation factor 1-alpha* (*f α* ; 5´- GATATCGCCCTGTGGAAGTTC-3´, 3´- GCTGCTGGAGCGAATGTTAC -5´) was used as reference. RT-PCR fragments were resolved using agarose gel electrophoresis.

Supporting information

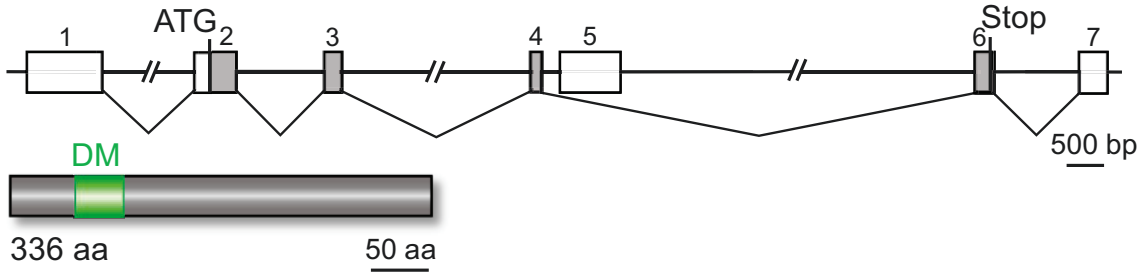


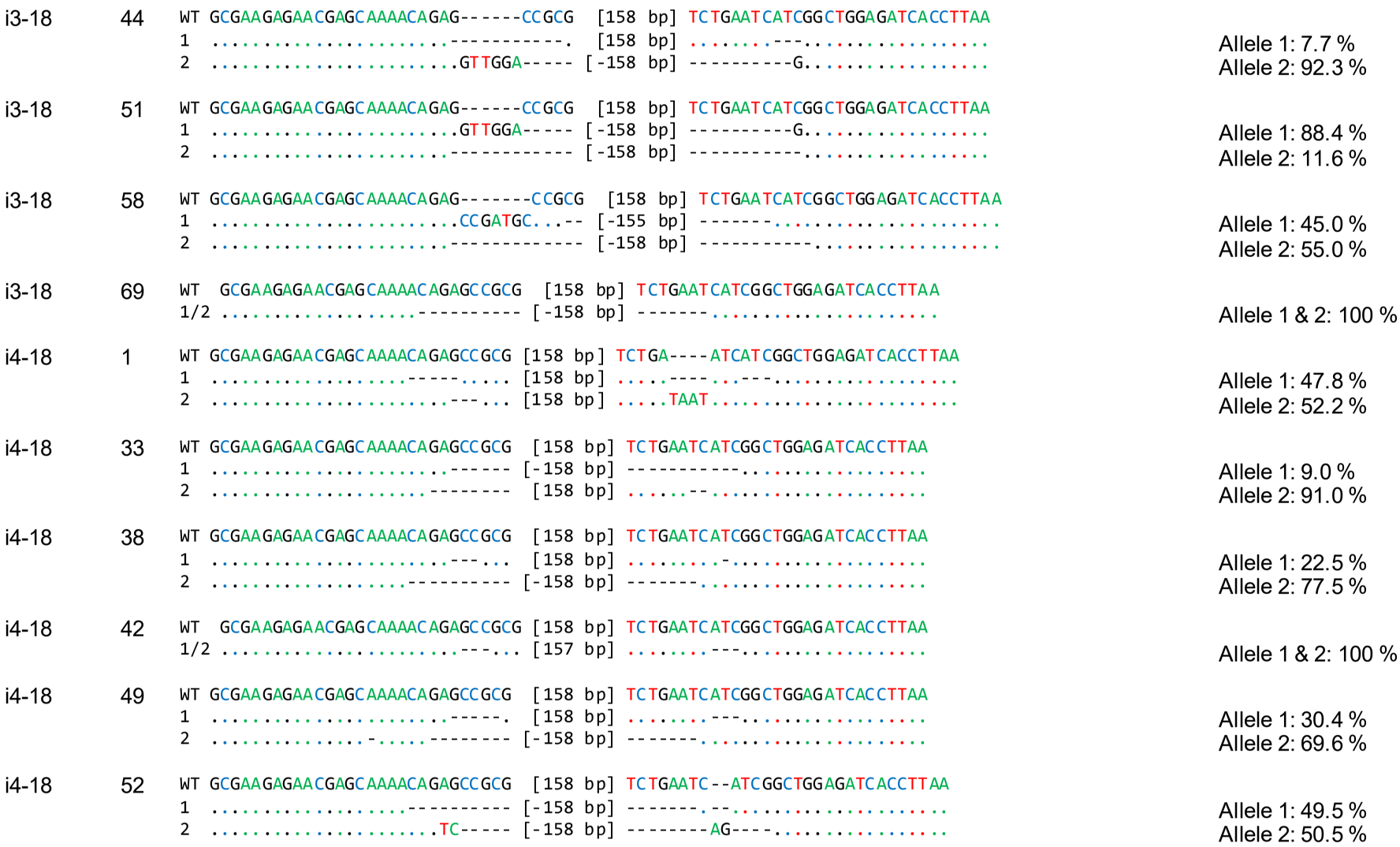
Figure S1. A Scheme of the genomic organization of the male-specifically spliced *dsx* gene. Boxes are exons and interconnected lines are introns. Translation start and stop are indicated. Gray boxes indicate the ORF (open reading frame). Translated male protein is 336 aa long, with the DNA binding motif (DM = Doublesex/MAB-3) domain and the male-specific stop in exon 6.

