Untersuchungen zur genetischen Spezifizierung des Arbeiterinnenverhaltens bei der Honigbiene

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Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Haan, im Februar 2025 _____

(Jana Seiler)

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Kapitel I

Allgemeine Einleitung

Die Sozialstruktur des Tierreichs zeigt eine beeindruckende Vielfalt, die von einfacher Aggregation bis hin zu hochkomplexen sozialen Systemen reicht. Ein besonders faszinierendes Beispiel für diese Vielfalt stellt die Eusozialität und ihr Vertreter die Honigbiene (*Apis mellifera*) dar. Sie zeigt neben einer extremen Arbeitsteilung eine ausgeprägte Form von Altruismus, die bei kaum einem anderen sozialen Insekt oder Wirbeltier in derselben Intensität zu finden ist. Während bei den meisten Tierarten die Bildung von Gruppen in erster Linie den Vorteil gesteigerter Nahrungssuche oder besseren Schutzes vor Prädatoren hat, wie beispielsweise bei Delfinschulen, Pinguingruppen oder einigen Schildkrötenarten, weist die soziale Organisation der Honigbiene eine weit über diese hinausgehende Komplexität auf (Hamilton, 1964). Der Superorganismus Honigbiene ist eine hochspezialisierte soziale Gesellschaft, in der jede Biene eine ganz bestimmte Rolle übernimmt. So obliegt der Königin die Fortpflanzung und die Drohnen übernehmen dabei die Begattung der Königin. Hervorzuheben an dieser Stelle ist das altruistische Verhalten der Arbeiterinnen - sie tragen von der Brutpflege, über Nestverteidigung, bis zur Nahrungssuche zum Erhalten des Bienenstocks bei, pflanzen sich jedoch nicht selbst fort (Johnson, 2010; Lindauer, 1952; Rösch, 1925, 1930). Dieses Verhalten stellt eine Besonderheit der Eusozialität dar, die höchste Stufe sozialer Organisation, bei der es zur Aufteilung in reproduktive und nicht-reproduktive Kasten kommt. Die Honigbiene, mit dieser klar definierten Kastenstruktur und genetischen Zugänglichkeit, stellt einen idealen Modellorganismus dar, um die genetischen und neuronalen Grundlagen sozialen Verhaltens zu untersuchen. Diese Dissertation trägt dazu bei, die molekularen Mechanismen und neuronalen Verbindungen zu entschlüsseln, die der Regulation des Sozialverhaltens zugrunde liegen.

Die soziale Organisation von Honigbienenkolonien: ein komplexes System aus Arbeitsteilung und Kommunikation

Die Honigbiene lebt in einem hochorganisierten Sozialverband, in dem die Individuen klar definierte Aufgaben übernehmen (Oster & Wilson, 1978; Rösch, 1925; Seeley, 1995; Winston, 1987). Die Population gliedert sich in drei grundlegende Gruppen: die

männlichen Drohnen und die weiblichen Bienen, die sich wiederum in Königinnen und Arbeiterinnen unterteilen.

Die Drohnen haben im Bienenvolk eine Hauptaufgabe: die Befruchtung einer Königin. Diese Aufgabe spiegelt sich auch in ihrer Morphologie wider. Für eine Begattung im Flug, den sogenannten Hochzeitsflug, besitzen die Drohnen eine starke Flugmuskulatur und ausgeprägte Fortpflanzungsorgane. In den paarig angelegten Gonaden reifen mehrere Millionen Spermien heran, die bei der Paarung freigesetzt werden (Woyke, 1960). Nach der Begattung sterben die Drohnen und erfüllen damit ihre Aufgabe im Reproduktionszyklus des Bienenvolkes (Baudry et al., 1998; Schlüns et al., 2005).

Die Bienenkönigin gilt als Fortpflanzungszentrum eines Bienenvolkes. Als einziges fruchtbares Weibchen ist sie für die Eiablage verantwortlich. Dafür fliegt die Königin einmal zu Beginn ihres Lebens zum Hochzeitsflug aus und lässt sich von bis zu 20 Drohnen befruchten (Woyke, 1955). Dadurch ist die Spermathek, dem Speicherort der Spermien, einer Königin ausreichend für ihre gesamte Lebensdauer von bis zu fünf Jahren ausrechend gefüllt (Gessner & Ruttner, 1977). Nach einer Verzögerung von etwa einer Woche beginnt die Königin bereits mit der Eiablage (Page & Erickson, 1988). Eine beständige Reizreaktion auf leere Wabenzellen, initiiert dabei das Legen der Eier. Während der Suche nach weiteren leeren Zellen, signalisiert die Königin zeitgleich den Arbeiterinnen ihre Anwesenheit mittels Absonderung des Königinnen-Mandibular-Pheromons (Queen mandibular pheromone; QMP) (Hoover et al., 2003; Slessor et al., 1988).

Die Arbeiterinnen sind die sterilen weiblichen Mitglieder eines Volkes und machen den Großteil des Stockes aus. Sie übernehmen eine Vielzahl an Aufgaben, welche durch ihr Alter und physiologischen Zustand beeinflusst sind (Robinson, 1992). Dieser zeitliche und physiologische Polyethismus gewährleistet eine effiziente Aufgabenteilung und maximiert die Produktivität des Volkes. Allerdings kann dieser aber aufgrund sich ändernder Umstände ausgesetzt werden, um das Überleben des Volks zu sichern (Haydak, 1963; Robinson, 2002; Seeley, 1982). Mit zunehmendem Alter ändern die Arbeiterinnen nicht nur ihre Aufgaben innerhalb des Volkes, sondern auch ihren Aufenthaltsort vom Zentrum des Bienenstocks, den Brutwaben, in die Peripherie, dem Lagerort für Pollen und Honig (Johnson, 2008; Seeley, 1982). Die jüngsten Arbeiterinnen, die bis zu drei Tage alt sind, zeigen zunächst ein eher inaktives

Verhalten und beginnen sich verschiedene Fähigkeiten anzueignen (Seeley, 1982). Zu den ersten Aufgaben, welche die Arbeiterinnen im Stock erfüllen, zählt die Reinigung der Wabenzellen. Ab einem Alter von etwa drei Tagen leisten die Arbeiterinnen, auch als Ammenbienen bezeichnet, einen essenziellen Beitrag zur Aufrechterhaltung des Stockes: Sie übernehmen die Pflege der Brut (Ribbands, 1953; Seeley, 1982). Zu diesem Zweck erfolgt eine Inspektion der Zellen sowie eine damit einhergehende Verarbeitung der sensorischen Informationen über den Inhalt der einzelnen Zellen (Siefert et al., 2021). Findet eine Arbeiterin eine hungernde Larve vor, erfolgt die Gabe des in der Hypopharynxdrüse gebildeten Futtersaftes in die Zellen der jeweiligen Larve (Deseyn & Billen, 2005). Neben der Brut werden auch die adulten Bienen im Stock von den Ammenbienen gefüttert. Dieser als Trophallaxis bezeichnete Nahrungsaustausch finden auch zwischen älteren Bienen statt (Crailsheim, 1992). Die Königin wird dabei hauptsächlich von den Ammenbienen gefüttert, die durch das ausgeschüttete QMP dazu angeregt werden, einen Hofstaat um die Königin zu bilden - eine Gruppe von Arbeiterinnen, die die Königin ständig umgibt und versorgt (Slessor et al., 2005). Durch stetigen Kontakt mit der Königin nehmen Arbeiterinnen das nicht-flüchtige QMP auf und fungieren als Bote im Bienenstock (Seeley, 1979; Velthuis, 1972). Das vielseitig wirkende QMP ist mitverantwortlich für die Unterdrückung der Reproduktionsorgane bei Arbeiterinnen und der Aufzucht einer neuen Königin (Conte & Hefetz, 2008; Hoover et al., 2003; Slessor et al., 2005). Dies sichert der Königin das Fortpflanzungsmonopol im Stock. Die vielseitigen Auswirkungen des QMP auf das Verhalten der Arbeiterinnen sind eng mit der Fähigkeit verbunden, die neurochemischen Prozesse im Gehirn dieser zu beeinflussen. Durch die Modulation der Produktion von Schlüsselneurotransmittern wie Dopamin wird durch QMP unter anderem das Belohnungssystem der Arbeiterinnen beeinflusst (Vergoz et al., 2007). Diese Veränderung des Belohnungssystems hat weitreichende Konsequenzen für das Lernverhalten der Arbeiterinnen. Während das QMP das aversive Lernen, wie zum Beispiel die Assoziation eines Duftes mit einem unangenehmen Reiz, bei jungen Bienen blockiert, bleibt das appetitive Lernen, welches beispielsweise bei der Verbindung von Duft und Belohnung aktiv ist, unbeeinträchtigt. Diese selektive Beeinflussung des Lernverhaltens hat zur Folge, dass junge Arbeiterinnen keine Abneigung gegen den Geruch ihrer Königin entwickeln (Vergoz et al., 2007). Stattdessen werden sie durch das QMP dazu angeregt, den Kontakt mit der Königin zu suchen (Slessor et al., 2005). Dieser Mechanismus stellt sicher, dass die Königin

als Zentrum des Bienenvolkes wahrgenommen wird und ihre Monopolstellung erhalten bleibt. Darüber hinaus verändert QMP spezifisch die Dopamin-Signalwege im Gehirn der Arbeiterinnen, die eine zentrale Rolle bei der Verhaltensregulation und motorischen Kontrolle spielen (Beggs et al., 2007; Beggs & Mercer, 2009). QMP, insbesondere der Hauptbestandteil Homovanillylalkohol, senkt den Dopaminspiegel im Gehirn signifikant, was die Expression von Dopaminrezeptorgenen wie *Amdop1* beeinflusst und die neuronalen Reaktionen auf Dopamin in den übergeordneten Integrationszentren für alle Sinneseindrücke, den Pilzkörpern (Mushroom Bodies; MB) verändert. Dadurch wird das Aktivitätsniveau der Arbeiterinnen gesenkt, was dazu führt, dass junge Arbeiterinnen sich auf koloniebezogene Aufgaben wie Brutpflege oder die Betreuung der Königin konzentrieren, anstatt sich auf Bewegung oder andere Aktivitäten zu fokussieren (Beggs et al., 2007). Diese Verhaltensänderungen gewährleisten eine effiziente Arbeitsteilung und die Organisation des Bienenvolks.

Mit ansteigendem Alter erweitern die Arbeiterinnen ihr Aufgabenspektrum und übernehmen zunehmend Tätigkeiten außerhalb des unmittelbaren Brutbereichs. Bienen im Alter von 12 bis 21 Tagen, auch "middle-aged bees", zeichnen sich durch ein besonders großes Aufgabenrepertoire aus (Johnson, 2008). Sie sind im gesamten Bienenstock anzutreffen und übernehmen den größten Anteil der Aufgaben, die zum Erhalt und zur Entwicklung des Volkes beitragen. Zu ihren Aufgaben zählen unter anderem der Bau von Waben, die Produktion von Honig, die Verarbeitung von Propolis und die Verteidigung gegen Prädatoren (Johnson, 2008). Die Arbeiterinnen konstruieren die sechseckigen Zellen, die als Lager für Honig, Pollen und Brut dienen. Dafür verarbeiten sie das Wachs, das von speziellen Drüsen in ihrem Abdomen produziert wird (Page & Peng, 2001). Propolis, ein harzartiges Gemisch, sammeln die Bienen von Pflanzen ein und verwenden es zur Desinfektion und zum Abdichten des Bienenstocks (Calderone & Page, 1988; Seeley, 1995). In dieser Lebensphase legen die Arbeiterinnen weite Strecken im Stock zurück und übernehmen zunehmend Aufgaben im Zusammenhang mit der Nahrungsversorgung. Sie nehmen Nektar von Sammlerinnen auf und speichern ihn in ihrem Honigmagen bis dieser in den Honigwaben eingelagert wird.

Mit Erreichen eines Alters von etwa drei Wochen spezialisieren sich die Arbeiterinnen auf die Aufgabe der Sammlerin. Sie verlassen den Bienenstock und sammeln Nektar, Pollen und Wasser (Calderone & Page, 1988; Johnson, 2010; Seeley, 1995).

Informationen über die Qualität der einer Nahrungsquelle wird im Stock über Trophallaxis an andere Sammlerinnen weitergegeben (Goyret & Farina, 2005; Korst & Velthuis, 1982). Durch die Ausführung verschiedener Tanzfiguren und die Dauer des Schwänzeltanzes können die Arbeiterinnen präzise Angaben über die Entfernung und die Richtung einer Nahrungsquelle machen (Riley et al., 2005).

Das *doublesex*-Gen als zentraler Regulator des Geschlechts-dimorphismus und geschlechtsspezifischen Verhaltens bei Insekten

Das Gen *doublesex* (*dsx*) hat sich in den letzten Jahrzehnten als ein entscheidender Faktor für die Regulation des Geschlechtsdimorphismus und geschlechtsspezifischen Verhaltens bei einer Vielzahl von Insektenarten erwiesen. Als nachgeschalteter Effektor der Geschlechtsbestimmenden Kaskade hat *dsx* weitreichenden Einflüsse auf die Morphologie und Physiologie, sowie auf das Verhalten.

Bei der Fruchtfliege *Drosophila melanogaster* beispielsweise steuert *dsx* nicht nur die Entwicklung der Geschlechtsorgane (Camara et al., 2019), sondern beeinflusst auch die Pigmentierung (Kopp et al., 2000; Rideout et al., 2010; Williams et al., 2008), die Morphologie der Beine (Devi & Shyamala, 2013; Rice et al., 2019) und die Ausbildung geschlechtsspezifischer Neuronenverbände, welche beispielsweise die Grundlage für das charakteristische Balzverhalten der männlichen Fruchtfliege bilden (Ghosh et al., 2019; Shirangi et al., 2016). Weibliche Fruchtfliegen zeigen hingegen eine veränderte Empfänglichkeit für männliche Balzsignale, die ebenfalls durch *dsx* reguliert wird (Kimura et al., 2008; Rideout et al., 2007). Auch bei anderen Insekten wie Käfern, Schmetterlingen und Mücken wurde eine enge Verbindung zwischen *dsx* und der Ausbildung geschlechtsspezifischer Merkmale festgestellt. So führt ein Mangel von *dsx* bei männlichen Seidenraupen zur Entwicklung von abnormalen Geschlechtsorganen (Xu et al., 2017), während bei Mücken das *dsx*-Gen die Fertilität und die Morphologie der Mundwerkzeuge beeinflusst (Mysore et al., 2015).

Während die Funktion von *dsx* bei vielen Insektenarten gut untersucht ist, sind die Erkenntnisse zur Honigbiene noch lückenhaft. Als zentraler Bestandteil der Geschlechtskaskade beeinflusst *dsx* die Entwicklung geschlechtsspezifischer Merkmale auf mehreren Ebenen. Bei der Honigbiene ist bekannt, dass Mutationen im *dsx*-Gen in genetischen Weibchen die Integrität der Eierstöcke beeinträchtigen und zu

Intersex-Formen führen können (Roth et al., 2019). Die Identität des *dsx*-Gens wird dabei maßgeblich durch das Gen *feminizer* bestimmt, welches die Transkription der männlichen und weiblichen Isoformen reguliert (Gempe et al., 2009; Roth et al., 2019; Seiler & Beye, 2024). Allerdings sind mögliche Auswirkungen auf andere morphologische Merkmale und das Verhalten, wie beispielsweise die Kastendetermination oder das Sozialverhalten, noch weitgehend unerforscht.

Vergleiche der *dsx*-Gene verschiedener Insektenarten haben gezeigt, dass die zugrundeliegende Proteinstruktur hochkonserviert ist (Biewer et al., 2015; Burtis & Baker, 1989; Cho et al., 2007). Der molekulare Mechanismus, durch den *dsx* diese vielfältigen Effekte erzielt, ist insbesondere bei *D. melanogaster* und *Caenorhabditis elegans* gut charakterisiert. Das Dsx-Protein enthält eine hochkonservierte DM-Domäne, die an die DNA bindet und als Transkriptionsfaktor fungiert (Burtis & Baker, 1989; Raymond et al., 1998; Shen & Hodgkin, 1988; Zhu et al., 2000). Die DM-Domäne besteht aus der ersten Oligomerisierungsdomäne (OD1) und zwei verschlungenen CCHC- und HCCC-Zinkfingerdomänen, die für die DNA-Bindung essenziell sind. Die geschlechtsspezifische Regulation erfolgt über eine zweite Oligomerisierungsdomäne (OD2), die im C-terminalen Bereich lokalisiert ist und in männlichen und weiblichen Isoformen unterschiedlich aufgebaut ist (An et al., 1996; Clough et al., 2014; Erdman et al., 1996; Erdman & Burtis, 1993; Zhu et al., 2000). Diese Isoformen entstehen durch alternatives Spleißen. Durch die Bindung an spezifische Zielgene kann Dsx die Expression geschlechtsspezifischer Merkmale induzieren oder unterdrücken. Bekannte Zielgene von Dsx sind beispielsweise *Yolk protein* (Burtis et al., 1991), *bric-a-brac* (Williams et al., 2008) und *Flavin-containing monooxygenase-2* (Luo & Baker, 2015) in *D. melanogaster* sowie *vitellogenin* in *Bombyx mori* (Suzuki et al., 2003, 2005). Interessanterweise binden sowohl die männliche als auch die weibliche Isoform von Dsx an identische DNA-Sequenzen, interagieren jedoch mit unterschiedlichen Kofaktoren, wie beispielsweise mit dem Produkt des Hox-Gens *abdominal-B*. Aufgrund dieser Interaktion mit einem Homeodomain-haltigen Transkriptionsfaktor hat die weibliche Dsx-Form das Potential, nahezu jede Art von Gewebe und geschlechtsspezifische Entwicklung zu regulieren (Ghosh et al., 2019). Diese Überschneidung von Effekten und der konservierte Aufbau des Proteins deutet auf einen gemeinsamen evolutionären Ursprung und eine ähnliche Funktionsweise in verschiedenen Insektenarten hin.

Methoden zur genetischen Manipulation in Honigbienen

Die genetische Manipulation von Insekten hat in den letzten Jahren aufgrund der Entwicklung des CRISPR/Cas9-Systems, welches eine präzise und effiziente Veränderung des Genoms ermöglicht, erhebliche Fortschritte gemacht (Bassett et al., 2015; Bassett et al., 2013; Chaverra-Rodriguez et al., 2018; Hammond et al., 2016). Bei der Honigbiene wurden bisher vor allem vollständige Gen-Inaktivierungen mittels CRISPR/Cas9 und des nicht-homologen Endverbindens (NHEJ) durchgeführt (Chen et al., 2021; Değirmenci et al., 2020; Kohno et al., 2016; Roth et al., 2019). Dieser Reparaturmechanismus führt durch zufällige Insertion oder Deletion von Nukleotiden an der Bruchstelle in der Regel zu Frameshifts (Gagnon et al., 2014; Sander & Joung, 2014). Während diese Ansätze wertvolle Einblicke in die Genfunktion liefern, besteht ein wachsendes Interesse an gezielten Genom-Modifikationen, die beispielsweise die Expression eines Gens an einem spezifischen Ort und zu einer bestimmten Zeit ermöglichen. Der bisherige Einsatz von Transposasen zur Integration fremder DNA in das Genom der Honigbiene, ermöglich zwar das Einbringen der Fremd-DNA, jedoch ist die Integration ohne eine spezifische Zielsequenz und mit einer schlechten Effizienz und Mosaizismus verbunden (Otte et al., 2018; Schulte et al., 2014).

In anderen Organismen hat sich die homologe rekombinatorische Reparatur (Homology-Directed-Repair; HDR) als leistungsfähige Methode zur Integration von DNA-Sequenzen an einen definierten Genom-Lokus erwiesen (Albadri et al., 2017; Aslan et al., 2017; Bosch et al., 2020). In Kombination mit CRISPR/Cas9 ermöglicht HDR die präzise Insertion von Genen oder die Korrektur von Mutationen. Durch die Nutzung von guide RNAs kann das Cas9-Enzym an eine gewünschte DNA-Sequenz geleitet werden, und die HDR-Maschinerie ersetzt diese Sequenz durch die gewünschte DNA-Konstruktion. Die erfolgreiche Integration von Fremd-DNA mittels HDR eröffnet neue experimentelle Möglichkeiten. Ein häufiges Vorgehen ist die Verwendung von Reportergenen wie dem Grün-Fluoreszenz-Protein (Green Fluorescent Protein; GFP), um die Expression des markierten Gens beispielsweise im Gehirn zu visualisieren (Auer et al., 2014; Billeter et al., 2006). Dies ermöglicht eine detaillierte Analyse von Genexpressionsmustern und Proteinlokalisationen.

Während die Integration von Fremd-DNA mittels HDR und die Visualisierung durch GFP wertvolle Einblicke in die Genfunktion und -expression ermöglichen, beschränken

sich diese Methoden oft auf statische Veränderungen des Genoms. Die vollständige Inaktivierung eines Gens durch CRISPR/Cas9 kann zwar Aufschluss über dessen Funktion geben, jedoch können die resultierenden phänotypischen und verhaltensbezogenen Veränderungen sowohl durch den direkten Verlust der Genfunktion als auch durch indirekte Effekte auf andere Prozesse verursacht werden. Um kausale Zusammenhänge zwischen spezifischen neuronalen Schaltkreisen und bestimmten Verhaltensweisen herzustellen und eine präzise funktionelle Analyse durchzuführen, ist es notwendig, die Genaktivität dynamisch und gezielt zu modulieren. Diese gezielte Modulation neuronaler Aktivität wurde in anderen Modellorganismen bereits erfolgreich mit optogenetischen Werkzeugen, wie beispielsweise der Einsatz des lichtaktivem Protein Channelrhodopsin-2, realisiert (Duebel et al., 2015; Pulver et al., 2011). Diese ermöglichen es, die Aktivität von Neuronen durch Licht zu kontrollieren. In Insekten, wie der Honigbiene, sind optogenetische Ansätze jedoch aufgrund der lichtundurchlässigen Cuticula nur bedingt geeignet.

Eine vielversprechende Alternative bieten Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Diese synthetischen, chimäre G-Protein-gekoppelte Rezeptoren, die in spezifische Neuronen exprimiert werden können, ermöglichen eine präzise pharmakologische Kontrolle neuronaler Aktivität (Armbruster et al., 2007; Chen et al., 2015; Coward et al., 1998; Roth, 2016). Eine häufig verwendete DREADD-Variante ist hM4Di, ein inhibitorischer Rezeptor, der bei Aktivierung durch den synthetischen Liganden, wie Clozapin-N-oxid (CNO) oder Compound 21 (C21) die neuronale Aktivität hemmt (Armbruster et al., 2007; Atasoy & Sternson, 2018; Roth, 2016). hM4Di ist ein G-Protein-gekoppelter Rezeptor, der auf dem muskarinischen M4-Rezeptor basiert. Wird hM4Di in eine Nervenzelle eingebracht und diese Zelle anschließend mit CNO behandelt, bindet CNO an den Rezeptor und aktiviert ihn. Diese Aktivierung führt zur Öffnung von Kaliumkanälen, was zu einer Hyperpolarisation der Zelle führt (Coward et al., 1998; Shan et al., 2022). Die Zelle wird somit weniger erregbar und ihre Frequenz der Aktionspotentiale sinkt.

Die Vorteile von hM4Di liegen in seiner Präzision, der zeitlichen Kontrolle und der Reversibilität (Michaelides & Hurd, 2016; Zhu & Roth, 2014). Durch die gezielte Expression von hM4Di in bestimmten neuronalen Populationen kann die Aktivität genau dieser Neuronen beeinflusst werden. Die Aktivierung erfolgt erst nach

Verabreichung des spezifischen Liganden, was eine präzise zeitliche Kontrolle ermöglicht. Zudem ist seine Wirkung reversibel, d.h. nach Absetzen des Liganden normalisiert sich die neuronale Aktivität wieder (Armbruster et al., 2007; Roth, 2016).

Die hM4Di-Technologie hat sich in zahlreichen Modellorganismen bewährt, darunter Mäuse (Alexander et al., 2009), Ratten (Ferguson et al., 2011), nicht-menschliche Primaten (Eldridge et al., 2016) und sogar Insekten wie der Fruchtfliege (Becnel et al., 2013). In diesen Organismen wurden DREADDs eingesetzt, um die Rolle verschiedener Hirnregionen bei einer Vielzahl von Verhaltensweisen und Prozessen zu untersuchen, darunter Lernen, Gedächtnis, Angst, Belohnung, Sucht, neurodegenerative Erkrankungen und soziale Interaktionen.

Neben hM4Di gibt es eine Vielzahl weiterer DREADD-Varianten, die unterschiedliche Wirkungen auf die neuronale Aktivität haben. So aktiviert beispielsweise hM3Dq bei Bindung des Liganden die Adenylatcyclase und führt so zu einer Erhöhung des cAMP-Spiegels und einer Aktivierung der Zelle (Armbruster et al., 2007; Atasoy & Sternson, 2018; Roth, 2016). Das hM4Di-DREADD-System stellt ein leistungsstarkes Werkzeug dar, um die Aktivität spezifischer neuronaler Populationen in lebenden Organismen präzise zu modulieren. Durch die Kombination mit anderen genetischen Werkzeugen und bildgebenden Verfahren eröffnet es neue Möglichkeiten, die neuronalen Grundlagen von Verhalten und Krankheit zu verstehen.

Um die Spezifität und Effizienz der neuronalen Manipulation weiter zu erhöhen, kann die DREADD-Technologie mit der HDR kombiniert werden. Diese Kombination von HDR und DREADDs eröffnet neue Möglichkeiten in der neuronalen Forschung. Sie ermöglicht eine noch präzisere Untersuchung neuronaler Schaltkreise und trägt dazu bei, die komplexen Zusammenhänge zwischen genetischen Faktoren und Verhalten besser zu verstehen.

Zielsetzung

Die Honigbiene *A. mellifera* stellt ein faszinierendes Modellorganismus dar, um die Auswirkungen der Genetik auf das komplexe Verhalten zu untersuchen. Das hochgradig soziale Leben der Honigbienen, charakterisiert durch eine strikte Arbeitsteilung, komplexe Kommunikation und kooperative Verhaltensweisen, ist ein Phänomen, dessen genetische Grundlagen noch weitgehend unerforscht sind (Crailsheim, 1998; Johnson, 2008). Die Frage, wie solche angeborenen Verhaltensweisen im Gehirn der Arbeiterinnen während ihrer Entwicklung festgelegt werden, ist bisher ungeklärt.

Mit dem Regulator *dsx*, einem Gen, für das bereits in anderen Spezies gezeigt wurde, dass es neben einem Einfluss auf die Geschlechtsdifferenzierung nach neuesten Studien zu urteilen auch einen Einfluss auf soziale Interaktionen bei Insekten hat (Beckers et al., 2017; Kimura et al., 2008; Rideout et al., 2007; Siwicki & Kravitz, 2009), liegt uns ein Kandidatengen vor, das prädestiniert ist, die genetische Grundlage des ausgeprägten Sozialverhaltens bei der Honigbiene *A. mellifera* zu untersuchen.

Ein zentrales Ziel dieser Arbeit ist die Etablierung einer robusten Genomeditierungsmethode in *A. mellifera*, um gezielt das Gen *dsx* zu markieren. Hierzu soll die CRISPR/Cas9-basierte HDR-Methode optimiert und erfolgreich eingesetzt werden, um verschiedene Konstrukte, darunter mCD8-P2A und HA-Myc-Tag, in den *dsx*-Locus von *A. mellifera* zu integrieren. Diese Konstrukte dienen vor allem der Markierung von *dsx*-exprimierenden Zellen, können aber auch zur Untersuchung von Protein-Protein-Interaktionen genutzt werden.

Ein weiteres Ziel ist die Untersuchung der funktionellen Bedeutung von *dsx* für das Sozialverhalten. Durch gezielte Manipulation der Dsx-Zinkfingerdomäne sollen Arbeiterinnen mit einer Funktionsverlustmutation im Gen *dsx* erzeugt werden. Die Verhaltensphänotypen dieser mutierten Arbeiterinnen werden mithilfe eines computergestützten Tracking-Systems (Blut et al., 2017) charakterisiert, um verschiedene Aspekte ihrer sozialen Interaktionen zu quantifizieren. Dazu gehören nicht nur grundlegende kooperative Verhaltensweisen wie die Brutpflege, sondern auch die Fähigkeit, den Input anderer Arbeiterinnen wahrzunehmen und zu verarbeiten. Um dieses Verhalten besser zu verstehen, soll auch der Ort, an dem das Verhalten spezifiziert wird, das Gehirn, genauer analysiert werden. Dazu soll zum einen die neuronale Entwicklung der Honigbiene *A. mellifera* mittels histologischer

Analyse der Gehirne der erzeugten *dsx*-mutierten Arbeiterinnen bestimmt werden. Zum anderen soll die Verteilung der *dsx*-exprimierenden Neuronen im Arbeiterinnengehirn mittels GFP-Knock-in-Strategie kartiert und die Funktion, der von diesen Neuronen beeinflussten, neuronalen Netzwerke untersucht werden.

Damit der Einfluss der *dsx*-exprimierenden Neuronen und die dazugehörigen neuronalen Schaltkreise auf das Verhalten noch genauer bestimmt werden kann, soll das hM4Di-DREADD-System für die Honigbiene *A. mellifera* etabliert werden. Dies soll eine präzise räumliche und zeitliche Kontrolle der *dsx*-Aktivität ermöglichen.

Zusammengenommen werden die Ergebnisse dieser Dissertation unser Verständnis der molekularen und neuronalen Mechanismen, die dem einzigartigen sozialen Verhalten der Honigbiene *A. mellifera* zugrunde liegen, erheblich erweitern. Durch die Analyse der Funktion des *dsx*-Gens werden wir wertvolle Erkenntnisse über die Evolution komplexer sozialer Systeme und die genetische Grundlage der Verhaltensvielfalt gewinnen.

Kapitel II: Manuskripte

Manuscript I

Highly efficient site-specific integration of DNA fragments into the honeybee genome using CRISPR/Cas9

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Investigation

Highly efficient site-specific integration of DNA fragments into the honeybee genome using CRISPR/Cas9

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Abstract

Functional genetic studies in honeybees have been limited to transposon mediated transformation and site directed mutagenesis tools. However, site- and sequence-specific manipulations that insert DNA fragments or replace sequences at specific target sites are lacking. Such tools would enable the tagging of proteins, the expression of reporters and site-specific amino acid changes, which are all gold standard manipulations for physiological, organismal, and genetic studies. However, such manipulations must be very efficient in honeybees since screening and crossing procedures are laborious due to their social organization. Here, we report an accurate and remarkably efficient site-specific integration of DNA-sequences into the honeybee genome using clustered regularly interspaced short palindromic repeat/clustered regularly interspaced short palindromic repeat-associated protein 9-mediated homology-directed repair. We employed early embryonic injections and selected a highly efficient sgRNA in order to insert 294 and 729 bp long DNA sequences into a specific locus at the *dsx* gene. These sequences were locus-specifically integrated in 57% and 59% of injected bees. Most importantly, 21% and 25% of the individuals lacked the wildtype sequence demonstrating that we generated homozygous mutants in which all cells are affected (no mosaicism). The highly efficient, locus-specific insertions of nucleotide sequences generating homozygous mutants demonstrate that systematic molecular studies for honeybees are in hand that allow somatic mutation approaches via workers or studies in the next generation using queens with their worker progeny. The employment of early embryonic injections and screenings of highly efficient sgRNAs may offer the prospect of highly successful sequence- and locus-specific mutations also in other organisms.

Keywords: *Apis mellifera*; CRISPR/Cas9; homology-directed repair; methods; knock-in; gene editing

Introduction

Honeybees are equipped with remarkable behavioral abilities, morphological, and physiological features that associate their social organization in colonies. A honeybee colony typically consists of thousands of worker bees, a single queen, and hundreds of males (drones). The worker bee caste displays a rich behavioral repertoire (Seeley 1982; Seeley and Visscher 1985; Page and Erber 2002; Johnson 2008; Robinson *et al.* 2008), sophisticated cognitive abilities (Menzel 2001, 2012), and communication abilities (Frisch *et al.* 1967; Riley *et al.* 2005) that are devoted to the maintenance of the colony. The queens display behaviors related to reproduction that include egg-laying and mating behavior. The development into either queens and workers is the outcome of female-determining and caste-determining signal (Vleurinck *et al.* 2016; Roth *et al.* 2019). The female determination signal is provided by heterozygosity at the *complementary sex determiner* (*csd*) locus (Beye *et al.* 2003, 2013). The *doublesex* (*dsx*) gene is a further downstream component of the sex determination pathway regulating reproductive organ development (Roth *et al.* 2019). The differential feeding with worker diet or royal jelly during larval development determines the differentiation into either the worker and queen caste (Haydak 1970; Asencot and Lensky 1988; Kucharski *et al.* 2008; Leimar *et al.* 2012; Buttstedt *et al.* 2016; Maleszka 2018), a process that is a prominent example of developmental plasticity. A systematic dissection of the molecular processes of development and behavior in the honeybee are still limited in part due to the lack of site-specific gene manipulation tools that would enable targeted insertions of reporters and site-specific manipulations of gene functions.

So far, genes can be transgenetically expressed from endogenous and nonendogenous promoters using piggyBac-mediated transformations (Schulte *et al.* 2014; Otte *et al.* 2018). Or, endogenous genes can be site- but not sequence-specific mutated using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-system (Kohno *et al.* 2016; Roth *et al.* 2019; Değirmenci *et al.* 2020; Chen *et al.* 2021). However, locus- and sequence-specific insertion of DNA sequences would ensure in deep analyses of genes and their molecular and organismal function. To meaningfully apply such tools in honeybees, this requires highly efficient methods, which reduce laborious screening procedures. Each reproductive female, the

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queen, needs to be maintained in distinct colonies together with at least few thousand worker bees in containments which is mandatory due to the genetic manipulations (Schulte *et al.* 2014; Otte *et al.* 2018).

Recent work in other species showed that providing a donor DNA together with CRISPR/Cas9 can induce homology-directed repair (HDR) resulting in the insertion of donor DNA (Gratz *et al.* 2014; Port *et al.* 2015; Hammond *et al.* 2016; Chen *et al.* 2021). This process requires that homologous sequences of a specific locus are provided to left and right of the fragment that need to be inserted. As a donor, double strand DNA (Paix *et al.* 2017) as well as single strand DNA (Quadros *et al.* 2017) can be used. Donor DNA can be a circular plasmid or a linear fragment (Gratz *et al.* 2014; Paix *et al.* 2017). The sizes of the homology arms can range between 15 bp and 1.5 kb (Gratz *et al.* 2014; Paix *et al.* 2017; Li *et al.* 2019).

In this study, we demonstrated highly efficient, locus-specific integration of DNA sequences in honeybees via CRSPR/Cas9-mediated HDR. The presented procedure offers the prospect of systematic dissection of molecular and organismal gene function and the expression of reporter genes from endogenous gene promoters. Homozygous mutants are so frequently obtained even enabling functional studies in the injected generation (Roth *et al.* 2019).

Materials and methods

Donor DNA, sgRNA

The 794-bp long Myc + HA DNA fragment was synthesized as a single-stranded DNA fragment (IDT Integrated DNA Technologies, Coralville, IA: Megamer Single-Stranded DNA Fragments). We composed the coding sequence as such so that 5 repeats of the c-Myc-tag (EQKLISEEDL) (Evan *et al.* 1985; Kaltwasser *et al.* 2002) and 5 repeats of the hemagglutinin (HA)-tag (YPYDVPDYA) can be expressed which we fused with a Gly–Ser–Gly (GSG) linker sequence (Supplementary Fig. 1a) (Szymczak-Workman *et al.* 2012). The *mCD8*+P2A fragment was synthesized as double-stranded molecule and was 1,229 bp long (GeneStrands, Eurofins, Ebersberg, Germany). We combined the *mCD8*, GSG linker, and 2A peptide (P2A) coding sequence (Supplementary Fig. 1b) (Szymczak-Workman *et al.* 2012). The coding sequence of the alpha chain of the mouse lymphocyte antigen CD8 was derived from the Addgene data base (Addgene plasmid # 17746; http://n2t.net/addgene:17746; RRID: Addgene_17746). We adjusted all coding sequences to the codon usage of the honeybee (*Apis mellifera*) (https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=44477).

The sequences of the homologous arms were derived from the exon 2 sequence of the *dsx* gene [NCBI; gene ID: 725126; Reference Sequence: NC_037642; Assembly: Amel_HAv3.1 (GCF_003254395.2)]. Arm lengths were ~250 bp long, a size that gave high integration rate despite their rather small size in a previous study (Li *et al.* 2019).

The synthesized *mCD8*+P2A donor sequence was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Braunschweig, Germany) and the following oligonucleotide primers (forward primer: GTTGCAGAACGAGGAATCGGGGGAAAG; reverse primer: TGATCTTACACTTCTCGCAGGTACAAGTACG; Custom DNA Oligos, Eurofins). The amplicon was and purified with EZNA Cycle Pure kit (Omega Bio-Tek Inc., Norcross, GA) before injections.

The *dsx*-sgRNA1 was synthesized as described previously (Roth *et al.* 2019).

Microinjection and bee handling

Fertilized honeybee eggs were injected 0–1.5 h after egg deposition (Beye *et al.* 2002; Schulte *et al.* 2014; Roth *et al.* 2019) with 53-mm injection needles (Hilgenberg, Malsfeld, Germany). Approximately 200 pg Cas9 Protein (New England Biolabs, Ipswich, MA), 18.5 pg *dsx*-sgRNA1, and donor DNA were injected into each embryo.

Rearing of hatched larvae was performed (Roth *et al.* 2019) by supplying 170 mg of the worker larval diet "Diet 7" [53% royal jelly, 4% glucose, 8% fructose, 1% yeast extract, and 34% water (Kaftanoglu *et al.* 2010; Kaftanoglu *et al.* 2011; Roth *et al.* 2019)] under restricted humidity conditions (Schmehl *et al.* 2016).

DNA preparations, PCRs, and sequencing

Genomic DNA was isolated with the innuPREP DNA Mini Kit (Analytik Jena, Jena, Germany). PCRs were run under standard conditions (Hasselmann and Beye 2004) using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and oligonucleotide primers (forward primer: GATTCGTAATAATTCCTGTGC; reverse primer: CTTCCGCTACTCTTACTTTGAC; Custom DNA Oligos, Eurofins). For the Sanger sequencing (Mix2Seq Kit, Eurofins) amplicons were cloned into pGEM-T Easy Vector (Promega, Madison, WI).

Results

We used a previously published injection and CRISPR/Cas9 procedure (Schulte *et al.* 2014; Otte *et al.* 2018; Roth *et al.* 2019) of honeybees to insert 2 DNA fragments via CRISPR/Cas9-mediated HDR. The linear DNA fragment Myc+HA (Fig. 1a; Supplementary Fig. 1a) consisted of 5 repeats of a c-Myc-tag (Evan *et al.* 1985; Kaltwasser *et al.* 2002) and 5 repeats of an HA-tag (Wilson *et al.* 1984; Lee and Luo 1999), which we fused with a GSG linker (Szymczak-Workman *et al.* 2012). The other linear DNA fragment *mCD8*+P2A (Fig. 1a; Supplementary Fig. 1b) had a *mCD8* (Liaw *et al.* 1986; Lee and Luo 1999), a GSG linker and a P2A sequence (Szymczak-Workman *et al.* 2012).