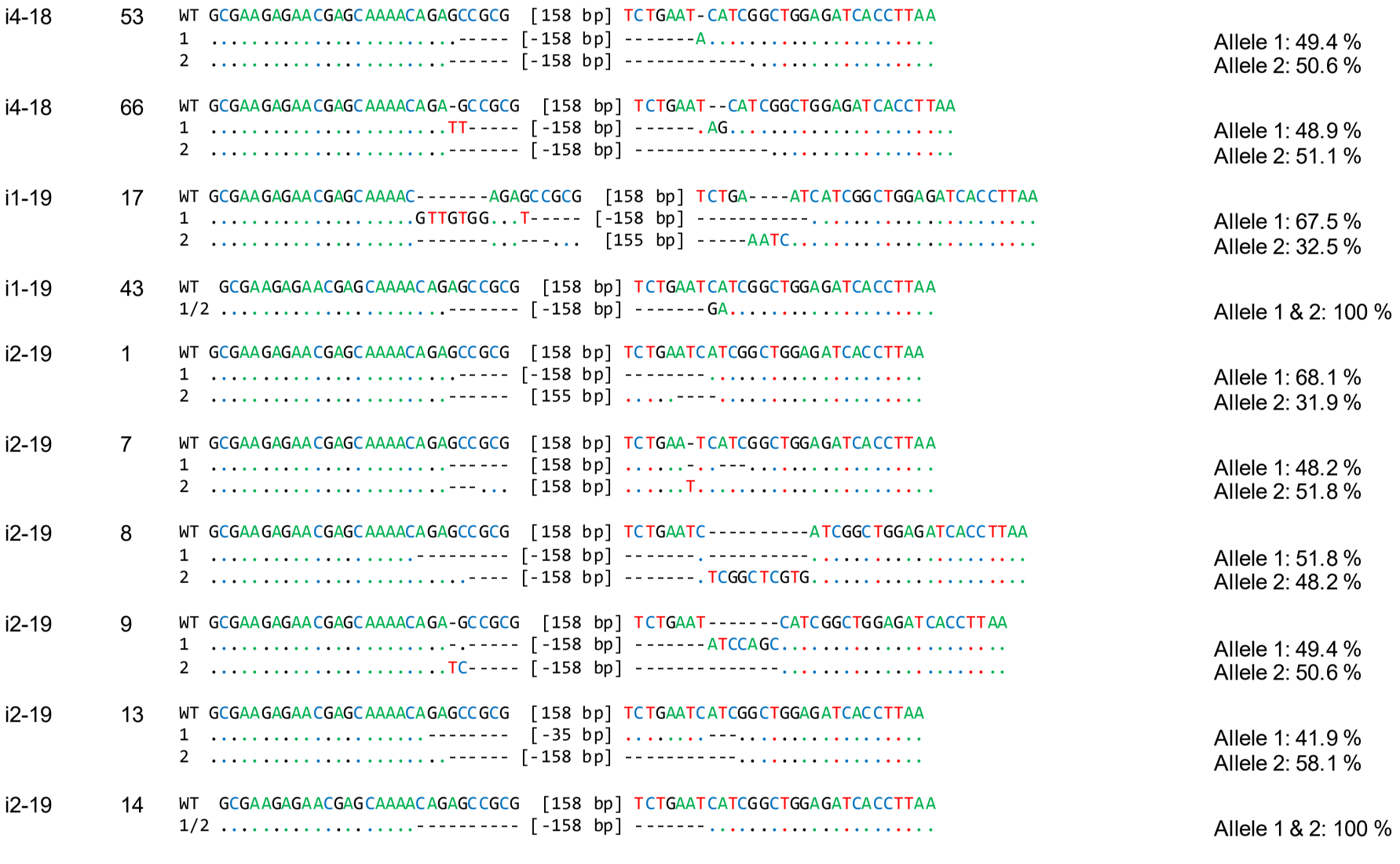
Table S2. Number of genotype identified in CRISPR/Cas9 targeting the *dsx* gene in *Apis mellifera*. We screened for homozygous stop mutations using deep amplicon sequencing (Roth et al. 2019). Total and respectively for injection replicates from 2018 (*n* = 3) and 2019 (*n* = 2). One replicate consists of individuals injected on 3 consecutive days. $dsx^{stop/stop}$ = homozygous stop mutation; heterozygous = stop mutation on one allele; mosaic = three and more allele variants; wildtype = no mutation.

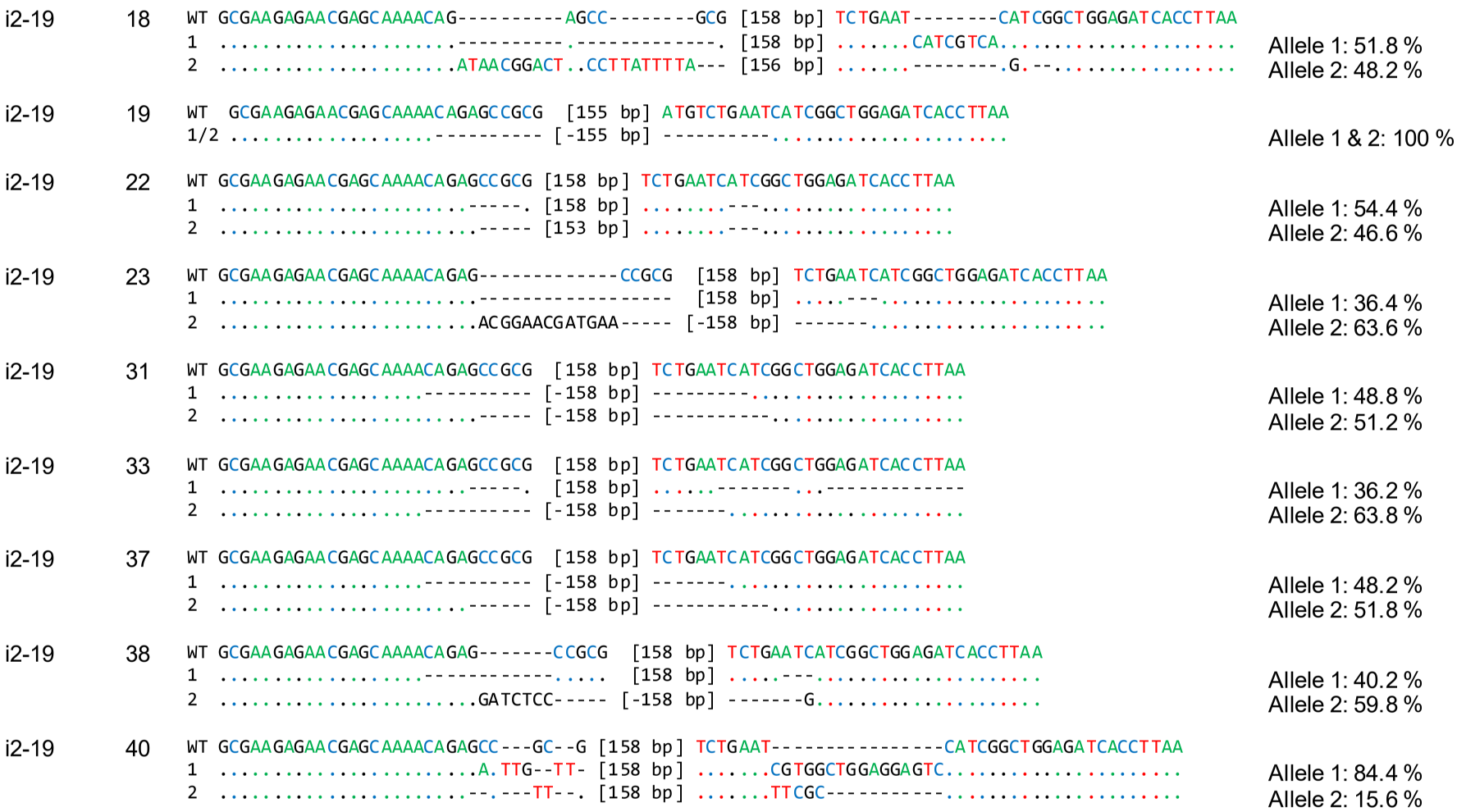
Genotype	No. (%) of adult worker bees	No. (%) of adult worker bees 2018	No. (%) of adult worker bees 2019
$dsx^{stop/stop}$	67 (58.3)	32 (65.3)	35 (53.0)
Heterozygous	26 (22.6)	11 (22.4)	15 (22.7)
Mosaic	15 (13.0)	4 (8.2)	11 (16.7)
Wildtype	7 (6.1)	2 (4.1)	5 (7.6)
Total	115	49	66

Table S3. Genotype sequences of *dsx*$^{stop/stop}$ mutant worker bees. The target sites of sgRNA1 and 2 were sequenced using amplicons. Individuals were screened for homozygous stop mutation on both alleles. This includes individuals that carry the 170 bp deletion and individuals where both sgRNA target sites induced a double strand break causing a stop due to indels. Wildtype (WT) sequence as references for each individual. Frequency of each allele displayed. Variants with 6 % or lower were excluded.