To induce a homologous repair in the *dsx* gene locus (Fig. 1b) we expanded the above fragments with 250 bp to the left and to the right using the nucleotide sequences upstream and downstream of the designated cleavage site of the Cas9 protein (Fig. 1a). We selected *dsx*-sgRNA1 which has highly efficient in directing mutations using our standard procedures. This sgRNA induced in up to 100% of the injected individuals mutations (Roth *et al.* 2019). Approximately 200 pg Cas9 protein and *dsx*-sgRNA1 at a molar ratio of 1:1 together with donor DNA were injected into 0–1.5 h old embryos. We injected 20–30 pg per embryo for the Myc + HA DNA fragment and 15–20 pg for *mCD8* + P2A DNA fragment. In respect to DNA concentrations, we followed thereby results from previous donor DNA based experiments as a guideline (Schulte *et al.* 2014; Otte *et al.* 2018). Embryos were reared and bees were collected and genotyped at larvae stage. PCR amplifications at the *dsx* locus [these oligonucleotide primers were not matching sequences in our donor DNA f (Fig. 1b)] revealed that 8 out of 14 (57%) individual bees carried the dsx^{Myc+HA} and 40 out of 68 (59%) bees the $dsx^{mCD8+P2A}$ allele (Fig. 1c and Table 1) suggesting a substantial integration rate. Next, we asked whether the insertions were homozygous which we examined by the presence of the inserted sequence in our bees. In 3 out 14 (21%) dsx^{Myc+HA} bees and in 17 out of 68 (25%) $dsx^{mCD8+P2A}$ bees we amplified sequences with insertions (to the level of detection). This result suggests that more than 20% of the mutated bees were homozygous and that mosaicism was absent. Further, we found 5

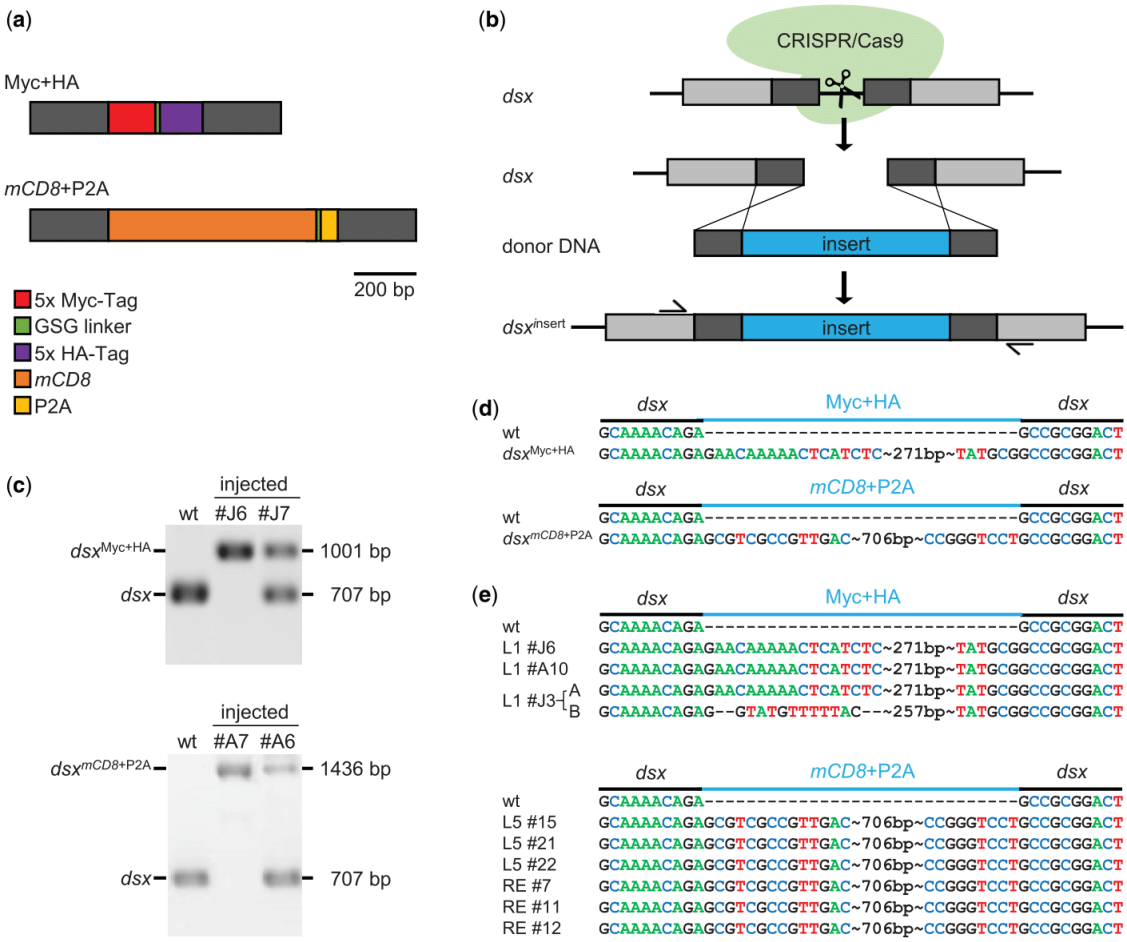

Fig. 1. The site-specific insertion of linear DNA fragments into the *dsx* gene of the honeybee. a) Schematic presentation of the DNA fragments employed. b) Scheme of the CRISPR/Cas9-mediated HDR. The blue box indicates the new DNA fragment that needs to be integrated. The black boxes indicate the homologous arms to the left and right. Gray boxes show the remaining part of the exon. Arrows above a box indicate the position of the oligonucleotide primers for amplifications. c) Amplicons from different individuals were analyzed by size in 1% agarose gel. Black and white reversed pictures of ethidium bromide stained gels are shown. d) The expected nucleotide sequence after locus-specific insertions. wt sequences of *dsx* gene above and the expected sequence after insertion for comparison with (e). e) The detected nucleotide sequences at the target site of our homozygous mutated individuals. wt, wildtype individual (noninjected).

Table 1. The DNA fragment insertions into the *dsx* gene.

Fragment		No. of bees with			No. of mutated bees
		No insert/no insert	Insert/no insert	Insert/insert	
dsx^{Myc+HA}	%	43	36	21	57
	N	(6/14)	(5/14)	(3/14)	(8/14)
$dsx^{mCD8+P2A}$	%	41	34	25	59
	N	(28/68)	(23/68)	(17/68)	(40/68)

out of 14 (36%) dsx^{Myc+HA} bees and 23 out of 68 (34%) $dsx^{mCD8+P2A}$ bees with and without an insert (Fig. 1c and Table 1). The later results indicate that the DNA fragment was inserted in only 1 allele or in a subgroup of cell.

To validate the PCR-based genotyping results, we determined the nucleotide sequence of the amplicons from the homozygous individuals with an insert/insert genotype. Three to 11 independent clones for each of the 9 individuals were sequenced

Table 2. Nucleotide sequences of the designated target site of homozygous individuals.

Fragment	Individual	No. of clones showing correct integration	
dsx^{Myc+HA}	L1 #J3	%	43
		N	(3/7)
	L1 #J6	%	100
		N	(7/7)
	L1 #A10	%	100
		N	(3/3)
$dsx^{mCD8+P2A}$	L5 #15	%	100
		N	(8/8)
	L5 #21	%	100
		N	(8/8)
	L5 #22	%	100
		N	(11/11)
	RE #7	%	100
		N	(5/5)
	RE #11	%	100
		N	(8/8)
	RE #12	%	100
		N	(8/8)

(Table 2). We found that all individuals (9 out of 9; 100%) carried the sequence of Myc + HA or *mCD8* + P2A at the designated locus (Fig. 1d and e) demonstrating targeted insertions. For 1 allele of individual J3 the sequence of the DNA fragment did not follow expectation suggesting that other mutations can rarely occur during this integration process. Thus, we conclude that 8 out 9 individuals (89%) had correctly inserted DNA sequences at the designated target site of the *dsx* gene demonstrating the power of this approach.

Discussion

Our results demonstrate that locus-specific insertions of new sequences of more than 700 bp are now very feasible in the honeybee (Table 1). Fifty-seven percent (ss donor) and 59% (ds donor) of our injected individuals carried the insert. Eighty-nine percent of individuals had the sequence correctly inserted at the designated target site (Table 2). Furthermore, our results showed that homozygous mutants with a $dsx^{insert/insert}$ genotype were quite frequent in our mutated bees (Table 1 and Fig. 1e) suggesting that this technique will have broad applications for systematic molecular and organismal studies (see further below). We suggest that that at least 3 factors have substantially contributed to this efficiency that possibly can also be applied to other organisms. First, a preselected sgRNA and optimized Cas9/sgRNA concentrations that induce mutations at very high frequency, which was up to 100% of individuals in our case (Roth *et al.* 2019). Second, early embryonic injections before the first cleavage of the nucleus after 3.5 h (Schnetter 1934), which was in our case 1–3 h after egg deposition. Third, the appropriate length of the homologous sequence of our donor fragment, which was in our case 250 bp.

The efficiency of locus-specific insertions of sequences establishes the CRISPR/Cas9-mediated HDR as a powerful, new genetic tool for honeybee studies. This tool extends the existing tool box that so far consist of site directed mutagenesis (Kohno *et al.* 2016; Roth *et al.* 2019) and transposon mediated expression of transgenes (Schulte *et al.* 2014; Otte *et al.* 2018). The locus-specific insertions of Myc, HA, or *mCD8* coding sequence into the open reading frame of an endogenous gene will enable labeling of gene products in tissues which can detected by commercially available antibodies and immunostainings. Or, such tool can induce site-specific changes of nucleotides and hence amino acids that will greatly support a deep understanding of the gene's function. This tool also offers the prospect to express molecular reporters in a subset of cells and tissues (Wang *et al.* 2019).

In *Drosophila melanogaster* the number of individuals carrying integrations is usually employed to determine integration rates, which includes individuals with mosaicism in the germline (Port *et al.* 2015). Hence, our results, which rely only on entirely mutated individuals, may indicate an even more efficient integrations in honeybees. Especially for honeybees, efficient integrations with no mosaicism are very important. Crossing experiments are laboriously in honeybees. This is because worker bees and queens need to be maintained in colonies. Genetic manipulations add further to this difficulty, since these colonies needs to be kept in a strict containment for safety reasons. These conditions limit the number of queens that can be reared and screened for the desired insertion. Now with this high insertion and homozygous rate, the methods establish a convincing approach to obtain locus- and sequence-specific manipulations in queens and hence, after instrumental inseminations, also in the worker progeny. If such next generation approach is not desirable, the high frequency and the absence of mosaicism offer the alternative route of a somatic mutation approach. We previously showed that the mutated embryos can be reared to worker bees and examined (Roth *et al.* 2019).

Studies in *D. melanogaster* reported that successful CRISPR/Cas9-mediated integration rates range between 11% (Gratz *et al.* 2014) and 26% per injected individual (Port *et al.* 2015) when using coinjected Cas9 encoding plasmids. Rates can be substantially improved up to 88% per injected individual (Gratz *et al.* 2014, 2015; Port *et al.* 2015) when using transgenetically expressed Cas9 protein under the control of the *vasa* or *nos* promoters. A study in the mosquito *Anopheles gambiae* report on integration rates of 11% and 19% per injected embryo using coinjected Cas9 encoding plasmids (Hammond *et al.* 2016). Our results in honeybees now suggest that we can obtain similarly high rates by injections of Cas9 proteins without transgenetically expressing Cas9 proteins. The transgenic expression is usually not achievable for most nongenetic model organisms.

Other considerable efficiency variations within the same species can be possibly attributed to the length of the homologous arms (Li *et al.* 2019) and to the use of PCR fragments instead of donor plasmids (Paix *et al.* 2017). Lengthening the insert usually leads to reduction in the integration rate (Paix *et al.* 2017). Since our results suggest very high integration rates, it is possibly that larger inserts can also be integrated into the honeybee genome.

Hence, the technique and improvements described here, may help to develop site-specific manipulations of genomes in other organisms as well.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplemental material is available at G3 online.

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Conflicts of interest

None declared.

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Supplementary data

A) Sequence of the Myc+HA fragment. The underlined sequence represents 5x Myc tag, the italicized sequence represents GSG linker and the thick sequence represents 5x HA tag.

GTTGCAGAACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGACTACGTT
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TGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACAGAGAACAAAAACTCATCTCGGAGG
AGGATCTGGAGCAAAAGTTGATATCCGAGGAAGACCTCGAACAAAAGCTGATTTCGGAAGAAGATTTG
GAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATCTCCGAAGAGGACTTG*GGATCCGG*
*A***TACCCATACGATGTTCCAGATTACGCTTACCCGTACGACGTGCCTGACTACGCATACCCTTATGATG**
TCCCGGACTACGCGTATCCTTACGATGTGCCTGACTACGCGTACCCTTACGACGTTCCGGATTATGCG
GCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGA
TAGCAAAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAA
AGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAG
AGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCA

B) Sequence of the *mCD8*+P2A fragment. The underlined sequence represents *mCD8*, the italicized sequence represents GSG linker and the thick sequence represents Protein 2A.

GTTGCAGAACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGACTACGTT
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TGTCGCTGAACCTGCTGCTCCTCGGTGAGTCGATTATCCTGGGTAGCGGAGAAGCTAAGCCTCAAGCA
CCGGAACTCCGGATCTTCCCAAAGAAAATGGACGCGGAACTCGGTCAAAAGGTGGACCTGGTATGCGA
AGTGTTGGGCTCCGTTTCGCAAGGATGCTCGTGGCTCTTCCAAAACTCCAGCTCCAAACTCCCGCAAC
CTACGTTCGTTGTCTACATGGCTTCGTCCCACAACAAGATCACGTGGGACGAGAAGCTGAATTCGTCG
AAACTGTTCTCGGCGATGAGGGACACGAATAATAAGTACGTTCTCACGCTGAACAAGTTCAGCAAGGA
AAACGAAGGCTACTACTTCTGCTCGGTCATCAGCAACTCGGTGATGTACTTCAGCTCGGTCGTGCCTG
TCCTCCAAAAAGTGAACTCGACGACGACGAAGCCTGTGCTGCGGACGCCTTCGCCTGTGCACCCTACG
GGAACGTCCCAACCTCAAAGGCCGGAAGATTGCCGGCCTCGGGGCTCGGTGAAGGGCACGGGATTGGA

CTTCGCGTGCGATATTTACATCTGGGCTCCTTTGGCGGGAATCTGCGTGGCGCTCCTGCTGTCCTTGA
TCATCACGCTCATCTGCTACCACTCGCGG*GGATCCGGA***GCGACGAACTTCTCGCTGTTGAAGCAAGCT**
GGAGACGTGGAAGAAAACCCGGGTCCTGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACAC
GTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAG
ACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCATCGG
CTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAA
GATCA

Figure S1: Sequences of Myc+HA and *mCD8*+P2A.

Author's contribution: Manuscript I

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Author's contribution to Manuscript I

- Experimental design
- sgRNA synthesis
- Implementation of microinjections
- In-vitro rearing
- Genotyping of mutant honeybees
- Data analyses: sequencing data
- Authoring the manuscript

Manuscript II

Dedicated developmental programing for group-supporting behaviors in eusocial honeybees

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EVOLUTIONARY BIOLOGY

Dedicated developmental programing for group-supporting behaviors in eusocial honeybees

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The evolutionary changes from solitary to eusocial living in vertebrates and invertebrates are associated with the diversification of social interactions and the development of queen and worker castes. Despite strong innate patterns, our understanding of the mechanisms manifesting these sophisticated behaviors is still rudimentary. Here, we show that *doublesex* (*dsx*) manifests group-supporting behaviors in the honeybee (*Apis mellifera*) worker caste. Computer-based individual behavioral tracking of worker bees with biallelic stop mutations in colonies revealed that the *dsx* gene is required for the rate and duration of group-supporting behavior that scales the relationship between bees and their work. General sensorimotor functions remained unaffected. Unexpectedly, unlike in other insects, the *dsx* gene is required for the neuronal wiring of the mushroom body in which the gene is spatially restricted expressed. Together, our study establishes dedicated programming for group-supporting behaviors and provides insight into the connection between development in the neuronal circuitry and behaviors regulating the formation of a eusocial society.

INTRODUCTION

The evolutionary transition from solitary to social living in vertebrates and invertebrates led to sophisticated social behaviors. During the past 50 to 150 million years of evolution, sociality in some species became so elaborate that individuals in the group forego reproduction and changed their behavioral performance to embrace collective behavior, while others specialize in reproduction leading to the development of two castes, queen and workers (*1*, *2*). From behavioral activities of hundreds and sometimes ten-thousands of worker individuals, new properties have emerged at the collective level such as shared brood care, warfare, collective thermoregulation, nest building, and farming, which contributed to the spectacular ecological success of the eusocial species (*2*). The collective tasks and functions cannot be performed by any single individual alone. They require a group and inherited behavioral patterns performed by individual workers. However, understanding how these sophisticated and innate behaviors for social organization manifest through genetically encoded developmental programs remains poorly understood.

Substantial progress has been made over the past several decades in elucidating the genetic basis of behavior. Although a vast number of genes are likely needed for the performance of a behavior, from those in the neurons driving the behaviors through to the development and movement of appropriate anatomical structures (*3*–*6*), key developmental genes have been identified that act during development to program the capacity of specific behaviors.

A gene in vertebrates for such developmental behavioral programming is the forkhead-domain transcription factor gene *FoxP2*, which is possibly involved in speech ability in humans and approaching behaviors in mice (*7*, *8*). In the invertebrate *Drosophila melanogaster*, the *doublesex* (*dsx*) and the *fruitless* (*fru*) gene control the development of different anatomical and molecular sex–specific neuronal populations in the central nervous system (CNS) that underlie sex-specific behaviors, such as male courtship and aggression, as well as female receptivity and post-mating behaviors (*9*, *10*). Thus, a major question has been whether such dedicated development programs also exist for behaviors underlying social living.

The evolutionary rise of sociality is associated with an expansion of the behavioral repertoires and a dynamic interplay of social interactions leading to cooperation. Diverse cues likely control this richness of behaviors in a social and context-specific manner. Given the complexity of these social behaviors, an important question in behavioral biology and genetics has been which aspects of the behaviors are genetically specified to establish group-living features. Other questions from the neurobiology and sociobiology field concern the neural circuitry representation and the cognitive requirements for these social behaviors. These later questions also stem from the debate whether the control of social behaviors in groups requires advanced cognitive and elaborate sensory processing abilities compared to species living solitarily (*11*–*13*).

To understand these behaviors at the molecular and cellular levels, we must examine eusocial insects with strong innate but elaborate behavioral patterns that can be genetically manipulated. The honeybee (*Apis mellifera*) is the ideal eusocial insect for these studies as it provides a combination of elaborate and well-described innate behaviors in the worker caste (*14*, *15*), computer-based behavioral tracking in small colonies (*16*), and powerful methods for genetic manipulations (*17*–*19*) to examine underlying mechanisms.

Of the dozens of behaviors the honeybees display, the behaviors of the worker bees at the nurse stage (usually at the age of 7 to 12 days) are best suited for this study as their behaviors are robust, occur frequently, involve more than seven behavioral tasks, and are required to collectively rear the brood (*14*, *15*). For example, the nurse bees walk on the comb, repeatedly inspecting the hexagonally structured cells. The bees then choose to take up and handle food (nectar or pollen) to feed the developing larvae involving hypopharyngeal gland (HPG) secretions or to clean the empty cells on the comb (*14*, *15*, *20*, *21*). The nurse bees also continually share food with other colony members through mouth-to-mouth transfer (trophallaxis) (*20*, *21*), which

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involves the expandable part of the gut called the "honey stomach" (*22*, *23*). When worker bees get older, their behaviors gradually shift to other behaviors, such as honeycomb construction or foraging outside for nectar and pollen (*14*, *15*). Juvenile hormone, vitellogenin protein, and differential gene expression are thought to play crucial roles in regulating this age-dependent polyethism (*24*–*27*). Genetic variation, experience, and physiological state influence the preference to engage in particular behaviors (*28*–*30*). However, how the capacities for such social behaviors are molecularly and cellularly programmed during caste development (*31*, *32*) is not known.

In advanced eusocial insect colonies, sophisticated innate behaviors establishing sociality are limited to the worker caste, not to queens. This led to our hypothesis that a dedicated developmental program for social behaviors will be found in the pathway determining the differentiation into the worker castes. Previous work showed that the worker caste is determined by the combined action of the sex determination cascade and a nutrition-derived signal (*19*, *33*–*38*). Different complementary sex determiner (Csd) proteins from heterozygous genotype direct female splicing of the *feminizer* (*fem*) transcripts, which express the active Fem proteins (Fig. 1A and fig. S1) (*33*–*35*). The nutrition signal can only be implemented in females if the Fem protein is expressed given rise to worker ovary characteristics (*19*), suggesting that sex determination and nutrition signal are intertwined to regulate worker caste differentiation. One downstream component the Fem gene regulates is the *dsx* gene. Fem mediates female-specific splicing of the *dsx* transcripts, which then express the female Dsx isoform protein (Dsx^F; Fig. 1A and fig. S1). Dsx proteins are part of the structurally and functionally conserved *doublesex* and *mab-3*–related transcription factor family (*Dmrt*) and are critical for sex-specific differentiation throughout the animal kingdom (*39*, *40*) and sexual behaviors in insects (*9*, *41*). Dsx^F is required for the worker-characteristic differentiation of the worker ovary, suggesting that Dsx^F is a component of the worker caste developmental program (*19*). The worker bees do not perform sexual behaviors. However, we found that *dsx* is also expressed in the worker's brain (*42*) suggesting that the gene may have another role unrelated to sexual reproduction but related to promoting social living behaviors in the worker caste. We, therefore, set out to define the function of the *dsx* gene in specifying worker-specific behaviors, brain organization, and peripheral chemosensory mechanisms.

RESULTS

dsx^F is spatially and worker-specifically expressed in the brain

As it is unclear where Dsx^F is expressed in the honeybee brain, we used CRISPR-Cas9–mediated homologous repair to insert myrGFP and the endopeptidase P2A coding sequence into the beginning of the *dsx* coding sequence (Fig. 1B and table S1). The resulting allele, dsx^{myrGFP}, produces wild-type (wt) Dsx^F and membrane-bound green fluorescent protein (GFP) proteins in the same cell.

In worker bees, which derived from $dsx^{myrGFP/+}$-inseminated queens, we detected GFP labeling in distinct brain areas and a selected population of neurons with known behavioral functions (Fig. 1, C to F, and movie S1 to S3) (*43*–*45*). In the antennal lobe (AL), we found that olfactory sensory neurons (OSNs) along sensory tract 1 (T1, arrowhead) of the antennal nerve and in cortical regions of olfactory glomeruli (arrows) were GFP labeled (Fig. 1, G to I) (*46*, *47*). Neurons in the subesophageal ganglion (SEG) were also GFP labeled (arrow, Fig. 1, J to L). We also detected a prominent GFP-labeled cluster with relatively large somata (arrow, which we name dsx-S1) in between and underneath the medial and lateral calyx of the mushroom bodies (MBs) (Fig. 1, M to O). Neurites from this cluster project ventrally and branch out diffusely in the superior lateral protocerebrum (SLP). We found a thin commissure below the vertical lobe (VL) that might be associated with the diffuse arborizations from both dsx-S1 clusters in the SLP on both sides (arrows; Fig. 1, P to R). We found that distinct Kenyon cell (KC) populations were labeled in the MB calyx in which multisensory input is processed and integrated. We found a large bundle of GFP-labeled KC neurites in the peduncle (PED) of the MB (arrow; Fig. 1, S to U) with arborizations in the basal ring and labeling of the inner compact layer of cell bodies of class I KCs in the medial and lateral calyx (arrowhead, m- and l-CA; Fig. 1, S to U). The neurites of this population of KCs proceed along the PED and bifurcate into the medial and vertical lobe of the MB. Strong labeling in the uppermost layer of the vertical lobe shows that most of these neurons are inner compact class I KCs (arrow; Fig. 1, V to X). This group of KCs receives multisensory input in the basal ring of the MB calyx from olfactory and visual projection neurons of the antennal and optic lobes, respectively (*44*, *46*). In addition to this prominent layer in the VL, a small layer of axonal projections from class II (clawed) KCs cells was also GFP labeled in the ventral most part of the VL (γ lobe) (arrowhead; Fig. 1, V to X), which have dendrites spanning over larger regions within all MB calyx subdivisions. These results suggest that within the MBs, the *dsx* gene is specifically expressed in basal ring–associated class I (spiny) KCs and a subset of class II (clawed) KCs.

We then generated dsx^{myrGFP} queens by injecting eggs as above and rearing larvae to queens (*18*, *19*). We selected the mutated queens with no mosaicism by deep sequencing of amplicons of the target sites from which we obtained seven $dsx^{myrGFP/stop}$ and a $dsx^{myrGFP/myrGFP}$ queens. We compared the pattern of GFP-labeled cells to that of the worker brain. At the gross level, we observed that the queens had similar patterns of GFP-labeled cells as worker bees across optical sections (Fig. 2 and movies S4 and S5). For example, we found the same pattern of axons from OSNS in the T1 tract (arrowheads; Fig. 2, A and B), in the cortical regions of AL glomeruli (arrows; Fig. 2, A and B), in the SEG (Fig. 2, C and D), and in the PED (arrow) with arborizations in the basal ring of the MB calyx (arrowhead; Fig. 2, E and F). However, quantitative examinations of the layer of class I KC axons labeled in the VL suggest that it is larger in worker bees compared to queens (Fig. 3, A to E). The length [Mann-Whitney *U* (MWU) test, $z = 2.66$, $P = 0.008$] and area size ($z = 2.31$, $P = 0.02$) but not the width of KC class I GFP-labeled cells in VL sections were expanded in workers compared to queens (Fig. 3F). The overall length of the VL was not different. In contrast, the overall area size was slightly larger in worker bees (MWU test, $z = 2.08$, $P = 0.04$, Fig. 3F). This expansion in worker bees cannot be explained by the effect of the stop codon in one allele in the $dsx^{myrGFP/stop}$ queens because the value of the $dsx^{myrGFP/myrGFP}$ queen did not differ or was even smaller than the values of the $dsx^{myrGFP/stop}$ queens (one-sample Wilcoxon signed-rank test, length: $z = 2.03$, $P = 0.04$; area: $z = 0.68$, $P = 0.5$; Fig. 3F). Hence, these results suggest a caste-specific dimorphism of *dsx* expressing (dsx^+) cells in the brain. The *dsx*-expressing class I

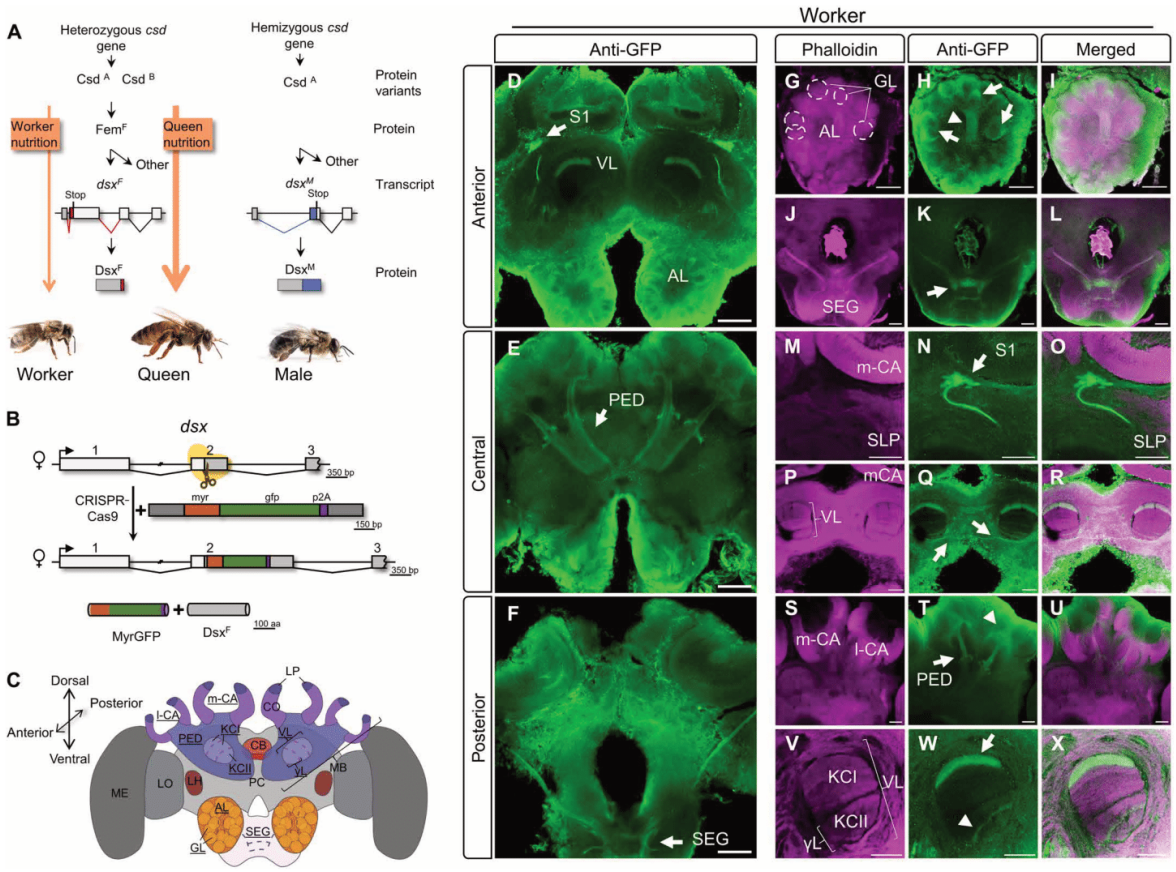

Fig. 1. The *dsx*^{myrGFP} cells in the brains of worker bees. (**A**) Sex-determination and worker nutrition signals together determine worker characteristics. ^{F}/red color, female-specific; ^{M}/ blue, male-specific products. (**B**) Targeted insertion of myrGFP. (**C**) Scheme of anatomy of the worker bee brain. MB, mushroom body. LO, lobula. LH, lateral horn. CA, calyx. PED, peduncle. CO, collar. LP, lip. CB, central body. AL, antennal lobe. VL, vertical lobe. γl, γ-lobe. GL, glomerulus. l-CA, lateral calyx. m-CA, medial calyx. KC I/II, projection areas of class I and II KCs in the VL. SEG, subesophageal ganglion. PC, protocerebrum. ME, medulla. Underlined; structures shown below. (**D** to **F**) Overview of *dsx*^{myrGFP} expression. Anterior to posterior optical sections through the central brain. Anti-GFP staining. S1, dsx-S1 cluster. (D) 30 μm; (E) 69 μm; (F) 33 μm thickness. (**G** to **X**) Double labeling shown for specific brain areas; anti-GFP (green) and neuropil phalloidin staining (magenta). [(G) to (I)] *dsx*^{myrGFP} in OSNs of sensory tract 1 (T1) of antennal nerve (arrowhead) and in olfactory glomeruli (GL) (arrows). Dashed circles; GL. Thickness, 27 μm. [(J) to (L)] Neuronal arborizations labeled in SEG (arrow). Thickness, 33 μm. [(M) to (O)] dsx-S1 soma (arrow) underneath m-CA and l-CA projecting into SLP. Thickness, 18 μm. [(P) to (R)] Commissure (arrows) *dsx*^{myrGFP} labeled underneath VL. Thickness, 42 μm. [(S) to (U)] Labeling of class I KCs and projections into PED (arrow). Arrowhead; cell bodies in l-CA and dendritic arborizations. Thickness, 96 μm. [(V) to (X)] Axonal *dsx*^{myrGFP}-labeled projections of class I (arrow) in the uppermost layer and class II KCs (arrowhead) in gamma lobe of VL. Thickness, 12 μm. Scale bar, 100 μm [(D) to (F)], 50 μm [(G) to (X)].

KCs form a larger bundle of axons with arborizations in the VL of workers relative to queens. There may be other caste dimorphic anatomy between workers and queens that we could not detect at this resolution.

We next examined expressions in other tissues of the worker bees using semiquantitative reverse transcription polymerase chain reaction (RT-PCRs). We found that in adult bees *dsx*^{F} (Fig. 1A and fig. S1) is consistently transcribed in the brain, ganglia, abdomen, legs, gonads, and fat body, while in the pupae, it is found in the brain, ganglia, thorax, gonads, and fat body (fig. S2). These results suggest that the *dsx* gene in the worker bees is female-specifically regulated, stage-specific, and spatially restricted within the brain and between tissues.

Brood rearing–related behaviors dysfunction in *dsx*$^{stop/stop}$ worker bees

A key aspect of eusocial living is the collective rearing of the brood. The worker bees at the nurse stage engage into different behavioral tasks to do so. They inspect single cells on the comb, eventually leading to larval feeding, food take up (which we refer to as food handling), or cell-cleaning behaviors, depending on the content of cells (*14*, *15*, *20*, *21*). The nurse bees also share food with other bees

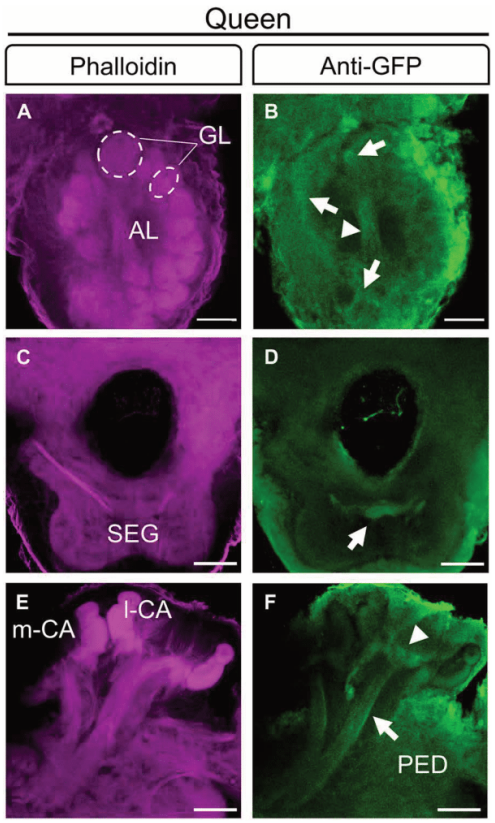

Fig. 2. The *dsx*^{myrGFP} cells in the brains of bee queens. Double labeling with anti-GFP (green) and neuropil staining with phalloidin (magenta). (**A** and **B**) AL: *dsx*^{myrGFP} expression in axonal projections of OSNs in cortical regions of glomeruli (arrows) and sensory tract 1 (T1) of the antennal nerve (arrowhead). Dashed circles in (A) indicate individual olfactory glomeruli labeled with phalloidin. Thickness, 45 µm. (**C** and **D**) Arborizations of *dsx*^{myrGFP}-expressing neurons (arrow) in the SEG. Thickness, 48 µm. (**E** and **F**) Neurites from inner compact class I KCs with arborizations in the basal ring (arrowhead) of the calyx and neurites in the PED of the MB (arrow). Scale bar, 50 µm [(A) and (B)], 100 µm [(C) to (F)]. Thickness, 27 µm.

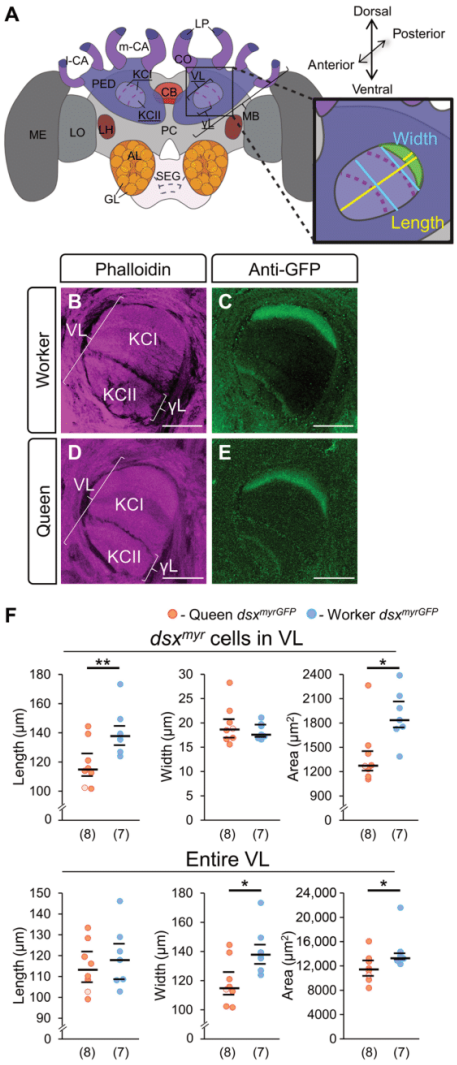

Fig. 3. Comparison of *dsx*^{myrGFP}-positive neurites in the MB vertical lobes of queen and worker bees. (**A**) Scheme of the brain with inset showing details on quantitative analyses of projection areas in the uppermost (basal ring associated) layer of the vertical lobe (VL) in queens and workers. The length, width, and area of *dsx*^{myrGFP}-positive neurites of the KC projections were measured using the position of the largest expansion of the structure as indicated. (**B** to **E**) Examples of *dsx*^{myrGFP} expression in the VL of workers and queens. GFP was detected using anti-GFP (green) and the neuropil labeled with phalloidin (magenta). The single queen with the *dsx*$^{myrGFP/myrGFP}$ genotype is shown as a dot with a lighter color (details see "dsx^{F} is spatially and worker-specifically expressed in the brain"). Thickness, 9 and 24 µm. (**F**) Quantitative comparison of the *dsx*^{myrGFP}-expressing uppermost (basal ring associated) VL layer and of the entire VL in the brain of queens and workers. The equivalent depth of optical sections was determined by characteristic landmarks in the brain. Length *dsx*^{myrGFP}: $P = 0.008$, $z = 2.66$, MWU test. Width *dsx*^{myrGFP}: $P = 0.73$, $z = 0.35$, MWU test. Area *dsx*^{myrGFP}: $P = 0.02$, $z = 2.32$, MWU test. Length of the entire VL: $P = 0.58$, $z = 0.58$, MWU test. Width VL: $P = 0.03$, $z = 2.2$, MWU test. Area of the entire VL: $P = 0.04$, $z = 2.08$, MWU test. Scale bar, 50 µm [(B) to (E)]. *$P < 0.05$; **$P < 0.01$.