Replicate	id	Alignment of *dsx* sequences	Frequency
i2-18	13	WT GCGAAGAGAACGAGCAAAACA-GAGCCGCG [158 bp] TCTGAATC-A----TCGGCTGGAGATCACCTTAA 1 .....T-T------ [-158 bp] ---------.----..... 2 .....-...----- [158 bp] .....T.CGCC.....	Allele 1: 75.5 % Allele 2: 24.5 %
i2-18	22	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTG------AATCATCGGCTGGATCACCTTAA 1 .....----. [158 bp] ...------..... 2 .....-.....[158 bp] ....ATCGTG.....	Allele 1: 50.0 % Allele 2: 50.0 %
i2-18	43	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA 1 .....--.... [158 bp] .....G.--..... 2 .....------ [-158 bp] ----------.....	Allele 1: 24.6 % Allele 2: 75.6 %
i2-18	58	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA 1 .....---------- [-158 bp] --------..... 2 .....------ [-158 bp] ----------.....	Allele 1: 56.1 % Allele 2: 43.9 %
i2-18	60	WT GCGAAGAGAACGAGCAAAACAGA---GCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA 1 .....TCTC----- [-158 bp] -------..... 2 .....--------- [-158 bp] ----------.....	Allele 1: 45.2 % Allele 2: 54.8 %
i3-18	7	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA 1 .....---------- [-158 bp] -------..... 2 .....------ [-158 bp] ----------.....	Allele 1: 50.3 % Allele 2: 49.7 %
i3-18	29	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGA---------ATCATCGGCTGGAGATCACCTTAA 1 .....------ [158 bp] .....T--------G..... 2 .....----.....[158 bp] .....ATCGTGGAG.....	Allele 1: 49.2 % Allele 2: 50.2 %
i3-18	30	WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA 1 .....-------- [158 bp] ---------..... 2 .....------ [-158 bp] ----------.....	Allele 1: 26.7 % Allele 2: 73.3 %


i3-18 44
WT GCGAAGAGAACGAGCAAAACAGAG------CCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....----------. [158 bp] ....---.....
2 ....GTTGGA----- [-158 bp] ----------G.....
Allele 1: 7.7 %
Allele 2: 92.3 %
i3-18 51
WT GCGAAGAGAACGAGCAAAACAGAG------CCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....GTTGGA----- [-158 bp] ----------G.....
2 ....---------- [-158 bp] ----------.....
Allele 1: 88.4 %
Allele 2: 11.6 %
i3-18 58
WT GCGAAGAGAACGAGCAAAACAGAG-------CCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....CCGATGC..-- [-155 bp] -------.....
2 ....---------- [-158 bp] ----------.....
Allele 1: 45.0 %
Allele 2: 55.0 %
i3-18 69
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1/2 ....---------- [-158 bp] -------.....
Allele 1 & 2: 100 %
i4-18 1
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGA----ATCATCGGCTGGAGATCACCTTAA
1 ....-----... [158 bp] .....----..---.....
2 ....---... [158 bp] .....TAAT.....
Allele 1: 47.8 %
Allele 2: 52.2 %
i4-18 33
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....------ [-158 bp] ----------.....
2 ....-------- [158 bp] .....--.....
Allele 1: 9.0 %
Allele 2: 91.0 %
i4-18 38
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....---... [158 bp] .....-.....
2 ....---------- [-158 bp] -------.....
Allele 1: 22.5 %
Allele 2: 77.5 %
i4-18 42
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1/2 ....---... [157 bp] .....---.....
Allele 1 & 2: 100 %
i4-18 49
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ....-----. [158 bp] .....---.....
2 ....-.....-------- [-158 bp] -------.....
Allele 1: 30.4 %
Allele 2: 69.6 %
i4-18 52
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATC--ATCGGCTGGAGATCACCTTAA
1 ....---------- [-158 bp] -------.--.....
2 ....TC----- [-158 bp] --------AG----.....
Allele 1: 49.5 %
Allele 2: 50.5 %


i4-18 53
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAAT-CATCGGCTGGAGATCACCTTAA
1 .....-----
[-158 bp] -------A.....
2 .....------
[-158 bp] ------------.....
Allele 1: 49.4 %
Allele 2: 50.6 %
i4-18 66
WT GCGAAGAGAACGAGCAAAACAGA-GCCGCG [158 bp] TCTGAAT--CATCGGCTGGAGATCACCTTAA
1 .....TT-----
[-158 bp] ------.AG.....
2 .....-------
[-158 bp] -------------.....
Allele 1: 48.9 %
Allele 2: 51.1 %
i1-19 17
WT GCGAAGAGAACGAGCAAAAC-------AGAGCCGCG [158 bp] TCTGA----ATCATCGGCTGGAGATCACCTTAA
1 .....GTTGTGG...T-----
[-158 bp] ----------.....
2 .....-------..---...
[155 bp] -----AATC.....
Allele 1: 67.5 %
Allele 2: 32.5 %
i1-19 43
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1/2 .....-------
[-158 bp] -------GA.....
Allele 1 & 2: 100 %
i2-19 1
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....-----
[-158 bp] --------.....
2 .....------
[155 bp] .....----.....
Allele 1: 68.1 %
Allele 2: 31.9 %
i2-19 7
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAA-TCATCGGCTGGAGATCACCTTAA
1 .....------
[158 bp] ......-..---.....
2 .....---...
[158 bp] ......T.....
Allele 1: 48.2 %
Allele 2: 51.8 %
i2-19 8
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATC----------ATCGGCTGGAGATCACCTTAA
1 .....---------
[-158 bp] -------.----------.....
2 .....----
[-158 bp] -------.TCGGCTCGTG.....
Allele 1: 51.8 %
Allele 2: 48.2 %
i2-19 9
WT GCGAAGAGAACGAGCAAAACAGA-GCCGCG [158 bp] TCTGAAT-------CATCGGCTGGAGATCACCTTAA
1 .....-.-----
[-158 bp] -------ATCCAGC.....
2 .....TC-----
[-158 bp] --------------.....
Allele 1: 49.4 %
Allele 2: 50.6 %
i2-19 13
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....--------
[-35 bp] .....---.....
2 .....------
[-158 bp] ----------.....
Allele 1: 41.9 %
Allele 2: 58.1 %
i2-19 14
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1/2 .....----------
[-158 bp] -------.....
Allele 1 & 2: 100 %


i2-19 18
WT GCGAAGAGAACGAGCAAAACAG----------AGCC--------GCG [158 bp] TCTGAAT--------CATCGGCTGGAGATCACCTTAA
1 .....----------.-------------. [158 bp] ....CATCGTCA.....
2 .....ATAACGGACT..CCTTATTTTA--- [156 bp] .......---------G.--.....
Allele 1: 51.8 %
Allele 2: 48.2 %
i2-19 19
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [155 bp] ATGTCTGAATCATCGGCTGGAGATCACCTTAA
1/2 .....---------- [-155 bp] ----------.....
Allele 1 & 2: 100 %
i2-19 22
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....-----. [158 bp] .....---.....
2 .....-----. [153 bp] .....---.....
Allele 1: 54.4 %
Allele 2: 46.6 %
i2-19 23
WT GCGAAGAGAACGAGCAAAACAGAG----------CCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....---------- [158 bp] .....---.....
2 .....ACGGAACGATGAA----- [-158 bp] -------.....
Allele 1: 36.4 %
Allele 2: 63.6 %
i2-19 31
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....---------- [-158 bp] ---------.....
2 .....-----. [-158 bp] ----------.....
Allele 1: 48.8 %
Allele 2: 51.2 %
i2-19 33
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....-----. [158 bp] .....-------..----------
2 .....---------- [-158 bp] -------.....
Allele 1: 36.2 %
Allele 2: 63.8 %
i2-19 37
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....---------- [-158 bp] -------.....
2 .....------ [-158 bp] ----------.....
Allele 1: 48.2 %
Allele 2: 51.8 %
i2-19 38
WT GCGAAGAGAACGAGCAAAACAGAG-------CCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 .....----------..... [158 bp] .....---.....
2 .....GATCTCC----- [-158 bp] -------G.....
Allele 1: 40.2 %
Allele 2: 59.8 %
i2-19 40
WT GCGAAGAGAACGAGCAAAACAGAGCC---GC--G [158 bp] TCTGAAT----------CATCGGCTGGAGATCACCTTAA
1 .....A.TTG--TT- [158 bp] .....CGTGGCTGGAGGAGTC.....
2 .....--.---TT--. [158 bp] .....TTCGC----------.....
Allele 1: 84.4 %
Allele 2: 15.6 %