(trophallaxis behavior) that is eventually used to feed the larvae. To understand whether the *dsx* gene developmentally specifies aspects of these brood rearing–related behaviors, we generated homozygous *dsx* stop mutants (*dsx*$^{stop/stop}$) in worker bees using the CRISPR-Cas9 method (*19*). We introduced mutations before the essential DNA binding domain, the DM domain, which consists of two intertwined Zinc finger (ZnF) motifs (Fig. 4, A and B, and fig. S1) (*39*). Eggs were injected, and larvae were reared in the laboratory to adults (somatic mutation approach) (*19*). We obtained $n = 67$ (58%) adult *dsx*$^{stop/stop}$ worker bees with no mosaicism, which we characterized by deep sequencing of the amplicons of the target site (tables S2 and S3). The survival of *dsx*$^{stop/stop}$ and the reared control wt worker bees were not different at the adult stage ($P > 0.25$, df = 1, Fisher's exact; table S4), suggesting that the biallelic stop mutations did not induce lethality. We examined the behaviors of *dsx*$^{stop/stop}$ and wt laboratory-reared control worker bees in small colonies on a brood comb in

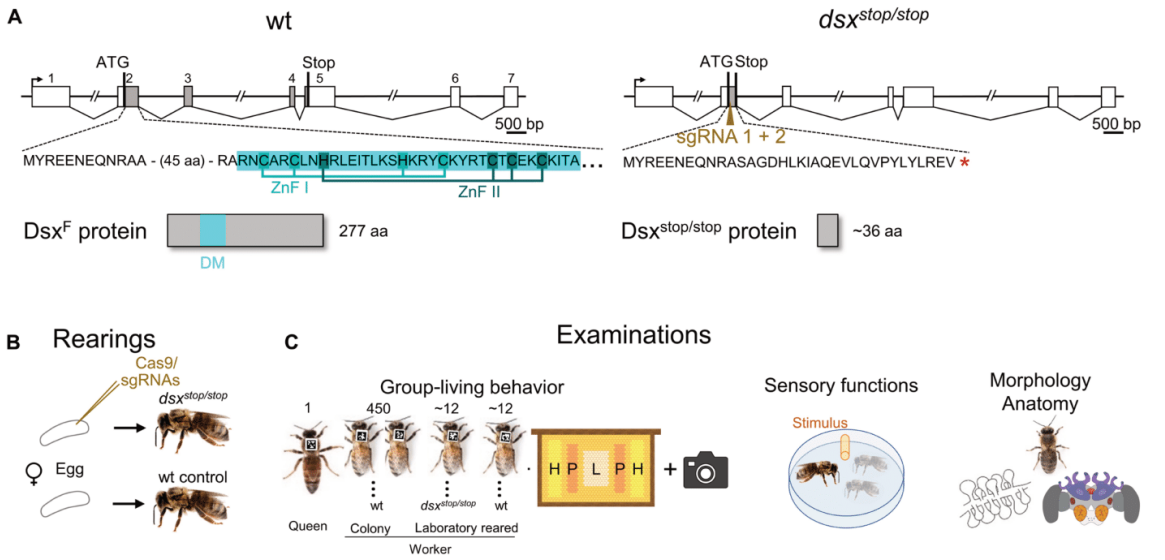

Fig. 4. The generation and examination of *dsx*$^{stop/stop}$ mutant worker bees. (**A**) Scheme of the genomic organization and coding sequence of the *dsx* gene in wt and *dsx*$^{stop/stop}$ bees. Boxes are exons, and interconnected lines indicate female splice variants (male splicing; fig. S1). Gray boxes indicate open reading frame (ORF) with translation starts and stops. The first part of the amino acid (aa) sequence and the resulting protein are presented. ZnF I and II, the two zinc finger motifs of the DM domain (DM, blue box). The brown-colored arrow indicates the target site of the two sgRNAs. (**B**) The generation of the experimental worker bees. Female embryos were reared in the laboratory to the adult stage. Genotyping identified biallelic *dsx*$^{stop/stop}$ mutants with no mosaicism. (**C**) The examinations. Left, experimental bees with 450 wt worker bees and a queen were computer-based tracked on a brood comb using 2D barcodes and a camera ($n = 5$ biological replicates). Brood comb had cells with larvae (L), pollen (P), and sugar solution (H). Middle, testing of sensorimotor functions in petri dish assays. Left, morphological/anatomical analyses of mutant bees, including the HPG and the brain.

which distinct areas with the same number of cells contained either larvae, pollen, or sugar solution, while others were left empty. This comb mimics the condition of a brood comb in a colony but with a standardized amount of work to enable behavioral quantification among biological replicates (fig. S3 and table S5). The *dsx*$^{stop/stop}$ and wt laboratory-reared worker bees were introduced into a group of 450 wt colony-reared worker bees together with a queen (Fig. 4C). When the bees were 7 to 9 days old, each bee in the group was tracked on the brood comb using individualized two-dimensional (2D) bar codes, which were computer- and video-based recorded (Fig. 4C and table S6) (*16*). The larvae provided in the cells on the comb were reared in all five replicate experiments, suggesting that the nurse bees fulfilled this collective task (table S7).

While walking on the comb, worker bees inspect cells by inserting their head into a cell for less than 5 s to detect possible work (*15*). We examined the rates to understand whether the *dsx* gene specifies inspection behaviors. We observed that the rate was markedly reduced by approximately twofold in the *dsx*$^{stop/stop}$ versus wt worker bees for the inspection of food-containing cells and cells that were empty (Fig. 5A, table S8, and movies S6 and S7; Mann-Whitney, $z > 2.02$, $P = 0.04$). This result suggests that the initiation of these cell inspections is impaired. However, the inspection rate for cells containing a larva was not reduced. This lack of an effect cannot be explained by the impairment of larval feeding behavior, which could inflate the inspection rate (a portion of inspection behaviors leads to feeding behavior) because the rate of cell inspection and larval feeding behavior together did not differ between *dsx*$^{stop/stop}$ and wt bees (fig. S4). Collectively, these results suggest that the *dsx* gene is required for the behavior of inspecting empty and food-containing cells.

After inspecting a cell, a worker bee possibly initiates the behavioral tasks depending on the content of the cell. These task behaviors are larval feeding, food handling, or cleaning of cell. Our observations indicate that the rate at which worker bees performed larval feeding was reduced by a twofold median estimate (Fig. 5B, table S9, and movies S8 and S9; Mann-Whitney, $z = 1.9$, $P = 0.05$). Food handling and cell cleaning behavior was not affected. This result suggests that the initiation of larval feeding behavior was specifically impaired. To determine whether the *dsx* gene specifies the sustainment of these behaviors, we examined the duration of the behavioral task. We observed a twofold reduction in the duration of food handling behavior (from an average of 13.5 s to less than 7 s) in *dsx*$^{stop/stop}$ versus wt controls (Fig. 5C and table S10). The other behavioral tasks were not affected, suggesting that the sustainment of food handling behavior was specifically misfunctioned. We did not observe any impairment of the movement patterns during the behaviors (movies S6 to S9), suggesting that the motor programs were unaffected. These results indicate that the *dsx* gene is specifically required to initiate larval feeding and sustain food-handling behavior.

The nurse bees are responsible for consuming and processing pollen to produce easily digestible jelly (*22*). The nurse bees often share this jelly and liquid food from their stomachs with other bees. The transferred food is often used to feed the larvae. Usually, this food sharing requires a

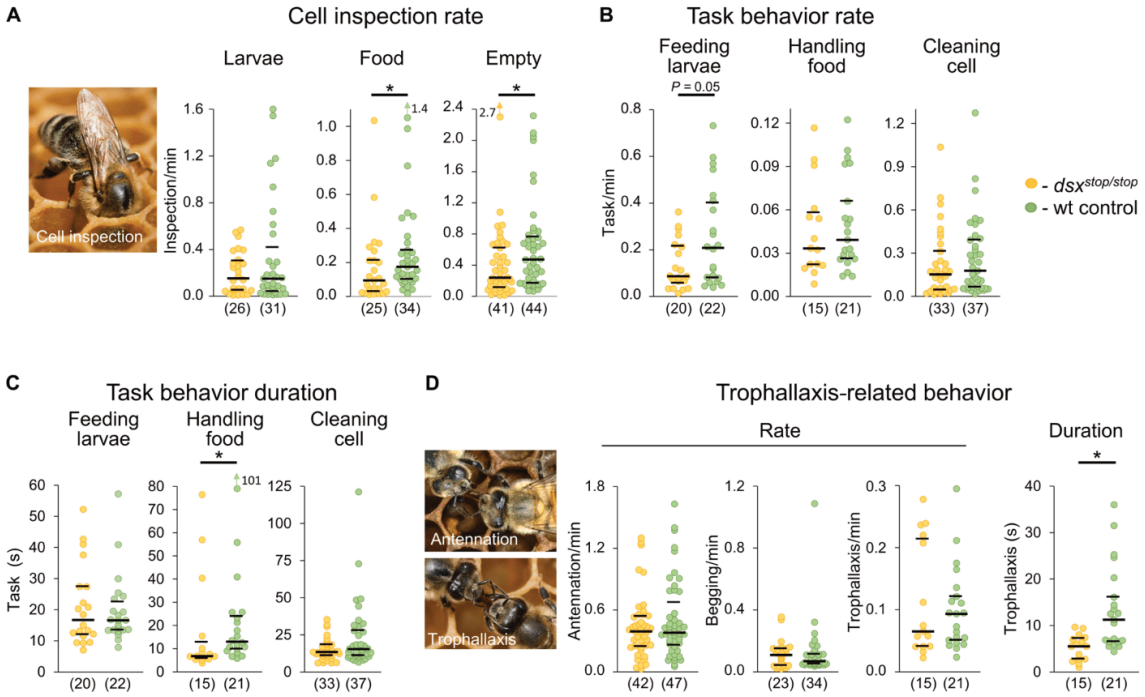

Fig. 5. *dsx* gene activity is required for the initiation and sustainment of brood rearing–related behaviors. (**A** to **D**) Comparison of $dsx^{stop/stop}$ versus wt worker bees. (A) The rate of cell inspections for the different cell types: cells with larvae ($P = 0.67$, $z = 0.83$, MWU test), cells with food ($P = 0.04$, $z = 2.04$, MWU test), or empty cells ($P = 0.04$, $z = 2.03$, MWU test). (B) The rate of feeding the larvae behavior ($P = 0.05$, $z = 1.93$, MWU test), handling of food behavior ($P = 0.53$, $z = 0.65$, MWU test), and cleaning of empty cell behaviors ($P = 0.29$, $z = 1.05$, MWU test). (C) The duration of larval-feeding behaviors ($P = 0.82$, $z = 0.23$ MWU test), handling of food behavior ($P = 0.02$, $z = 2.28$, MWU test), and cleaning of empty cell behavior ($P = 0.21$, $z = 1.25$, MWU test). (D) Rate of antennation ($P = 0.65$, $z = 0.52$, MWU test), begging ($P = 0.71$, $z = 0.67$, MWU test), and trophallaxis behaviors ($P = 0.95$, $z = 0.06$, MWU test). The duration of trophallaxis behavior ($P = 0.001$, $z = 3.23$, MWU test). The median (middle line) and quartiles are presented. *n* values are shown in parentheses. min, minutes; s, seconds. *$P < 0.05$.

sequence of behaviors in which the workers can quit the behavior at decision points or move on in the behavioral sequence. The sequence usually starts with antennation behavior, which is possibly followed by begging behavior and which perhaps results in food-sharing behavior (trophallaxis behavior) (movies S10 to S12) (*22*, *23*). To understand whether the *dsx* gene specifies these behaviors, we examined the rate of begging behaviors and the length of trophallaxis behaviors in the $dsx^{stop/stop}$ versus wt control bees (movies S13 to S18). We observed that the rates of antennation, begging, and trophallaxis behavior did not differ (Fig. 5D and table S11; Mann-Whitney test, $z < 0.06$, $P > 0.7$). However, the duration of the trophallaxis behavior in $dsx^{stop/stop}$ versus wt bees was substantially reduced from average 11 to less than 6 s, suggesting a dysfunction (Fig. 5D and table S12; Mann-Whitney, $z = 3.2$, $P = 0.001$;). The movement patterns during the behaviors showed no abnormalities (movies S13 to S18). These results suggest that the *dsx* gene is also required to sustain trophallaxis behavior.

Movement behavior, maturation, and sensorimotor functions are not affected

To understand whether the impairments in behavior result from general defects, we examined walking behavior, morphology, and stimulus perception. The walking distance (Fig. 6A and table S13; MWU test, $z = 1.24$, $P = 0.22$) and the visiting behaviors of the comb areas did not differ between the $dsx^{stop/stop}$ and wt worker bees (Fig. 6A and table S14; MWU test, $z < 1.37$, $P > 0.17$). This result suggests that cell inspection and task behavior impairments cannot be explain by altered movement and visit area patterns on the comb. In addition, the time the $dsx^{stop/stop}$ bees spent in different areas of the comb was not affected as revealed from wt bee comparison (Fig. 6A and table S15). Possibly, there is a trend of the $dsx^{stop/stop}$ bees spending less time than the wt bees in the area of the food (MWU test, $z = 1.86$, $P = 0.06$), which may reflect the twofold decline of cell inspections in the food area, which will affect the time spent but not the visit rate. External morphological defects cannot explain the behavioral dysfunctions in $dsx^{stop/stop}$ bees. The triangular-shaped head morphology, the sex-specific antennal/abdominal segments, and the overall body morphology of the $dsx^{stop/stop}$ and wt worker bees did not differ upon close and quantitative inspections (Fig. 6B, fig. S5, and table S16; Fisher's exact test, $P = 1$, df = 1; MWU test, $z = 0.16$, $P = 0.87$).

Bees maturate to the nurse bee stage in the colony usually when they are 7 to 12 days old. To determine whether the $dsx^{stop/stop}$ worker bees entered the nurse stage, we examined whether the bees developed HPGs and ascini, which produce secretions for the feeding of the larvae (*48*). All the examined $n = 23$ $dsx^{stop/stop}$ worker bees had developed HPGs similar to those of the wt control

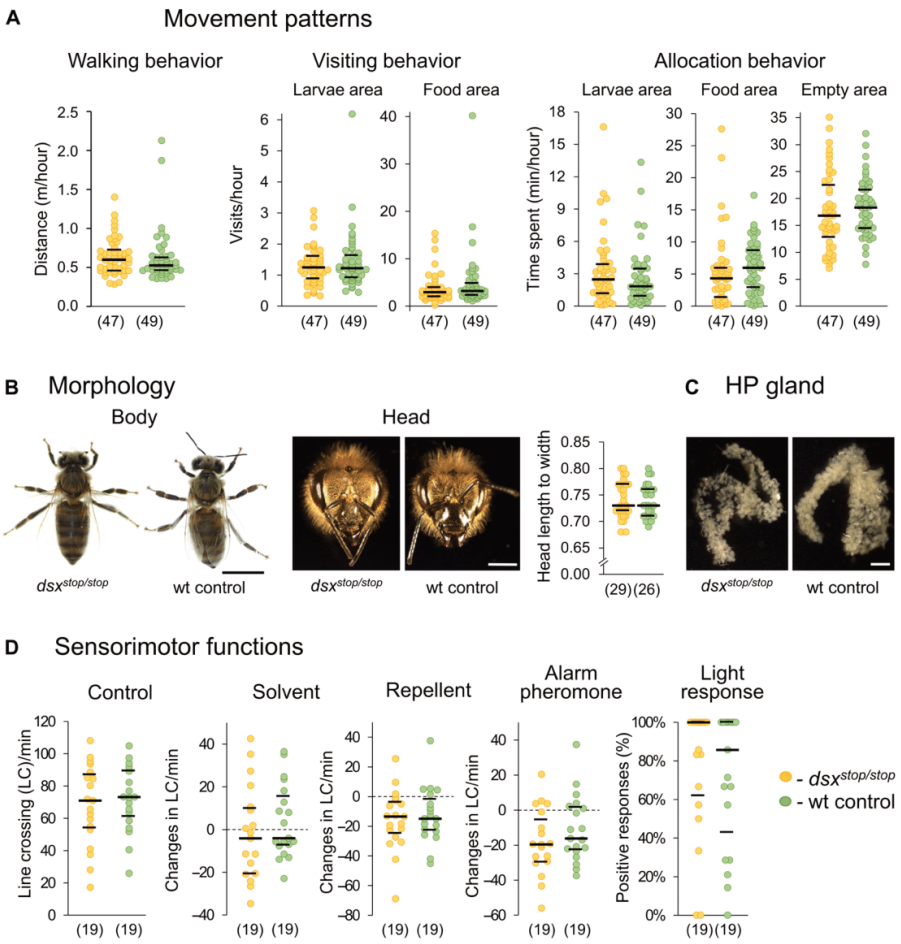

Fig. 6. *dsx* activity is not required for movement behaviors, gross morphology, maturation, or gross sensory functions. (**A**) The walking distance of $dsx^{stop/stop}$ and wt worker bees on the comb ($P = 0.22$, $z = 1.24$, MWU test). The rate of visit behaviors in different comb areas (larvae, $P = 0.85$, $z = 0.19$; food, $P = 0.17$ $z = 1.36$, MWU test). The allocated time to areas (larvae, $P = 0.33$, $z = 0.97$; food, $P = 0.06$, $z = 1.86$; empty, $P = 0.36$, $z = 0.92$, MWU test). (**B**) Example of body (scale bar, 5 mm) and head morphology (scale bar, 1 mm). To the right, head length relative to head width ($P = 0.87$, $z = 0.16$, MWU test). (**C**) Example of the HPGs. (**D**) Sensorimotor function examinations using line crossings (LC). No stimulus (control): $P = 0.64$, $z = 0.48$, MWU test. Changes in LC in response to the solvent isopropanol ($P = 0.3$, $z = 1.05$, MWU), the repellent benzaldehyde ($P = 0.77$, $z = 0.31$, MWU test), and the alarm pheromone IPA ($P = 0.3$, $z = 1.07$, MWU test). The $dsx^{stop/stop}$ mutant and wt worker both responded to benzaldehyde ($P < 0.01$, $z > 2.6$) and IPA ($P < 0.03$, $z > 2.25$, one-sample Wilcoxon signed-rank test). The responses to light pulse ($P = 0.67$, $z = 0.48$, MWU test). The median (middle line) and quartiles are presented. *n* values are shown in parentheses. min, minutes.

worker bees (table S16). The HPGs were composed of acini, densely packed along the collecting ducts (Fig. 6C), suggesting that the $dsx^{stop/stop}$ mutant bees had entered the nurse stage. Furthermore, as previously reported (*19*), we observed gross malformations of the worker-characteristic reproductive organs that differ from queens (fig. S6 and table S16). The gross malformations were observed in 50% of the $dsx^{stop/stop}$ mutants, suggesting that the expressivity of the mutant phenotype varies (fig. S6 and table S16).

To understand whether gross motor and sensory functions were compromised, we examined response to different odors and light (Fig. 6D and fig. S7), we demonstrated that loss of *dsx* function mutation did not affect the response to the repellent benzaldehyde (*49*) (MWU test, $z = 0.31$, $P = 0.77$), the alarm pheromone component, isopentyl acetate (IPA) [(*50*); MWU test, $z = 1.07$, $P = 0.3$], or light (MWU test, $z = 0.48$, $P = 0.67$). As a control, we showed that the $dsx^{stop/stop}$ worker bees behaviorally responded to the stimulus benzaldehyde and alarm pheromone (test against zero, one-sample Wilcoxon signed-rank test, benzaldehyde: $z > 2.6$, $P < 0.01$ and IPA: $z > 2.25$, $P < 0.03$). These quantitative results indicate that gross motor and sensory functions were not affected in $dsx^{stop/stop}$ worker bees. These results suggest that general abilities of stimuli perception and walking behaviors were intact in the $dsx^{stop/stop}$ worker bees. We conclude that beside the development of the usually sterile reproductive organ, the *dsx* gene is specifically required to initiate and sustain specific worker behaviors.