i2-19 42
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTG-------AATCATCGGCTGGAGATCACCTTAA
1 ..... [158 bp] ....AATCATT.....
2 ..... [-158 bp] ----------.....
Allele 1: 19.7 %
Allele 2: 80.3 %
i2-19 50
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [158 bp] .....---.....
2 ..... [-158 bp] ----------.....
Allele 1: 35.1 %
Allele 2: 64.9 %
i2-19 176
WT GCGAAGAGAACGAGCAAAACAGA----------GCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------.....
2 ..... [-158 bp] -------.....
Allele 1: 54.8 %
Allele 2: 45.2 %
i3-19 1
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAA--TCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] ----..AA.....
2 ..... [-158 bp] ----------.....
Allele 1: 29.3 %
Allele 2: 70.7 %
i3-19 2
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAAT-------CATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------AAACCGA.....
2 ..... [-158 bp] ----------.....
Allele 1: 57.4 %
Allele 2: 42.6 %
i3-19 10
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------.....
2 ..... [158 bp] ...---.....
Allele 1: 60.4 %
Allele 2: 39.6 %
i3-19 17
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------.....
2 ..... [158 bp] .....---.....
Allele 1: 7.4 %
Allele 2: 92.6 %
i3-19 19
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------.....
2 ..... [-158 bp] ----------.....
Allele 1: 29.9 %
Allele 2: 70.1 %
i3-19 20
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] --------.....
2 .....C.....----..... [158 bp] .....---.....
Allele 1: 66.5 %
Allele 2: 33.5 %
i3-19 26
WT GCGAAGAGAACGAGCAAAACAGAGCCGCG [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
1 ..... [-158 bp] -------.....
2 ..... [-158 bp] --------.....
Allele 1: 49.5 %
Allele 2: 50.5 %

```
i3-19   27   WT GCGAAGAGAACGAGCAAAACAGAGCCGCG  [158 bp] TCTGAATCATCGGCTGGAGATCACCTTAA
             1  .....................--------  [158 bp] .....---.....................          Allele 1: 34.9 %
             2  ...................---------- [-158 bp] -------......................          Allele 2: 65.1 %

i3-19   41   WT GCGAAGAGAACGAGCAAAACAGAGCCGCG  [158 bp] TCTGAAT-------CATCGGCTGGAGATCACCTTAA
             1  ...................---------- [-158 bp] --------------......................   Allele 1: 48.4 %
             2  ........................----- [-158 bp] -------AACAAGA......................   Allele 2: 51.6 %
```

Table S4. Survival of in-vitro reared injected individuals compared with wildtype controls. Shown for 5 replicates i2, i3, i4 2018 and i2 and i3 2019 (i = injection round, consisting of injections on three consecutive days). Total and % for each group. Fisher´s exact (df = 1) to test if CRISPR/Cas9 injections influence survival. Number and % of identified homozygous *dsx* stop mutants ($dsx^{stop/stop}$) of the hatched injected adults, genotyped at the age of 11 to 14 days. Bees were genotyped after sensorimotor tests and morphological analyses, along this path individuals died or lost the 2D identification barcode, causing their exclusion from the study and not being genotyped (total injected individuals that were excluded *n* = 18, 2.45 %).

replicates	group	No. 2-day old larvae	No. hatched adults, min. 24 h old	% hatched adults, min. 24 h old	*P*-value Fisher´s exact, df = 1	No. $dsx^{stop/stop}$ of injected adults	% of $dsx^{stop/stop}$ of injected adults
i2 2018	injected	111	18	16.2 %	0.21	8	50 %
	control	213	50	23.5 %		0	0 %
i3 2018	injected	171	31	18.1 %	0.31	15	48.4 %
	control	211	48	22.8 %		0	0 %
i4 2018	injected	92	20	21.7 %	0.49	11	55 %
	control	129	23	17.8 %		0	0 %
i2 2019	injected	217	53	24.4 %	0.39	19	35.8 %
	control	236	67	28.4 %		0	0 %
i3 2019	injected	141	49	34.8 %	0.44	14	28.6 %
	control	118	47	38.8 %		0	0 %
Total	injected	732	171	23.1 %	0.25	67	39.2 %
	control	907	235	25.9 %		0	0 %

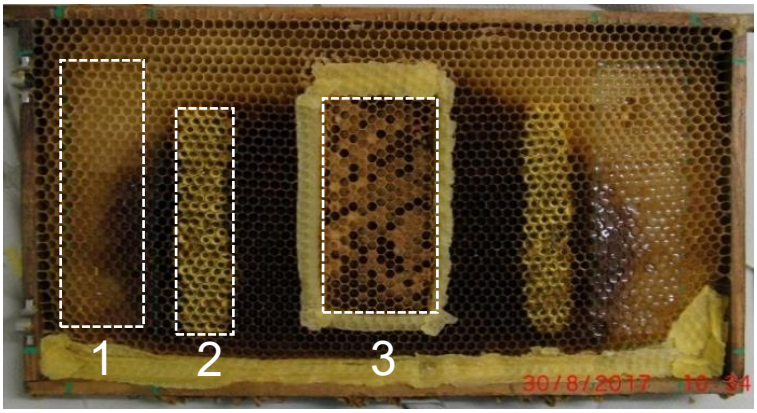


Figure S5. Standardized comb with honey (1), pollen (2) and brood (3) area. Brood area consists of 151 larvae of L3-L4 larval stage and is located in the middle of the comb. Honey and pollen are provided in two areas of the same size with respectively one on each side of the brood area. Honey areas consist in total of 550 cells each filled with 200 µl sugar solution. Pollen areas are filled with a total of 30 g grinded pollen. Mean area sizes see Table S6.

Table S6. Size of areas on the standardized combs used for tracking for each replicate. SD = standard deviation.

replicate	brood area (cm^2)	pollen area (cm^2)	honey area (cm^2)
i2-2018	56.72	32.33	74.31
i3-2018	46.50	29.70	76.49
i4-2018	75.39	34.09	74.81
i2-2019	69.34	27.90	71.12
i3-2019	61.12	32.44	72.00
mean	61.81	31.29	73.75
SD +/-	10.02	2.20	1.95

Table S7. Detection rate. Was determined using speed data. For every hour, the percentage of detection is calculated and over 24 hours the average measured. Individuals that were not detected were sorted out. No. of bee Id identified of the tracking was stopped after 72 h. SD = standard deviation.

replicate	% Detection rate	# Bee Id
i2-2018	0.79	465
i3-2018	0.70	461
i4-2018	0.75	460
i2-2019	0.91	461
i3-2019	0.83	447
mean	0.80	458.8
SD +/-	0.07	6,14

Table S8. Maintenance of the 151 larvae in the brood area for each replicate. Was measured in counting capped brood/pupae. SD = standard deviation.

replicate	% of maintained larvae
i2-2018	53.6
i3-2018	68.2
i4-2018	70.2
i2-2019	63.6
i3-2019	58.3
mean	62.78
SD +/-	6.2

Table S9. Number of inspection cells (IC) and work in cells (WIC) per minute for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. SD = standard deviation. MWU = Mann-Whitney U-test.