Developmental organization of the brain's central integrative center is impaired

We next asked whether the *dsx* gene is a developmental regulator that specifies the anatomy of the worker's brain. We examined the anatomy of 26 $dsx^{stop/stop}$ worker bee brains. We used f-actin staining with phalloidin to highlight synaptic neuropils and examined stacks of confocal images of worker bee brains. We found that 23% of the 26 mutants had malformations at a gross observation level. This observation is significantly different to the 29 laboratory-reared wt worker bees that never had such malformations ($P < 0.01$, df = 1, Fisher's exact test; table S17 and Fig. 7). We repeatedly observed ($n = 5$) spheroidal neuropil-like structures preferentially located on the inside of the MB calyx cup that is typically occupied by cell bodies of KCs (arrowheads in Fig. 7, B to I) together with general malformations of the MB calyx (Fig. 7, D and E; table S18; and movies S1 and S19 to S22). The example in Fig. 7G shows that these neuropilar abnormalities contain microglomerular structures, which comprise clusters of synaptic neuropils at atypical locations. In $n = 2$ individuals, we observed additional structures of very dense neuropil in the lateral horn area (arrowheads in Fig. 7, J to M; table S18; and movies S1, S19, and S23). These results suggest that the *dsx* gene is required for the developmental organization of neuropils and wirings in the MB area. We examined whether these malformations increase the dysfunction of the behaviors. However, the behaviors of these MB malformed workers were in the range of the other mutant workers (fig. S8). Because the expressivity of *dsx* mutant phenotype varies greatly from strong to mild (table S16) (*19*, *51*), we next examined the volume of the calyces (CA) in the other $dsx^{stop/stop}$ worker bee with no gross MB malformations to find more subtle changes. We found a tendency that the volume of the CAs were slightly smaller relative to wt (Fig. 7N, MWU test, medial CA: $z = 1.28$, $P = 0.2$; lateral CA: $z = 1.25$, $P = 0.21$). There may be other subtle changes that we cannot detect at this level of analysis, which is limited by a lack of resolution at the cellular level.

The brood rearing–related behaviors on the comb are primarily controlled by the sensation of chemical cues from the local environment. Chemosensory receptors are used to perceive molecules from the environment. They are expressed in the OSNs of antennal sensilla, for which we found *dsx* expression in the sensory axons within the AL (Fig. 1, G to I). To understand whether the transcription factor gene *dsx* specifies mechanisms of olfactory and/or gustatory reception, we examined the expression of odorant receptor (OR), odorant-binding protein (OBP), gustatory receptor (GR), and chemosensory protein (CSP) genes in the antennae of mutant bees. We observed that none of the OR, OBP, GR, CSP genes or other genes ($n = 9361$) were differentially transcribed between $dsx^{stop/stop}$ and wt worker bees, as revealed by RNA sequencing (RNA-seq) analysis (fig. S9). These results suggest that *dsx* does not control the transcription of chemosensory receptor–encoding genes. We conclude that the *dsx* gene is not used to specify worker behaviors at the level of the chemosensory receptor genes.

DISCUSSION

The *dsx* gene acts as a developmental regulator to specify worker behaviors

The behavioral patterns that build the basis of eusociality are usually neither taught by adult individuals nor are they learned by the young. They are inherited—programed into the development of the individual by genes that elicit specific behavioral patterns in the adults. Because eusocial behavior is inherited, there must be genes whose products govern molecular and cellular mechanisms as well as neuronal circuitry properties that innately program behavior of individuals in such a way that coordinated behavior of a colony acting as a single organism emerges. However, it was unknown whether there are dedicated developmental programing that specifies the worker-specific innate behaviors into adult bees. By introducing biallelic frameshift mutations into worker bees leading to translation stops, we showed that the activity of the *dsx* gene is necessary for inspection, brood rearing, food handling, and food exchange behaviors. These activities and task behaviors are not displayed by the queen caste and the males. They contribute to the collective brood rearing at the group level. Whereas these behaviors were specifically impaired, the general sensorimotor functions of the worker bees were unaffected, suggesting that the *dsx* gene dedicatedly programs the identity of the worker-specific behaviors into the worker caste. Previous work identified molecular determinants that act during the shift from inside to outside the nest behaviors. For example, juvenile hormone and vitellogenin play crucial roles in the shift in this age-dependent polyethism (*24*–*27*). Other data focused on behavioral states and the transcriptome patterns in the brain (*6*, *27*, *52*). For example, the authors found massive transcriptionally differences of 5839 genes between in-hive and foraging bees (*27*). Other types of data rely on the use of genetic variation to study social behaviors (*30*, *53*) and identified by quantitative trait mapping an inherited basis and genomic loci harboring multiple genes. However, in no case that there has a developmental gene been identified that specifies the identity of these worker-specific behaviors into the worker caste. The rate and duration of task and task-related behaviors specified contribute to collective brood-rearing work, which defines a eusocial society. Hence, our result suggest that we have characterized a key developmental regulator for the programing of social worker behaviors.

We further showed that the *dsx* gene's activity (*19*) acts as a developmental regulator in the worker bee's brain. Dsx^F thereby controls the proper developmental organization of the MB, with *dsx* mutants showing extra neuropil structures and malformations of the MB calyx neuropil in a proportion of mutants, suggesting that the *dsx* gene is involved in the developmental guidance of neurons in the MB calyx. Because other *dsx* mutant phenotypes show varying expressivity (table S16) (*19*, *51*), more subtle effects might be present in the MB, but detecting those may require higher resolution at the cellular level.

The sex-specific activities of the *dsx* orthologs are in invertebrates and vertebrates integral for sexual differentiation (*19*, *39*, *40*). There is also evidence in other insect species that the *dsx* gene shows plasticity in its regulation of traits depending on internal nutritional states (e.g., horn and mandible formation in the dung and stag beetles, respectively) (*54*, *55*). However, in honeybees, *dsx* is part of the caste determination. Dsx^F together with the nutrition signal regulate the caste-specific development of the worker and possibly the queen ovary (*19*). Hence, a key question is how the *dsx*-dependent identity of the worker-specific behaviors is possibly realized in the nervous system. Here, we demonstrate that Dsx^F-expressing neurons form anatomic differences in workers versus queens. The dsx^+ basal ring–specific class I KCs in the MBs are caste dimorphic. This result suggests that worker bees have larger neuronal projections in the VL with more connecting dsx^+ KC class I neurons than queens. Hence, our results demonstrate that *dsx* is a developmental regulator of the

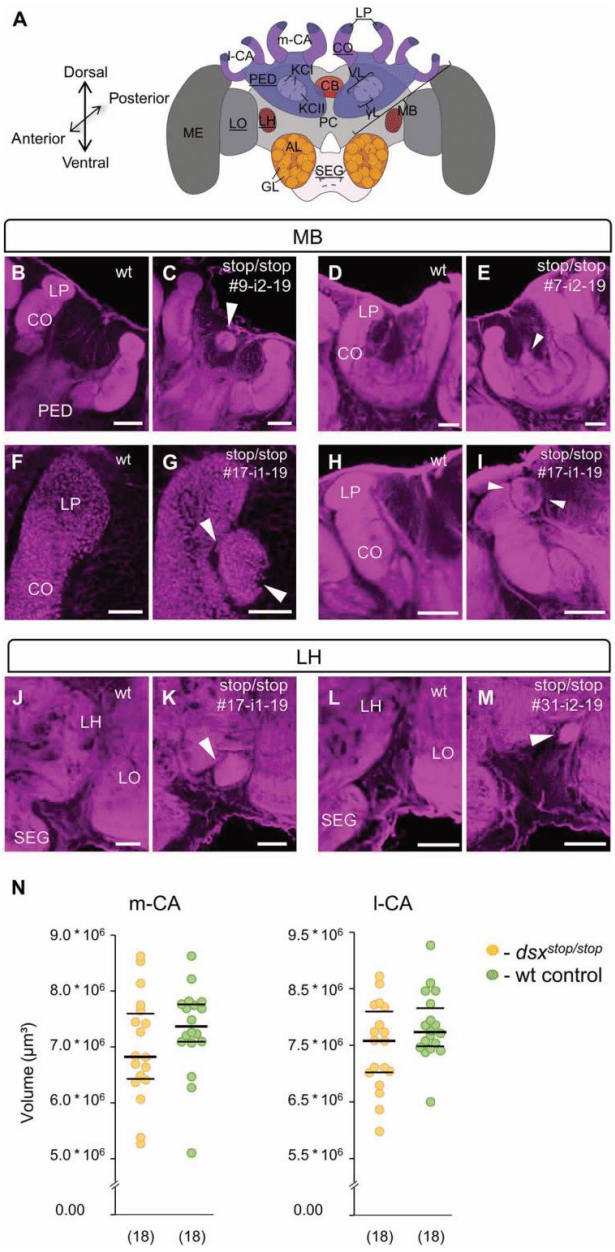

Fig. 7. The *dsx* gene is required for proper differentiation of the MB and LH area. (**A**) Scheme of the anatomy of the worker bee brain. Underlined labels highlight the structures shown below. (**B** to **I**) Single optical sections of wt and different $dsx^{stop/stop}$ worker bees in the MB area. The brain tissue was stained using phalloidin. Abbreviations in labels are indicated in (A). Arrowheads mark additional structures or malformations. Further explanantions in the text. (**J** to **M**) Single optical sections of wt and two $dsx^{stop/stop}$ worker bee brains in the LH area. $dsx^{stop/stop}$ worker bees were independently mutated. The brain tissue was stained using phalloidin. Abbreviations in labels are indicated in (A). Arrowheads mark additional neuropil structures adjacent to the LH. Scale bars, 50 µm. (**N**) Volume measures of the CAs. MWU test, medial CA, $z = 1.28$, $P = 0.2$; lateral CA, $z = 1.25$, $P = 0.21$.

MB and is involved in establishing worker-specific anatomic identity in class I KCs of eusocial honeybees.

Selection and duration of behaviors are developmentally scalable features

Organisms living socially have a rich behavioral repertoire that contributes to different tasks in and outside the colony. Their control is mediated by stimuli that are precepted from the environment and social partners. Hence, an important question in behavioral biology and genetics is about which aspects of these behavioral capacities are genetically specified that establish the features for social living. Here, we show that specifically the rate and duration aspects of the behaviors, but not the movement patterns, are innately programed. The mutants approximately show a 50% reduction in the rate and duration of task and task-related behaviors. Because the collective tasks require many bees and repeated behavioral performances of a worker (for example, 52 cell inspections and 13 larval feedings are performed on average by a single worker bee per hour in our experimental setting), a 50% reduction in each worker is a substantial effect on the collective outcome and social organization. This further supports the notion that the *dsx* gene is a key developmental gene for the programming of group-supporting behaviors in the worker caste. Specifically, we showed that *dsx* specifies the rate a worker bee inspects a cell and finds work during the bees' walks on the comb (*15*, *56*). We also found evidence that *dsx* specifies the rate a worker bee feeds larvae with a mixture of protein-enriched pharyngeal gland secrets, pollen, and nectar (*21*, *22*), a behavioral capacity representing a hallmark of eusocial organization (*1*, *2*). All these aspects were specified for specific cues the worker bee encountered in the larval, food, or empty cells, suggesting that behavioral specifications are context specific.

Our results also suggest that the duration of food-related behaviors is specified, suggesting another level of innate control. We showed that the *dsx* gene specifies the duration of the food-handling behaviors in the cells (*15*) and the food exchange behaviors among colony members (*22*, *23*). How is the duration of the behavioral performances possibly specified? We suggest that sensory feedback control for the behaviors that are developmentally specified must exist. The perception of cues during the behaviors will control whether the behavior will be further performed, making the performances less rigid and adjustable to local demand (*57*).

Most of the work in the colony cannot be performed by a single bee alone. The workload in the colony requires small and repeated contributions from many bees that collectively fulfill the work. This raises the crucial question how a developmental program can specify behavioral capacities in individual bees in such a way that behaviors in a colony acting as a functional unit emerges. Previous work showed that single bees differ in their experience and genotype make up through genetic variation that determines which bee engage in a behavioral task for a given stimulus [response threshold (RT) model]. A bee below this threshold will behavioral respond to the task stimulus, while a bee above the threshold will not respond. This variation in RTs regulates the fraction of bees in the group that perform this task (*28*, *29*, *53*). Our results now showed that *dsx* developmentally specifies not all but the rate and duration of behavioral performances at some scale for a given stimulus and standard conditions used. Hence, the rate and duration aspects of task behaviors are developmentally programed at some scale. This establishes a programable mechanism for social living because the innate rate and/or duration of performing behavior quantitatively determines the strength of the social relationship the bees have with other bees and the work they share together. For example, the innate rate of brood feeding will specify how often a bee will care for the brood, while the innate duration of the trophallaxis will specify how long the food is transferred between social partners. Even small differences in this scalability will have profound consequences at the collective level because the repeated task engagements and the many worker bees involved amplify the effect. For example, even small changes of the innate scale of longer trophallaxis behaviors (in our experiment, six trophallaxis behaviors were performed per worker per hour) will alter the amount of liquid food circulating in the colony. How is the scalability of the behavioral performance possibly specified? We propose that the *dsx* gene may specify the intensity of neuron activation, the type of inhibitory and stimulatory regulation in a neuronal network, and/or the number of activated neurons (*58*) for stimulus processing leading to motor program control. Hence, our results show that selection and duration of the behaviors are innate, context dependent, and scaled by a dedicated developmental program uncovering mechanisms of innate specifications of eusocial living behaviors.

***dsx* gene operates in brain areas that integrate and evaluate sensory information**

How the behavioral control in eusocial societies is possibly represented at the level of neural circuitry is still rudimentary. Our reporter gene studies now showed that Dsx proteins operate in spatially highly restricted areas of the worker bee brain and at different sensory information processing and evaluation levels. This expression includes OSNs that project from the antennae to glomeruli in the AL and express OR proteins (*45*, *59*, *60*). The transcription factor gene, *dsx,* does not regulate the expression of the chemosensory receptor proteins, which excludes peripheral chemosensory mechanisms as a possible source for the specification of worker behaviors. However, *dsx* may still affect olfactory processing at the level of AL glomeruli. *dsx* may act on selected neurons in the SEG and influence information processing from other sensory systems on the mouthparts, i.e., gustatory and mechanosensory input (*45*, *59*). Most prominently, *dsx* operates developmentally in selected groups of KCs, the projections of which show caste-dimorphic differences in the VL. Most are class I (spiny) KCs of the inner compact type associated with the MB calyx basal ring. The basal ring receives multisensory input from both olfactory and visual modalities, and KCs in the basal ring may even integrate both modalities that are mapped in close vicinity. These findings suggest a potential role of *dsx* in specifying the neural circuits underlying multisensory information processing affecting behavioral decisions (*43*, *44*, *57*). A minor population of class II (clawed) KCs also expressed *dsx*. A notable feature of class II KCs is that their claw-like dendritic arborizations span over larger regions of all regions of the MB calyx, the olfactory (lip), visual (collar), and basal ring, indicating that these KCs also integrate different sensory modalities. Thus, *dsx*'s role in specifying innate behaviors may be associated with dsx^{+} neurons involved in processing of multisensory information. One possible mechanism of specification is the presence of a larger population of activated class I KCs in the worker bee (as the caste dimorphism suggests) that can activate a higher number of MB output neurons to activate a motor program. The *dsx* expression in the fat body may also indirectly affect the function of the brain and

behavioral control, as shown for the fruitless protein that is secreted from the male fat body and influences the CNS and the courtship song in *D. melanogaster* (*61*).

There is a long-standing debate about whether organisms living socially require higher cognitive and/or larger neuronal processing abilities than those living solitarily (*11*–*13*). The *dsx's* role in specifying behaviors in socially living worker bees and solitarily living *D. melanogaster* establishes a rare opportunity to evaluate this hypothesis by comparing the gene's function and expression in the brain of the two species. As in the honeybee, the *dsx* transcripts undergo in *D. melanogaster* sex-specific alternative splicing to encode either a male- or female-specific isoform (*62*). In *D. melanogaster*, *dsx* expression is highly regulated in both male and female flies, as shown by its temporally and spatially restricted expression patterns through development, with only a select group of neurons expressing *dsx* (*9*, *63*, *64*). A detailed analysis of *dsx* expression in both the male and female CNS found that *dsx*-expressing neuron clusters are sexually dimorphic in cell number and connectivity—None of the dsx^+ neuron clusters are sexually monomorphic (*64*). Many of these *dsx* higher-order neurons in the brain act as key sex-specific processing nodes of sensory information that are essential for the execution of sexual behaviors [reviewed in (*65*)]. However, unlike in *D. melanogaster* [and *Bombyx mori* (*66*)], *dsx* in honeybees also operates in KCs, suggesting a not yet described function and expansion to the MBs. This high-order center integrates sensory information and evaluates them for decision-making by comparing incoming sensory input with stored information that has been acquired via sensory integration, learning, and memory formation (*43*, *44*, *57*, *67*). Besides MB's historically defined role in learning, innate behavioral decisions and olfactory learning were recently shown in *D. melanogaster* to share circuitry of the MB (*68*, *69*). This suggests the potential that the prominent dsx^+ cells of the MB are possibly involved in the innate decision process that were impaired in the *dsx* mutants. The robust behavioral effects together with a selected population of labeled neurons now offer an opportunity to examine the representation and mechanisms of the *dsx*-dependent behavioral controls at the level of neural circuitry.

The *dsx's* role in specifying worker behaviors were co-opted from sexual behavior

It is largely unknown how innate behaviors required for social and eusocial organizations genetically evolve through developmental programs. Previous data informed us about the molecular rate, genome evolution, gene family expansions, signatures of selection, and changes in gene regulatory network at the genome scale (*70*–*72*) that are associated with the origin of sociality or eusociality.

In the solitary living species, the fruit fly *D. melanogaster* and the wasp *Nasonia vitripennis* (a hymenopteran sister group of the honeybee), the *dsx* gene is involved in specifying sexual behaviors (*9*, *41*, *63*, *73*). Parsimony evolutionary inference from the two species and the honeybee suggests that the *dsx* gene evolutionarily gained a new role in the honeybee lineage to specify social living behaviors in the worker bees. This new function has been evolutionary co-opted from its ancestral function, the specification of sexual behaviors. We conclude that co-opting a gene from its sexual to a social living–specifying function is an evolutionary path and mechanism involved in the origin of eusociality, which links with the gain of developmental control in the high-order center, the MB.

MATERIALS AND METHODS

Honeybee handling procedures

The honeybees were collected from *Apis mellifera carnica* colonies (western honeybee) at the bee yard of the Heinrich-Heine University, Düsseldorf, Germany. Female eggs were collected from naturally mated queens, which were maintained in small nuc colonies with five combs (Holtermann, Germany). To collect female eggs, the queens were caged in Jenter egg-collecting cages (Jenter queen rearing kit, Karl Jenter GmbH, Frickenhausen, Germany). For the tracking, we collected newly eclosed bees (0 to 24 hours old) from a brood comb that was maintained in an incubator at 34°C. The laboratory rearing of worker bees was done as previously reported (*19*). We grafted the newly hatched larvae into plastic cups (#4963, Heinrich Holtermann KG, Brockel, Germany) with worker diet # 7 (53% royal jelly, 4% glucose, 8% fructose, 1% yeast extract, and 34% autoclaved water) (*74*). To obtain bees with fully developed worker characteristics, we experimentally determined the amount of food provided, which was 170 µl per larvae. The larvae were kept at 34°C and 90% relative humidity. The latter was generated using saturated solution of K_2SO_4 (*75*). Before defecation, the larvae were transferred onto Kimwipe papers (Delicate Task Wipers, #066664, Kimberly Clark) and were kept in petri dishes for 2 days at 70% relative humidity, which we generated using saturated $NaCl_2$ solutions (*75*). After defecation, the prepupae were separated into plates with 24 wells (#92424, Peter Oehmen GmBH, Essen, Germany) in which filter papers were placed (15 mm, grade 413; VWR, International GmbH, Darmstadt, Germany). Once they started walking, they were marked and maintained in small cages together with eclosed wt workers coming from colonies (#20104; Imkereifachhandel Jasniak, Trossin, Germany) in which water and sugar paste supplemented with pollen (#7032; Heinrich Holtermann KG, Brockel, Germany) was provided. To obtain myrGFP-mutated worker or queen bees, we reared the queens as described (*17*, *76*). To obtain myrGFP worker bees, 12- to 19-day-old myrGFP queens were inseminated with wt drones using standard insemination techniques. The queens were treated with CO_2 1 day before insemination. Inseminated queens were maintained in small nucs ("Kieler Begattungskasten," Holtermann, Germany) with wt worker bees. The nucs were kept in a containment so that mutated animals were not able to escape into nature. To obtain newly eclosed $dsx^{myrGFP/+}$ worker bees, combs with capped cells were maintained in an incubator at 34°C.