IC/min	***dsx*$^{stop/stop}$**				**Wt control**				MWU	Z
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	
All cells	42	0.49	0.63	± 0.53	45	0.87	1.05	± 0.85	0.006	-2.74
Brood cells	26	0.15	0.20	± 0.18	31	0.15	0.35	± 0.46	0.67	-0.83
Food cells	25	0.09	0.18	± 0.23	33	0.17	0.27	± 0.29	0.04	-2.04
Empty cells	41	0.24	0.41	± 0.47	44	0.48	0.63	± 0.59	0.04	-2.03
WIC/min	***dsx*$^{stop/stop}$**				**Wt control**				MWU	Z
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	
All cells	39	0.17	0.27	± 0.27	39	0.39	0.42	± 0.34	0.02	-2.35
Brood cells	20	0.09	0.13	± 0.11	22	0.21	0.25	± 0.21	0.054	-1.93
Food cells	15	0.03	0.05	± 0.03	21	0.04	0.05	± 0.03	0.53	-0.65
Empty cells	33	0.15	0.22	± 0.24	37	0.18	0.27	± 0.27	0.29	-1.05

Table S10. Median and mean length of work in cell (WIC) in the respective area. Respectively for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. Measured over 24 h. SD = standard deviation. MWU = Mann-Whitney U-test.

WIC/min	***dsx*$^{stop/stop}$**				**Wt control**				MWU	Z
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	
All cells	39	13.42	16.17	± 7.67	39	17.84	20.27	± 10.05	0.04	-2.03
Brood cells	20	16.69	21.12	± 12.99	22	16.57	19.72	± 11.12	0.82	-0.23
Food cells	15	6.75	17.37	± 22.22	21	13.50	25.81	± 29.19	0.02	-2.28
Empty cells	33	13.42	15.03	± 7.33	37	15.37	22.76	± 21.63	0.21	-1.25

Table S11. Number of antennation, begging and Trophallaxis per minute. Median and mean length per trophallaxis, respectively for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. SD = standard deviation. MWU = Mann-Whitney U-test.

Frequency [per min]	***dsx*$^{stop/stop}$**				**Wt control**				MWU *P*-value	Z
	n	Median	Mean	± SD	*n*	Median	Mean	± SD		
Antennation	42	0.39	0.43	± 0.31	47	0.38	0.50	± 0.38	0.65	-0.52
Begging	25	0.11	0.11	± 0.09	34	0.07	0.12	± 0.18	0.71	-0.67
Trophallaxis	15	0.07	0.12	± 0.09	21	0.09	0.14	± 0.07	0.95	-0.06
Length [sec]	***dsx*$^{stop/stop}$**				**Wt control**				MWU *P*-value	Z
	n	Median	Mean	± SD	*n*	Median	Mean	± SD		
Trophallaxis	15	5.50	5.2	± 2.83	21	11.25	13.49	± 9.11	0.001	-3.23

Table S12. Median and mean time spent (minutes) in the respective area. Respectively for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. Measured over 24 h. SD = standard deviation. MWU = Mann-Whitney U-test.

Time spent in [min]	***dsx*$^{stop/stop}$ (*n* = 47)**			**Wt control (*n* = 49)**			MWU *P*-value	Z
	Median	Mean	± SD	Median	Mean	± SD		
Brood	4.67	5.48	± 3.52	3.66	4.71	± 3.69	0.13	-1.53
Food	4.90	7.15	± 6.13	6.27	6.69	± 4.19	0.66	-0.44
Empty	13.87	14.77	± 6.23	15.79	15.38	± 4.28	0.39	-0.87

Table S13. Median and mean visits per hour (visits/h) to the respective area. Respectively for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. Measured over 24 h. SD = standard deviation. MWU = Mann-Whitney U-test.

Visits per hour [visits/h]	***dsx*$^{stop/stop}$ (*n* = 47)**			**Wt control (*n* = 49)**			MWU *P*-value	Z
	Median	Mean	± SD	Median	Mean	± SD		
Brood	1.83	2.71	± 2.45	1.87	3.01	± 4.19	0.77	-0.62
Food	4.00	7.59	± 8.78	4.30	8.26	± 11.25	0.54	-0.29

Table S14. Median and mean speed (m/sec) measured over 24 h. Respectively for *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. SD = standard deviation. MWU = Mann-Whitney U-test.

Speed [m/sec]	***dsx*$^{stop/stop}$ (*n* = 47)**			**Wt control (*n* = 49)**			MWU *P*-value	Z
	Median	Mean	± SD	Median	Mean	± SD		
Speed	0.59	0.64	± 0.25	0.53	0.62	± 0.33	0.21	-1.26

Table S15. Fishers exact Test development of hypopharyngeal glands (HPG). Fisher exact test statistic value is 1**.**

Group	Wild-typically developed	Not present/deformed	Marginal Row Totals
dsx$^{stop/stop}$	23	0	23
Wildtype control	27	0	27
Marginal Column Totals	50	0	50 (Grand Total)

Table S16. Number of individuals for the analyzed morphology for head, hypopharyngeal gland (HPG), body morphology and abdominal and antennal segments, and dissected ovaries. Respectively for homozygous *dsx* stop-mutants (*dsx*$^{stop/stop}$). Head morphology was measured in calculating the ratio of head length to head width, body morphology in abdomen and antennae segments. For HPG presence was noted. Segments were counted: Worker wildtypes have 6 abdominal segments and 13 antennal segments. *P* = 1, df = 1, Fisher´s exact.

Analyzed structure	No. of *dsx*$^{stop/stop}$ mutants that showed wild type phenotype[1)]/ total tested	No. of wildtype controls that showed wildtype phenotype / total tested
head	29 / 29	26 / 26
Hypopharyngeal gland	23 / 23	27 / 27
Body morphology	17 / 17	11 / 11
Abdominal segments	17 /17	11 / 11
Antennal segments	24 / 24	26 / 26
Ovaries	6 / 12	5 / 5

[1)] All individuals were analyzed for wildtype phenotype. For HPG we noted presence or absence.

Table S17. Ratio of head length and width of adult *dsx*$^{stop/stop}$ and wildtype (wt) control worker bees. Length was divided by width to calculate ratio and measured using images taken of the decapitated head. MWU = Mann-Whitney U-test.

replicate	*dsx*$^{stop/stop}$ id	ratio	replicate	Wt control id	ratio
i1-2019	17	0.73	i1-2019	119	0,79
	43	0.76		122	0,76
i2-2019	1	0.77		149	0,69
	8	0.73	i2-2019	57	0,80
	9	0.80		96	0,72
	18	0.74		81	0,76
	19	0.72	i3-2019	52	0,75
	22	0.68		55	0,71
	31	0.80		62	0,72
	33	0.72		63	0,76
	40	0.71		66	0,73
	42	0.68		74	0,73
	50	0.72		90	0,71
i3-2019	1	0.73		99	0,73
	17	0.78	i3-2018	79	0.75
	19	0.79		83	0.73
	20	0.72		89	0.71
	27	0.76		92	0.77
	38	0.70		99	0.70
	41	0.71		100	0.71
i3-2018	7	0.75		106	0.71
	35	0.77		135	0.70
	47	0.72	i4-2018	80	0.79
	51	0.70		104	0.76
	58	0.71		117	0.76
	69	0.77		129	0.77
i4-2018	33	0.79			
	42	0.77			
	66	0.72			
median		0.73	median		0.73
mean		0.74	mean		0.74
MWU *P*-value		0.744			
Z		-0.29			

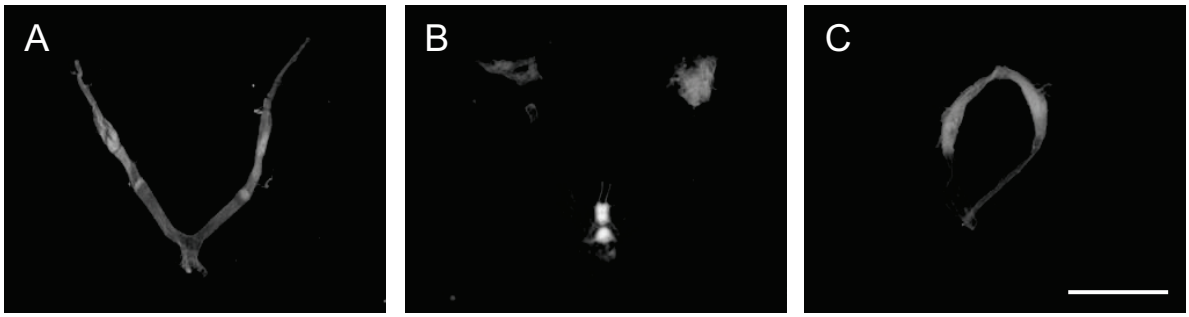


Figure S18. Ovaries dissected from female adult (10-13 days old) worker bees. A. Wildtype control reproductive organs. Oviducts about match the size/width of ovarioles. *n* = 7. B. *dsx*$^{stop/stop}$ worker bee. Intersex reproductive organs with enlarged oviducts and crumbled up ovarioles. C. *dsx*$^{stop/stop}$ worker bee. Ovaries with reduced size due to *dsx*$^{stop/stop}$ mutation. In 50 % of *dsx*$^{stop/stop}$ we observed differences in reproductive organ development (*n* = 6 out of 12), similar to what has been previously reported for reproductive organs in *dsx*-mutated pupae (Roth et al. 2019). Scale 500 µm.

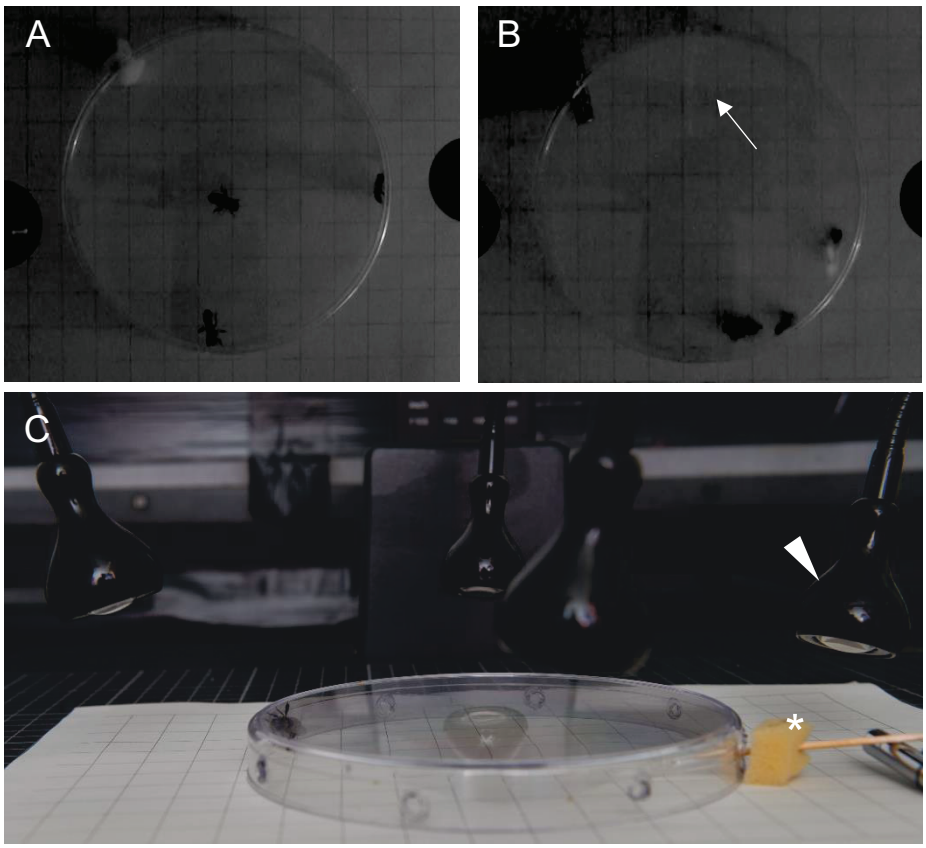


Figure S19. Setup for sensorimotor essays. Worker bees were tested in a petri dish (14 cm). Respectively wildtype control or *dsx*$^{stop/stop}$ worker bee was tested in the presence of two hive-reared wildtype bees. A. Setup for locomotion measurements and light responsiveness. B. Setup for repellent response. First a filter paper (arrow) was presented. Line crossings were measured. C. Essay setup from the side. Pseudo-queen introduced through hole on the side (asterisk) and LED lights for light responsiveness shown (arrow).

Table S20. Number and distribution of the malformations identified in *dsx*$^{stop/stop}$ worker bees. Seven individuals with neuronal malformation are listed, the associated malformation and the respective diameter range of the other neuronal structure. Total of affected structures listed at the end of the table.

Bee ID	Replicate	Genotype Allele1 /allele 2	Affected structure							Average size of other structure [µm]					
			Calyx left	Calyx medial	Calyx medial	Calyx right	LH left	LH right	Central Body	Calyx left	Calyx medial	Calyx medial	Calyx right	LH left	LH right
17	i1-19	Stop/stop			A*, B	A*, B	B					27.3-40.3	21.8-71.5	47.6	
43	i1-19	Stop/stop			D				B						
7	i2-19	Stop/stop			A	A						23.9	21.6		
9	i2-19	Stop/stop				A							41.6		
31	i2-19	Stop/stop					A							24.5	
19	i3-19	Stop/stop	A							20.0					
69	i3-18	Stop/stop	A	A	C	A			B	40.0	33.3		46.6		
total/average			2	1	4	4	2	-	2	30.0	33.3	30.5	40.6	36.1	0.0
A = other structure, A* = multiple			2	1	2	4	1	-	-	-	-	-	-	-	-
B = deformation			-	-	1	1	1	-	2	-	-	-	-	-	-
C = misplaced structure			-	-	1	-	-	-	-	-	-	-	-	-	-
D = loss of structure			-	-	1	-	-	-	-	-	-	-	-	-	-

Table S21. Fishers exact Test neuronal malformations detected in f-actin labeling. Fisher exact test statistic value is 0.005.

Group	Mutant	Wild-typic	Marginal Row Totals
dsx$^{stop/stop}$	7	21	28
Wildtype control	0	29	29
Marginal Column Totals	7	50	57 (Grand Total)

Table S22. Nucleotide sequences of the sgRNAs and myrGFP DNA fragment. For sgRNA1 and 2 the bold letters indicate the target site in the genome. For the myrGFP sequence the respectively 5´ and 3´ homologous sequence is shown with gray background. The start codon (ATG) of *dsx* is underlined and in green letters. Nucleotides depicted in red letters were added to the sequence to maintain the open reading frame. Myristoylation (myr) sequence in brown letters, GFP sequence in blue letters and the 2A peptide (P2A) sequence in dark green letters. The GS linker (between myr and GFP) and GSG linker (between GFP and P2A) are shown with orange background. Sequences for the insert were codon-optimized for a nucleotide distribution typical for the *A. mellifera* genome without generating changes in the amino acid sequence.