Genetic manipulation procedures

Eggs were collected every 1.5 hours using the Jenter egg collecting system (*17*). To induce stop codons, single-guide RNA 1 (sgRNA1) and sgRNA2 (table S1) were injected together with Cas9 protein (New England Biolabs, Ipswich, MA), which target base pair (bp) position 31 (sgRNA1) and 201 (sgRNA2) downstream of the start codon (fig. S10). Four hundred picoliters of the sgRNA/Cas9 mix was injected per egg (*19*) using Cas9 protein (375 ng/µl) and sgRNA1 and sgRNA2 at equal molar ratio. Needles were custom made (Hilgenberg, Malsfeld, Germany) as described in (*17*). The sgRNAs induced deletions of approximately 170 bp or mediated indels (sgRNA2), which both frequently produced frameshifts of the open reading frame and this before the essential DM domain of the *dsx* gene (*19*). Bioinformatic predictions suggest no off targets for these sgRNAs (*19*). Furthermore, the $dsx^{stop/stop}$ mutation were independently induced in each worker bee (thus representing independently mutated bees) making an off-target effect, which may also

be induced at some rate, as an explanation for the phenotype effect unlikely. To introduce myrGFP coding sequence, sgRNA1 together with DNA donor fragment were injected (table S18) following previously described procedures (*18*). The injected volume was as above, and concentrations were as follows: sgRNA1 (46.25 ng/μl), Cas9-protein (500 ng/μl), and 50 ng/μl of the myrGFP donor DNA. The donor DNA contained the *N*-myristoylation (myr) sequence, the GFP coding sequence, and the endopeptidase P2A coding sequence (table S1). Two nucleotides were introduced before the myr sequence to maintain the open reading frame. Gly-Ser-Gly coding linker was added between the myr/GFP and GFP/P2A sequences. The coding sequences were optimized for the codons used in the honey bee. Homologous (approximately 250 bp of the nucleotide sequences) were added to left and right to get target-specific insertion at the start codon of the *dsx* gene in exon 2 [National Center for Biotechnology Information (NCBI); gene ID: 725126; reference sequence: NC_037642; annotation: Amel_HAv3.1] (fig. S10 and table S1). This sequence was synthesized, cloned, and sequenced (standard gene, Eurofins, Ebersberg, Germany). The sequence was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Braunschweig, Germany) to generate the donor DNA for injection.

DNA, RNA, cDNA, and PCR procedures

DNA was extracted using the innuPrep Mini Kit (Analytic Jena, Jena, Germany). RNA was isolated using TRIzol reagent as described (*42*). First-strand cDNA synthesis was performed using Oligo(dT)$_{18}$ primer and the RevertAid reverse transcriptase kit following the instructions of the supplier (Thermo Fisher Scientific, Waltham, USA). Semiquantitative PCR amplifications were run under nonsaturating conditions and in technical triplicates for each bee sample using the housekeeping gene *elongation factor 1-alpha* (*Ef1*α; 5′-GATATCGCCCTGTGGAAGTTC-3′, 5′-GTAACATTCGCTCCAGCAGC -3′) as reference for quantitatively adjusting expression levels across different samples (*36*). Amplicons were resolved using standard agarose gel electrophoresis (*34*).

Genotyping and sequencing and procedures

For genotyping, we used PCR standard procedures using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA). Bees with a *dsx*$^{stop/stop}$ genotype were identified in a two-step process. Frame shift mutation was preselected by length polymorphism using hexachlorofluorescein-labeled amplicons. The amplicons were run on the ABI 3130XL Genetic Analyzer, and length differences were identified using Peak Scanner software (Applied Biosystems). Length (base pair) analysis was performed using Peak Scanner software (Thermo Fisher Scientific). Bees with a possible frame shift were deep sequenced. At least 50,000 reads per amplicon and individual were generated using Illumina MiSeq machine (Illumina, San Diego, USA). The sequencing data were analyzed using the web-based galaxy platform (https://usegalaxy.org/) to characterize frame shift mutations and mosaicism. Unrelated sequences, which made up to 6% of the sequences, were removed. The queens carrying myrGFP coding sequence were in the first step identified by genotyping using DNA extracts. PCR_1 amplified the sequence of the upstream insertion site (5′-GATTCGTAATAATTCCTGTGC-3′, 5′-CTGCGATGCCAGAAGGATATGTG-3′; Eurofins, Ebersberg, Germany). PCR_2 amplified the sequence of the downstream insertion site (5′-CTGCGATGCCAGAAGGATATGTG-3′; 5′-GTCAAAGTAAGAGTAGCGGAAG-3′). PCR_3 amplified the wt sequence (5′-GATTCGTAATAATTCCTGTGC-3′; 5′-GTCAAAGTAAGAGTAGCGGAAG-3′). The sequences of the targeted insertion sites were deep sequenced using Illumina MiSeq machine following the procedure described above. To perform RNA-seq of the antennas, we pooled RNA extracts from five bees for each of three biological replicates and genotype. Library preparation was performed using 500 ng of RNA and the "VAHTS Universal RNA-seq Library Prep Kit for Illumina V6 with mRNA capture module version 7.0" (Vazyme Biotech co.). Bead-purified libraries were normalized and finally sequenced on the HiSeq 3000/4000 system (Illumina Inc.) with a read setup of SR 1 × 150 bp. The bcl2fastq2 conversion software (v2.20.0.422) was used to convert the bcl files to fastq files as well as for the adapter trimming and the demultiplexing. Approximately 4 to 12 million single-end reads with a length of 150 bp were mapped to the *A. mellifera* transcriptome (NCBI Assembly Amel_HAv3.1) using the kallisto software tool (https://pachterlab.github.io/kallisto/about.html). Estimated read counts were normalized using the transcripts-per-kilobase-million method. Differences in gene expression were calculated using DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html). Genes were differentially expressed (differentially expressed genes, DEGs) if adjusted *P* values for multiple testing (P_{adj}) were < 0.05, and $\log_2$ fold change was greater than 1.5.

Behavioral examination procedures

To start the group experiments, 1-day-old laboratory-reared mutated and wt worker bees together with 1-day-old wt worker bees reared in the hive were assembled together with a queen into a group of approximately 500 bees (table S19). All bees were tagged using unique 2D barcodes that enable single bee computer-based tracking with the Bee Behavioral Annotation System [BBAS; (*16*)]. The bee group was maintained under dark condition and room temperature on a comb, which provided honey and pollen ad libitum. At day 6, the group of bees was transferred to a brood comb with food, which mimics the condition of the nurse bees on a brood comb in a colony. We standardized these brood combs in the biological replicates. To generate a standard brood comb, we filled cells with pollen or sugar solution at specific locations of the comb (fig. S3 and table S5). For the two pollen areas, we distributed 30 g of pollen among cells ("Echter Deutscher Spezial Blütenpollen," Werner-Seip-Biozentrum GmbH & Co. KG, Butzbach, Germany). The pollen in each cell was covered with 25 μl of sugar solution (70% w/v saccharose solution; "Ambrosia Futtersirup," Nordzucker AG, Braunschweig, Germany). The two honey areas consisted of 550 cells in which 200 μl of the sugar solution per cell was added. The brood area consisted of a piece of comb harboring 151 larvae of the third to fourth instar stage, which was located in the center of the comb (fig. S3 and table S5). Bees on the standardized comb were kept in an incubator overnight at 34°C. When worker bees were 7 to 9 days old, computer-based tracking of the bees was performed in the dark at room temperature for 48 hours (*16*).

For each bee, the BBAS generated information about the position on the comb in the X/Y/Z orientation four times per second together with a video. Average detection rate of the bees was 0.80 (table S6). If the detection of a bee was less than 10% in an hour, this hour was excluded. If the number of excluding hours exceeds 11 hours in a day, the bee was excluded for that day. We obtained $n = 47$ *dsx*$^{stop/stop}$ and $n = 49$ wt reared worker bees in five group replicates with bees $n \geq 5$ in each of the replicate (table S19). All the behavioral examinations of these worker bees were done randomized and blind,

as the observer or the experimenter had no knowledge about the genotype of the bee under study. We computed the time the bees' spent (min/hour) in the areas containing either food (pollen + honey) or larvae or areas that were empty. We computed the number of bees' visits (visits/hour) in the area containing food or larvae. We computed walking distance (m/h). This information of the moving behavior was extracted from the tracking data of the first 24 hours using C^{++} and Java scripts. The bees' trajectories were thereby not continuous. If only a single frame was missing in the trajectory, the gap was linearly interpolated. The single bee trajectories were kept separate. Sequences in the tracking data that possibly represent cell inspection, brood feeding, cleaning empty cells, food handling, antennation, begging, or trophallaxis behavior, we initially identified using machine-based trained "encounter" (*16*) and "head in cell" identifier; the latter we newly trained. Identifiers were implemented in the JAABA program (*16*, *77*). Cross-validation estimates from 10 cross-validation rounds (*16*) for our trained "head in cell" identifier showed that 91.8% of the frames were true positive, 87.3% true negative, 8.2% false positive, and 12.7% false negative. Subsequently, the bee behaviors were manually reanalyzed using the video records. We used VirtualDub software (VirtualDub-1.9.11, https://virtualdub.org/) and software addition (*78*) to label a single bee and this randomized and blinded in respect to the respective genotype, mutant versus wt laboratory-reared worker bees. We classified the encounter behavior by video observations from 60 min of tracking data into antennation, begging, or trophallaxis behavior (table S19). We classified the head into cell behavior from 150 min of tracking data (table S19) into cell inspection behavior if the time period was shorter than <5 s (*15*). For the head into cell behaviors with time periods larger or equal 5 s, we further classified the behavior into cleaning, food handling, and larval-feeding task behaviors on the basis of the cells the bees entered (*15*), which either contained food, larvae, or were empty.

Petri dish behavioral examination procedures

Polystyrene petri dishes (14-cm diameter; VWR, International GmbH, Darmstadt, Germany) were used as arenas for sensorimotor behavioral examinations. We introduced eight ventilation holes in the side walls and three of these in the lid with a diameter size of 5 mm diameter. The arenas were placed on a paper with black lines forming grids of 1.5 cm^2 (fig. S7A) (*79*). Each of the 10- to 13-days-old $dsx^{stop/stop}$ or wt laboratory-reared worker bees was tested in the arena together with two other wt worker bees from the tracking experiment. The experiment was run under red light condition under a laboratory hood. The worker bees were placed in the middle of the arena and were left 10 to 12 min before the assay started. Sensorimotor functions were tested using 100% isopropanol (our solvent control), the alarm pheromone component IPA (≥99%, water free, Sigma-Aldrich, Taufkirchen, Deutschland), or the repellent benzaldehyde (≥99%, Sigma-Aldrich, Taufkirchen Deutschland). The sequence of testing was as follows. A small strip of filter paper (75-mm diameter, grade 413; VWR, International GmbH, Darmstadt, Germany) was introduced for 1 min. Either 0.5 μl of isopropanol, IPA, or benzaldehyde was applied, which we led evaporate for 1 min before we introduced the filter paper for 1 min into the arena. We counted line crossing if the entire bee crossed a line of the grid. However, if a bee immediately turned around after crossing the line, we counted this event as a single crossing (*79*). Positive phototaxis was examined in the petri dish by counting whether or not the bee walked toward the light beam (*80*). Four LED light sources (220 lumen, 2700 K) were distributed around the arena (fig. S7B). Light pulse was given for 10 s using the light source with the largest distance toward the bee examined in the arena and then turned off for 10 s. This procedure was repeated six times. Arenas and the paper with the grid were replaced by a new one once another bee was examined. Video recordings of the behaviors were analyzed (60 fps, full HD, 44100 Hz; Casio Exilim Pro EX-F1) using VSDC Free Video Editor (Multilab LLC, https://videosoftdev.com/). The samples were randomized. The observer had no knowledge about the genotype of the bee.

Morphological and anatomical examination procedures

For the morphological and anatomical examinations, the bees were anesthetized on ice. Body, appendages, and heads were examined under the stereo microscope. Head morphology was quantified by measuring head length and head width as marked in fig. S5. Pictures were taken using a binocular (S8 APO, Leica) with a camera (UI-1240LE-C-HQ) and the software uEye Cockpit (IDS). To dissect the brain, the head was fixed on wax plate and covered with ice-cold honeybee saline buffer [130 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 15 mM Hepes, 25 mM glucose, and 150 mM sucrose (pH 7.2)] or with phosphate-buffered saline [(PBS) 145.3 mM NaCl, 8.4 mM $Na_2HPO_4 \times 2H_2O$, and 1.5 mM $NaH_2PO_4 \times H_2O$,(pH 7.4]. The head capsule was opened between the eyes to remove the brain. The brain was directly fixed in ice-cold 4% formaldehyde (v/v) in PBS (pH 7.4) at 4°C for a minimum of 24 hours. The brains were washed three times for 10 min in PBS, 10 min in PBS with Triton X-100 (2% PBS-T), and twice for 10 min in 0.2% PBS-T, followed by 1 to 2 hours of incubation in 0.2% PBS-T with 2% NGS (normal goat serum, Invitrogen, USA), which was done at room temperature on a shaker.

To obtain phalloidin staining (labeling f-actin) for anatomical examinations, fixed brains were incubated in 0.5% PBS-T with 5% NGS and 0.2 U of Alexa Flour 568 phalloidin (Molecular Probes, A-12380, Eugene, USA) for 2 days at 4°C. The brains were washed four times for 5 min in PBS and were dehydrated using an isopropanol series with PBS buffer (10, 30, 50, 70, and 90% and two times in 100% isopropanol) for 5 min on a shaker. The brains were cleared in methylsalycylate (MS; Sigma-Aldrich, Taufkirchen, Germany) and mounted in fresh MS solution. Malformations were reported when repeatedly observed in the same brain area.

To detect the GFP-labeled cells, we incubated the fixed brains in 0.2% PBS-T with 2% NGS and chicken anti-GFP (1:1000; Rockland Immunochemicals Inc., Limerick, PA, USA) for 4 days at 4°C. The brains were washed three times for 5 min in PBS. The brains were than treated with goat anti-chicken Alexa Fluor 488 (1:250; Thermo Fisher Scientific, Schwerte, Germany) and Alexa Flour 568 phalloidin in 0.2% PBS-T with 2% NGS for 2 days at 4°C. The brains were then washed three times for 5 min in PBS and dehydrated in an isopropanol series, cleared, and mounted in MS, as described above. All brains were stored at 4°C under dark condition until they were examined. Optical sections of the brain were generated with confocal laser scanning microscope (Leica TSC SP8 STED 3X, Leica Microsystems, Wetzlar, Germany) every 3.0 to 6.0 μm (*z*-stacks). Scans of brain were done with the 20× objective [multi/numerical aperture (NA) 0.75] using the Mosaic Merge function of the Leica Application Suite X 3.0.0 (LAS X, Leica Microsystems CMS, Wetzlar, Germany). We used the 40× objective (water/NA 1.10) for higher resolution scans. The $dsx^{myrGFP/+}$ worker progenies (n = 39) never showed the

disruption of brain anatomy (Fig. 1C and movies S1 and S2). To quantify the size of structure or staining, we used FIJI (ImageJ 1.53c; Wayne Rasband, National Institutes of Health, USA), LAS X, and, Imaris software (Oxford Instruments, Abingdon, United Kingdom, version 9.1.2).

Data analysis and statistics

Statistical analysis was performed using Systat (Systat Software GmbH, Erkrath, Germany) and IBM SPSS Statistics 27 software (IBM, Armonk, USA). MWU test was used for pairwise comparison. To test against zero (no change), one-sample Wilcoxon signed-rank test was used.

Supplementary Materials

The PDF file includes:
Figs. S1 to S10
Tables S1 to S19
Legends for movies S1 to S23

Other Supplementary Material for this manuscript includes the following:
Movies S1 to S23

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Supplementary data

Supplementary figures

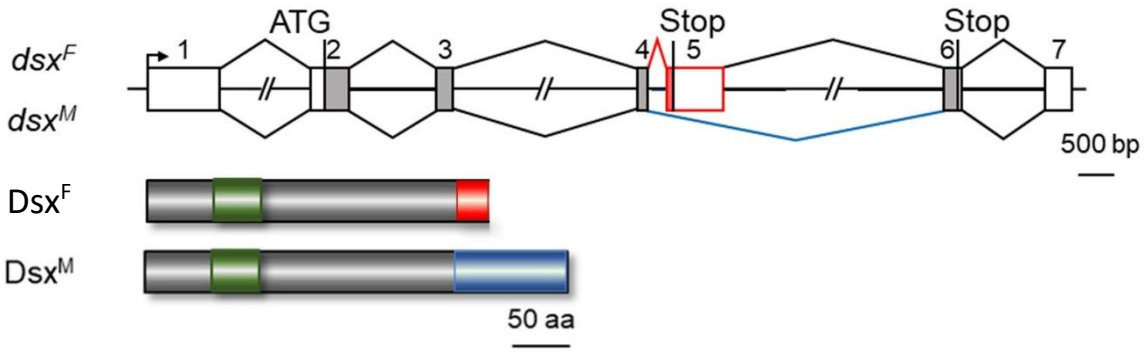

Figure S1. The sex-specific splicing scheme of the dsx gene in the honeybee. Above: boxes are the exons, and the interconnected lines indicate the splicing. Red lines/boxes indicate the female- specific inclusion of exon 5 via splice processing. The blue line indicates male-specific splicing and exclusion of female exon 5. Gray shaded boxes mark the ORF (open reading frame). Below: scheme of the protein structure highlighting the ZnF domain (DM domain; green) and the female- and male- specific peptide in red and blue, respectively. bp; base pairs. aa; amino acids.

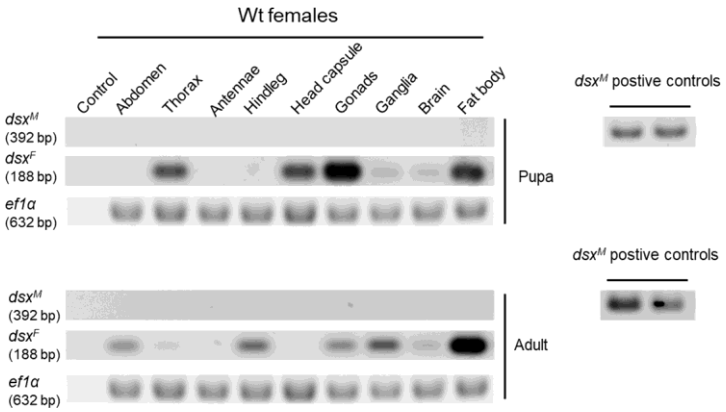

Figure S2: Sex-specific expression of dsx in worker bee tissues. Female (*dsx*^{F}) and male (*dsx*^{M}) dsx transcripts were examined in pupal stage 4 and 1-day-old adult worker bees. Amplicons from RT– PCRs were size-resolved and were semi-quantitatively adjusted across samples using *ef-1α* (*ef1α, elongation Factor 1α*) transcripts as a reference. Pictures are black and white inverted. Three biological each with three technical replicates were conducted: *ef1α: elongation Factor 1α*; Control: negative control for PCR. *dsx*^{M} positive controls: two cDNA samples were run as positive controls during *dsx*^{M} amplification experiments.