Molecule	Nucleotide sequence
dsx sgRNA1	**GAACGAGCAAAACAGAGCCG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCC GUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsx sgRNA2	**GUGCACGAUGUCUGAAUCAU**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCC GUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
myrGFP DNA fragment	GTTGCAGAACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGA CTACGTTTCGAAACACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGATTAGAGAGAG AGAGAGAAAGGACAACGATAGAGGGACAAACAACCGTTCAAACATTTCATTGAGATTGTTC TTTGTAATTATGAAAAGGCTGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAA AACAGAGGAATGGGCAATAAATGCTGCAGCAAAAGACAAGATCAAGAATTGGCTTTAGCGT ATCCAACAGGAGGTTACAAGAAATCGGATTATACGTTCGGACAAACACATATCAATTCTAG CGGCGGTGGAAATATGGGTGGAGTGTTGGGCCAAAAACATAACAATGGTGGATCGTTAGAT TCTAGATATACGCCAGATCCTAATCATAGAGGTCCATTGAAAATTGGAGGCAAAGGTGGAG TTGATATCATTAGACCTAGAGGATCTATGAGCAAAGGAGAAGAACTGTTCACAGGTGTTGT GCCAATCTTAGTTGAATTGGATGGCGATGTGAATGGACATAAATTCTCTGTGTCGGGTGAA GGAGAAGGCGATGCTACGTATGGTAAATTGACATTAAAATTCATTTGCACTACGGGAAAAC TGCCAGTGCCTTGGCCAACATTGGTTACGACTTTAACATATGGTGTGCAATGCTTCAGCAG ATATCCTGATCATATGAAACAACATGATTTTTTCAAATCTGCGATGCCAGAAGGATATGTG CAAGAAAGAACGATCTTTTTCAAAGATGATGGTAATTACAAAACAAGAGCTGAAGTTAAAT TCGAAGGAGATACGTTGGTGAATAGAATTGAATTAAAAGGTATCGATTTTAAAGAAGATGG AAATATTCTTGGTCATAAATTGGAATATAATTACAACAGCCATAATGTTTATATAATGGCT GATAAACAAAAAAATGGAATCAAAGTGAACTTCAAAATTAGACATAATATAGAAGATGGTT CGGTTCAATTAGCGGATCATTACCAACAAAATACACCAATTGGAGATGGTCCTGTTCTGTT GCCAGATAATCATTATTTAAGCACGCAATCTGCTTTGTCGAAAGATCCAAATGAAAAAAGA GATCATATGGTGTTACTTGAATTCGTTACAGCGGCTGGAATTACGCATGGTATGGATGAAT TATATAAAGGATCTGGTGCTACAAATTTCTCTTTGTTAAAACAAGCGGGAGATGTGGAAGA AAATCCAGGTCCTGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAG CGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAG ACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAA TCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACC TGCGAGAAGTGTAAGATCA

AACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGACTACGTTTC
GAAACACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGATTAGAGAGAGAGAGAGAAAGG
ACAACGATAGAGGGACAAACAACCGTTCAAACATTTCATTGAGATTGTTCTTTGTAATTATGA
AAAGGCTGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACAGAGCCGCGGAC
TTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGC
AAAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCA
AAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCATCGGCTGGAGATCACCTTAAAATCG
CACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGG
CAGCAAGTGATGCGGCAGAATATGAAGCTGAAAAGACACCTGGCACAGGATAAAGTCAAAGTA
AGAGTAGCGGAAGAG

Figure S23. Nucleotide sequences of exon 2 of *dsx* of *A. mellifera*. Sequence shown in 5´ to 3´. sgRNA 1 target sequence displayed in red, sgRNA 2 target sequence displayed in cyan. Site of double strand break indicated in bold and underlined. Start codon (ATG) indicated in green. Oligomerization domain 1 (OD1) indicated in yellow. Grey background marks the primers used for amplicon sequencing.

Table S24. Number of in-vitro reared *dsx*-mutants (injected), in-vitro reared wildtype control (wt control) and hive-reared wildtype worker bees (wildtype) per replicate. Genotype was determined after tracking. Number of hive-reared wildtype was adjusted to reach a total of 500 bees in the artificial hive. We tagged about 10 extra hive-reared wildtype bees to account for bees losing tags or die during the procedure.

replicate	group	*n*[1]	*n* evaluated tracking[2]	*n* evaluated encounter[3]	*n* evaluated cell insepection[4]
I2 2018	Injected	16	5	5	5
	Wt control	50	8	8	6
	Wildtype	444	-	-	-
I3 2018	Injected	28	7	5	6
	Wt control	42	8	6	7
	Wildtype	439	-	-	-
I4 2018	Injected	18	10	8	7
	Wt control	18	8	8	7
	Wildtype	474	-	-	-
I2 2019	Injected	38	16	15	15
	Wt control	57	16	16	13
	Wildtype	417	-	-	-
I3 2019	Injected	31	9	9	9
	Wt control	36	9	9	12
	Wildtype	443	-	-	-

[1] Total number of bees at the beginning of the tracking. Number decreases over the course of tracking, as bees for example lose their ID.
[2] Number of worker bees from which we obtained automatic generated tracking data.
[3] Number of worker bees which we manually evaluated for antennation, begging and trophallaxis behavior based on the automatic predicted trajectories.
[4] Number of worker bees which we manually evaluated for cell inspection behavior based on the automatic predicted trajectories.
[2,3,4] This number does not equal the total number of *dsx*$^{stop/stop}$/wildtype control worker bees that were tracked in the artificial hive, as some bees were or were not detected in the chosen time period.

Table S25. List of video material. For each behavior example a slow (frame-by-frame), fast (normal speed) and zoomed in video is available, for better resolution of short interactions. Fast videos are marked with “15fps”.

Video content	*dsx*$^{stop/stop}$	Wt control	description
Inspecting cell	V1	V2	The highlighted bee with the ID shows short cell inspection behavior (< 5 sec). The head is tilted inward of the cell, antennae not visible. Multiple cell inspections shown.
Work in cell	V3	V4	The highlighted bee with the ID shows long cell inspection behavior (> 5 sec). The head is tilted inward of the cell, antennae not visible.
Antennation	-	V5*	***Hive-reared wildtype.** Encircled bees approach each other. They face each other, come in contact with their antennae and start moving their antennae.
Begging	-	V6*	***Hive-reared wildtype.** Encircled bees approach each other. Antennae of bees are in contact and bee showing begging behavior (right) tilts its head toward other bee. Reaches with forelegs towards contact bee**.**
Trophallaxis	-	V7*	***Hive-reared wildtype.** Bees engaged in trophallaxis show intensive antennation while they are in oral contact. Occasionally bees lounge towards the contact be with their forelegs.
Antennation	V8	V9	The highlighted bee with the ID number 176 (mutant) / 78 (wt control) shows antennation behavior. The bees face each other (contact bee 479/146), and they are in contact with their moving antennae.
Begging	V13	V12	The highlighted bee with the ID number 29 (mutant) / 91 (wt control) shows begging behavior. The antennae of the bees are in contact, head tilted, marked bee X/91 reaches with forelegs towards the other marked bee and proboscis extended 160/40.
Trophallaxis	V10	V11	The highlighted bee with the ID number 42 (mutant) / 63 (wt control) shows trophallaxis behavior. The antennae of both bees are in contact, and the bee marked with arrow outstretches its proboscis towards the other marked bee 358/421. Bees are occasionally lounging towards the contact bee with their forelegs.
Brain z-stack	V13	V20	Bee ID #17. Multiple extra structures in the calyces, one in the area of the lateral horn and showed deformations of the lip. Fig. 6g, i.
Brain z-stack	V14		Bee ID #43. Major tissue loss with a missing calyx and the associated peduncle. Fig. 6c.
Brain z-stack	V15		Bee ID #7. Extra structures in the medial and lateral calyx.
Brain z-stack	V16		Bee ID #9. Extra structure in the lateral calyx. Fig. 6e.

Brain z-stack	V17	V20	Bee ID #31. Extra structure in the lateral horn area.
Brain z-stack	V18		Bee ID #19. Extra structure in the lateral calyx.
Brain z-stack	V19		Bee ID #69. Lack of a fully developed central complex and a medial calyx located further posterior in the brain.

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Authors contribution: Manuscript II

1st author

Author's contributions 90 %

- Experimental design
- sgRNA synthesis
- Implementation of microinjections
- In-vitro rearing
- Dissection of brain tissue, reproductive organs and HPG
- External morphology analyses
- Immunohistochemistry and microscope imaging
- Bee handling for bee tracking
- Application of behavior classifiers and evaluation
- Manual video analysis of tracking video segments and sensorimotor essays
- Data analyses: sequencing data, tracking data, statistics
- Authoring the manuscript

CHAPTER III

Summary

Advanced social insects display a unique form division of labor which is accomplished by sexual and caste dimorphisms. In the honeybee (*A. mellifera*) the female castes take upon vital tasks, as the queen reproduces, and the worker bees take care of the brood, hive, and foraging. To do so worker bees perform almost all tasks within the colony, requiring a task repertoire with up to 50 different tasks. Females and males are defined by cell-autonomous sex determination cascade. Caste-differentiation is regulated by differential nutrition, but little is known about caste is genetically specified during development. The transcription factor *doublesex* (*dsx*), a gene with female- and male-specific isoforms, specifies sexual dimorphisms and sex-specific behavioral traits in the fruit fly *Drosophila*. In this study I use CRISPR/Cas9 to target genes of the sex determining cascade. During sex determination in *A. mellifera feminizer* (*fem*) instructs female development and regulates sex-specific splicing of the downstream target *Am-dsx*. I show that worker bee pupae with a *fem*-mutation display large male-like reproductive organs and that a mutation of *Am-dsx* results in smaller and intersex reproductive organs. The gene *fem* is thus involved in the nutrition-depended genetic response for small size female development, *Am-dsx* however has a less pronounced effect on primary traits. Analyzing behavioral traits of *Am-dsx* mutants in a computer-based tracking I show that *Am-dsx* activity is essential for secondary caste-specific traits. I find that cell inspecting (CI) behavior, important for brood and cell care, is disrupted *in Am-dsx* mutants. Reduced frequencies of short CI (<5 sec) and long work in cell (WIC, > 5 sec) behaviors, indicate that *Am-dsx* activity is vital to program the initiation of such behaviors. Further, seems the sustainment of WIC tasks also to be dependent on *Am-dsx* activity, as time spent per WIC behavior is substantially reduced in *Am-dsx* mutants. As trophallaxis is significantly reduced and shorter in *Am-dsx* mutants, I conclude that *Am-dsx* activity is vital for developing traits specific for worker bees and essential for social group living. Behavioral defects are not due to disrupted locomotion, stimuli preferences or perception, but can rather be attributed to visible neuronal extra structures in the higher integration centers (mushroom bodies) I detected in 25 % of *Am-dsx* mutants. This is the first study to attribute the activity of a single gene, the *Am-dsx* gene, to the development of specific behavioral traits and neuronal specification in worker bees of *A. mellifera*, bringing research one step closer to understanding the origin of advanced social behavior.

Zusammenfassung

Soziale Insekten weisen eine einzigartige Form der Arbeitsteilung auf, die durch Geschlechts- und Kastendimorphismus erreicht wird. Bei der Honigbiene (*A. mellifera*) übernimmt die weibliche Kaste die lebenswichtigen Aufgaben, da die Königin für die Fortpflanzung zuständig ist und die Arbeitsbienen sich um die Brut, den Bienenstock und die Futtersuche kümmern. Dabei übernehmen die Arbeitsbienen fast alle Aufgaben innerhalb des Bienenvolkes, was ein Aufgabenrepertoire mit bis zu 50 verschiedenen Aufgaben erfordert. Weibchen und Männchen werden durch eine zellautonome Geschlechtsbestimmungskaskade definiert. Die Kastendifferenzierung wird durch unterschiedliche Ernährung reguliert, aber es ist wenig darüber bekannt wie die Kastendifferenzierung während der Entwicklung genetisch festgelegt wird. Der Transkriptionsfaktor *doublesex* (*dsx*), ein Gen mit Weibchen- und Männchen-spezifischen Isoformen, spezifiziert sexuelle Dimorphismen und geschlechtsspezifische Verhaltensmerkmale in der Fruchtfliege *Drosophila*. In dieser Studie verwende ich CRISPR/Cas9, um Gene der geschlechtsbestimmenden Kaskade gezielt zu mutieren. Während der Geschlechtsbestimmung in *A. mellifera* steuert, *feminizer* (*fem*) die weibliche Entwicklung und reguliert das geschlechtsspezifische Spleißen des nachgeschalteten Zielgens *Am-dsx*. Ich zeige, dass Puppen von Arbeiterinnen mit einer *fem*-Mutation männliche Fortpflanzungsorgane aufweisen und dass eine Mutation von *Am-dsx* zu kleineren und intersexuellen Fortpflanzungsorganen führt. Das Gen *fem* ist somit am Futter-abhängigen genetischen Signal für die geschlechtsspezifische Entwicklung beteiligt, *Am-dsx* hingegen hat eine weniger ausgeprägte Wirkung auf primäre Merkmale. Durch die Analyse von Verhaltensmerkmalen von *Am-dsx* Mutanten mit Hilfe eines computergestützten Tracking zeige ich, dass die *Am-dsx* Aktivität essenziell für sekundäre kastenspezifische Merkmale ist. Ich zeige, dass das Zellinspektionsverhalten (CI), das für die Brut- und Zellpflege wichtig ist, bei *Am-dsx* Mutanten gestört ist. Die verringerte Häufigkeit von kurzen CI- (< 5 Sekunden) und langen WIC-Verhaltensweisen (> 5 Sekunden) deutet darauf hin, dass *Am-dsx* Aktivität für die Programmierung der Initiation dieser Verhaltensweisen unerlässlich ist. Außerdem scheint die Aufrechterhaltung von WIC-Aufgaben ebenfalls von der *Am-dsx* Aktivität abhängig zu sein, da die für ein WIC-Verhalten aufgewendete Zeit bei *Am-dsx* Mutanten signifikant reduziert ist. Da die Trophallaxis bei *Am-dsx* Mutanten deutlich

reduziert und kürzer in ihrer Dauer ist, schließe ich daraus, dass *Am-dsx* Aktivität für die Entwicklung von spezifischen Arbeiterinnen-Merkmalen und das Leben in sozialen Bienen-Gruppen essenziell ist. Die Verhaltensdefekte sind nicht auf eine gestörte Fortbewegung, Stimuli-Präferenz oder -Wahrnehmung zurückzuführen, sondern vielmehr auf sichtbare neuronale, extra Strukturen der höheren Integrationszentren (Pilzkörper), die ich bei 25 % der *Am-dsx* Mutanten nachweisen konnte. Dies ist die erste Studie, in der die Aktivität eines einzigen Gens, des *Am-dsx*-Gens, mit der Entwicklung spezifischer Verhaltensmerkmale und neuronaler Spezifizierungen bei Arbeitsbienen von *A. mellifera* in Verbindung gebracht wird, was die Forschung dem Verständnis des Ursprungs fortgeschrittenen Sozialverhaltens einen wichtigen Schritt näherbringt.

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