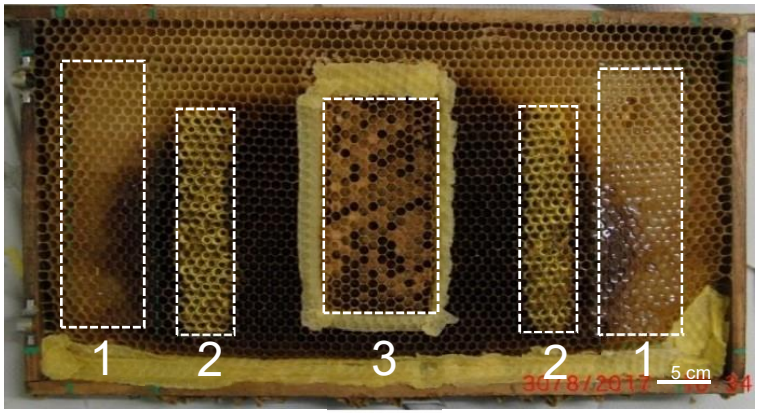

Figure S3. Brood comb to standardize workload for the worker bees in repeated tracking experiments. Sugar solution (1; mimicking honey), pollen (2) or larvae (3) were provided in the same areas across combs. The brood area consisted of 151 larvae at larval stages 3 and 4. 200 µl sugar solution per cell was provided. 30 g of ground pollen was split among the two pollen areas.

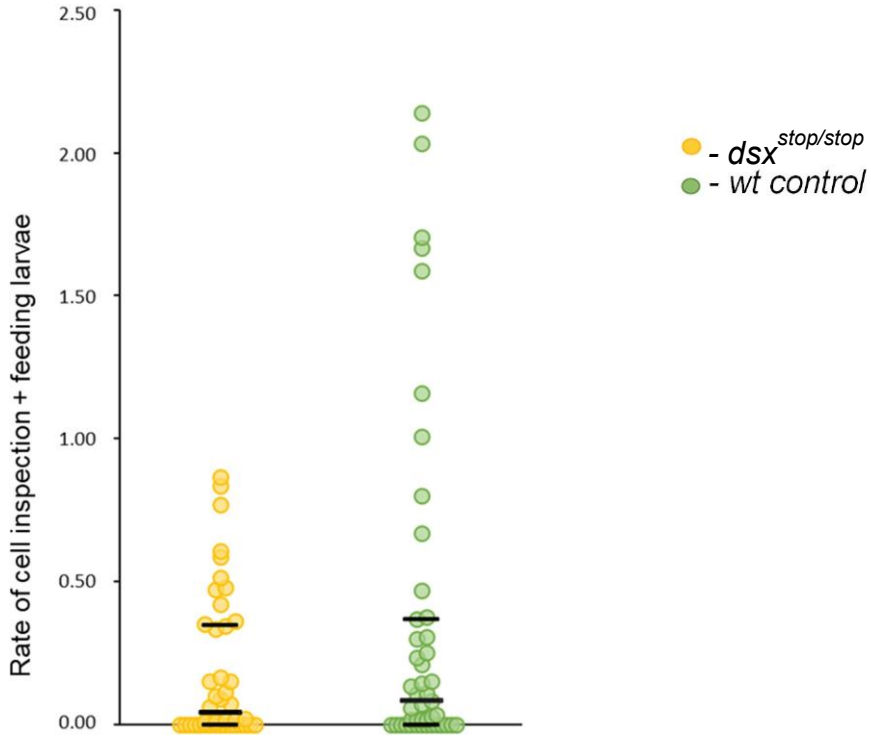

Figure S4. *dsx* gene activity is not required for the first entry into the larval cell. Cell inspection and larval feeding values are combined for this comparison ($P = 0.365$, $z = 0.907$; MWU test) (See Figure 5A and B).

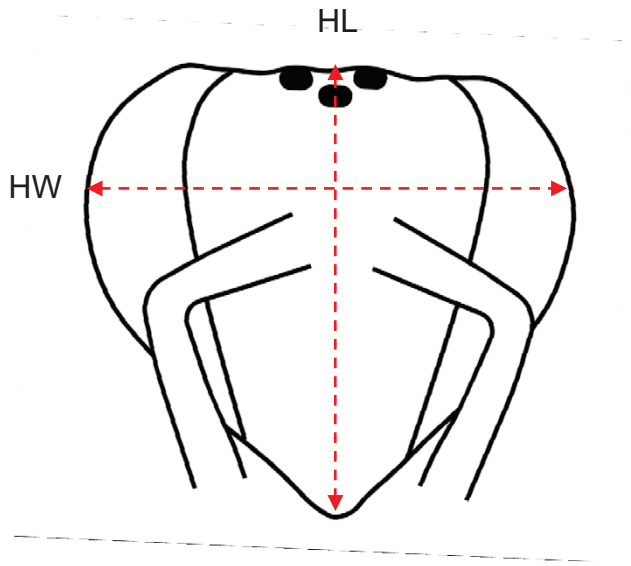

Figure S5. Measurements of honeybee head. Red lines represent the linear measurements of head length (HL) and width (HW).

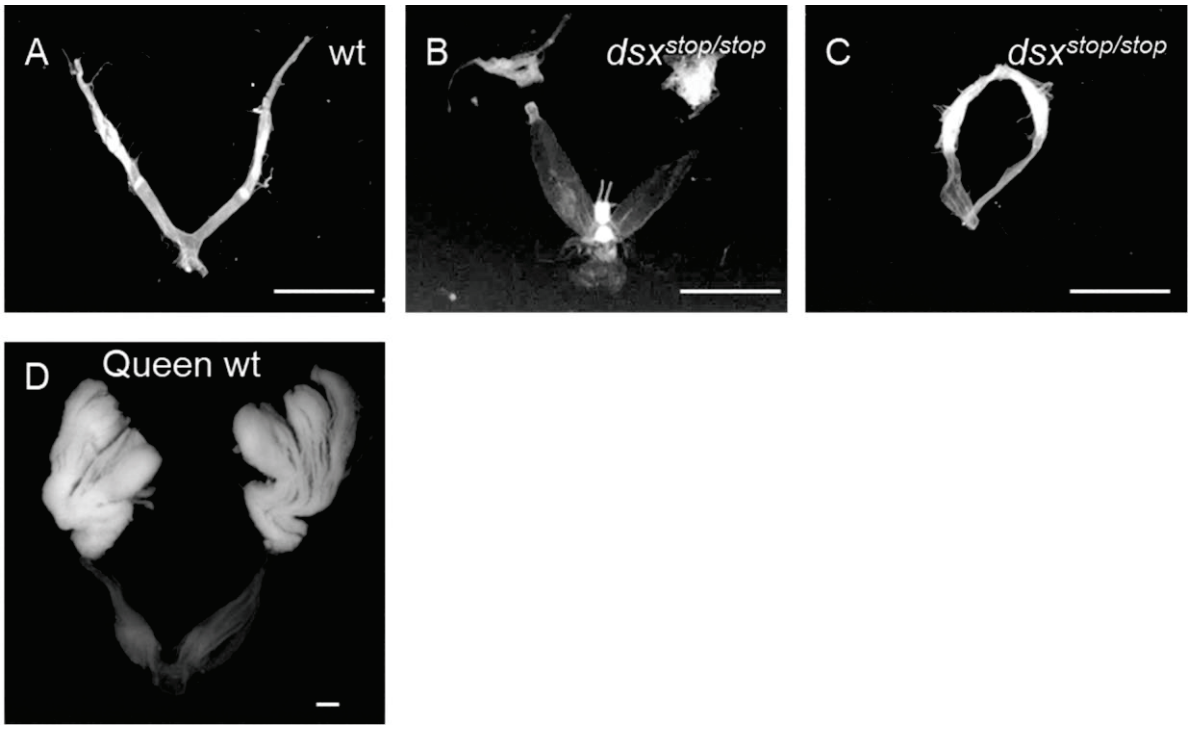

Figure S6. Worker ovaries from 10-13 days old worker bees. A. wt worker bee control **B.** *dsx*$^{stop/stop}$ worker bee with enlarged oviducts and intersex gonad. **C.** *dsx*$^{stop/stop}$ worker bee with reduced oviducts. **D.** Queen ovaries for comparison. Scale: 0.5 mm.

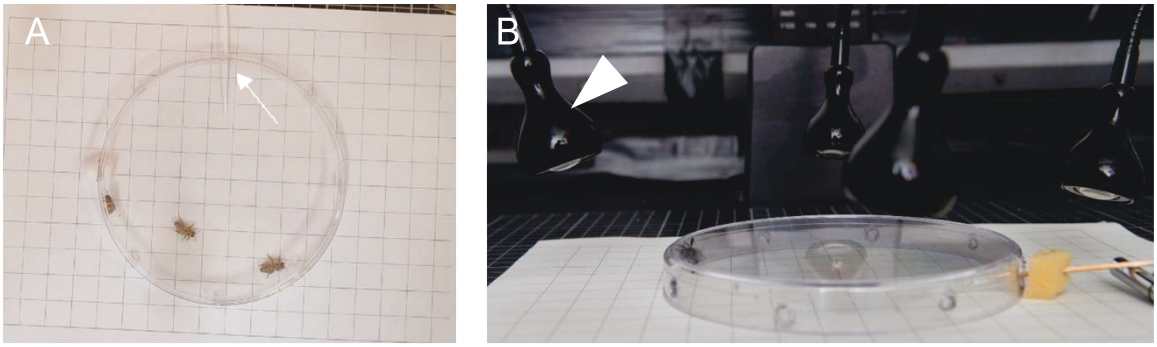

Figure S7. Petri dish behavioral assays for sensorimotor functions. Worker bees were examined in Petri dishes (14 cm). **A.** Example of *dsx*$^{stop/stop}$ worker bee odor sensorimotor examination in the presence of two hive-reared wt worker bees. Arrow shows the filter paper employed to provide the odor. **B.** Example of a wt worker bee examination of light responsiveness. The arrowhead marks the LED lamp.

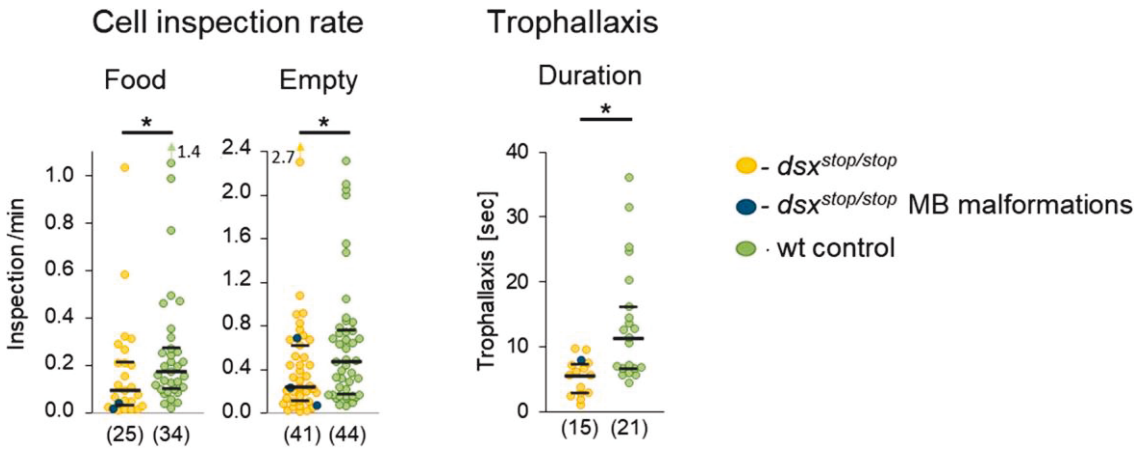

Figure S8. The dysfunctional task behaviors of mutant worker bees that have malformed MB brain structures. The median (middle line) and quartiles are presented. *n* values are shown in parentheses. min; minutes; sec; seconds; *; P < 0.05 (MWU test). Details see figure 5.

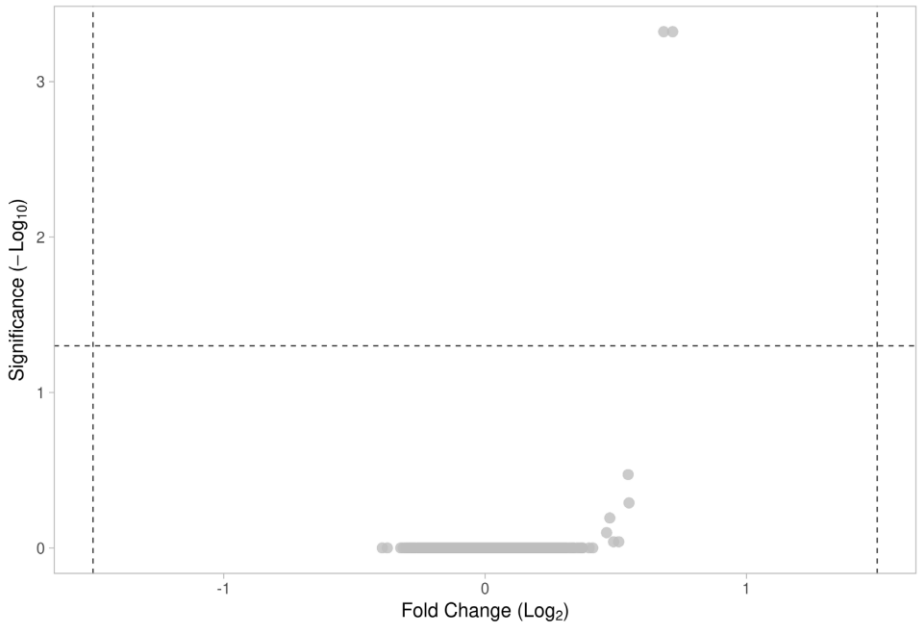

Figure S9. Differential gene expression in the antenna of *dsx*$^{stop/stop}$ versus wt worker bees. RNAseq was used, and 9361 genes were examined. Genes were differentially expressed (differentially expressed genes, DEGs) if adjusted P-values for multiple testing (Padj) were < 0.05 and log2 fold change was greater than 1.5 (dotted lines).

AACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGACTACGTTTCGAAAC
ACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGATTAGAGAGAGAGAGAGAAAGGACAACGATAG
AGGGACAAACAACCGTTCAAACATTTCATTGAGATTGTTCTTTGTAATTATGAAAAGGCTGTGAATCG
AGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACAGAGCCGCGGACTTGGCTCCCCAACAACCGAG
TGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGA
AGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGT
CTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTG
CGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGATGCGGCAGAATATGAAGCTGAAAAGACACC
TGGCACAGGATAAAGTCAAAGTAAGAGTAGCGGAAGAG

Figure S10. The nucleotide sequence elements of *dsx* exon 2 were employed in this study. The nucleotide sequence of exon 2 is shown, and sequence elements highlighted; single guide RNAs sgRNA1(red box) and sgRNA2 (brownish box) target sequence and the sites of their possible double- strand break (underlined); start codon (green colored box); sequence encoding the DM DNA binding domain (light blue colored box); target sequence of oligonucleotide primers (grey boxes) used for genotyping.

Supplementary table

Table S1. Nucleotide sequences of the sgRNAs and myrGFP DNA fragment.

Name	Nucleotide sequence
dsx sgRNA1 1)	**GAACGAGCAAAACAGAGCCG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCU AGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsx sgRNA2 1)	**GUGCACGAUGUCUGAAUCAU**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCU AGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
myrGFP DNA fragment 2)	GTTGCAGAACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGC CTCGACTACGTTTCGAAACACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGA TTAGAGAGAGAGAGAGAGAAAGGACAACGATAGAGGGACAAACAACCGTTCAAACATT TCATTGAGATTGTTCTTTGTAATTATGAAAAGGCTGTGAATCGAGGTTACCTATGT ATCGCGAAGAGAACGAGCAAAACAGAGGAATGGGCAATAAATGCTGCAGCAAAAGA CAAGATCAAGAATTGGCTTTAGCGTATCCAACAGGAGGTTACAAGAAATCGGATTA TACGTTCGGACAAACACATATCAATTCTAGCGGCGGTGGAAATATGGGTGGAGTGT TGGGCCAAAAACATAACAATGGTGGATCGTTAGATTCTAGATATACGCCAGATCCT AATCATAGAGGTCCATTGAAAATTGGAGGCAAAGGTGGAGTTGATATCATTAGACC TAGAGGATCTATGAGCAAAGGAGAAGAACTGTTCACAGGTGTTGTGCCAATCTTAG TTGAATTGGATGGCGATGTGAATGGACATAAATTCTCTGTGTCGGGTGAAGGAGAA GGCGATGCTACGTATGGTAAATTGACATTAAAATTCATTTGCACTACGGGAAAACT GCCAGTGCCTTGGCCAACATTGGTTACGACTTTAACATATGGTGTGCAATGCTTCA GCAGATATCCTGATCATATGAAACAACATGATTTTTTCAAATCTGCGATGCCAGAA GGATATGTGCAAGAAAGAACGATCTTTTTCAAAGATGATGGTAATTACAAAACAAG AGCTGAAGTTAAATTCGAAGGAGATACGTTGGTGAATAGAATTGAATTAAAAGGTA TCGATTTTAAAGAAGATGGAAATATTCTTGGTCATAAATTGGAATATAATTACAAC AGCCATAATGTTTATATAATGGCTGATAAACAAAAAAATGGAATCAAAGTGAACTT CAAAATTAGACATAATATAGAAGATGGTTCGGTTCAATTAGCGGATCATTACCAAC AAAATACACCAATTGGAGATGGTCCTGTTCTGTTGCCAGATAATCATTATTTAAGC ACGCAATCTGCTTTGTCGAAAGATCCAAATGAAAAAAGAGATCATATGGTGTTACT TGAATTCGTTACAGCGGCTGGAATTACGCATGGTATGGATGAATTATATAAAGGAT CTGGTGCTACAAATTTCTCTTTGTTAAAACAAGCGGGAGATGTGGAAGAAAATCCA GGTCCTGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCG TTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGAAGGTGCAAA CAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACGA TGTCTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA CCGTACTTGTACCTGCGAGAAGTGTAAGATCA

1) bold letters indicate the target site in the genome.

2) *dsx* gene homologous sequence are shown in gray boxes, myristoylation (myr) coding sequence in yellow box, GFP coding sequence in green box, 2A peptide (P2A) coding sequence in blue box and the GS and GSG linkers in orange boxes. The first two start codons are underlined. To maintain the open reading frame, nucleotides were inserted (red letters). Codon usage were optimized for the honeybee.

Table S2. The rate of generating *dsx*$^{stop/stop}$ worker bees.

Genotype	# (%) of adult worker bees
dsx$^{stop/stop}$	67 (59)
dsx$^{nonstop/stop}$ (mono allelic stop)	26 (23)
Mosaic	15 (13)
Wildtype	7 (6)
Total	115

Table S3. Nucleotide *dsx* exon 2 sequences (genotype) detected of the independently mutated and reared *dsx*$^{stop/stop}$ worker bees. Yellow box: mediated early stop codon. aa: amino acids.

Wildtype

>allele a/b

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCCGCGGACTTGGCTCCCCAACAACCGAGT
GGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGT
CCCAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGG
AATTGTGCACGATGTCTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTAC
TGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#13 i2-18

>allele a, aa 10

ATGTATCGCGAAGAGAACGAGCAAAACATGTGATCGGCTGGAGATCACCTTAAAATCGCA
CAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCG
GCAGCAAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCTACGCCTCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTAC
TGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#22 i2-18

>allele a, aa 66

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGTGATCATCGACTGGAGATCACCTTAAAATCGCACAAGAGGTA
CTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGT
G

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGTGATCATCGACTGGAGATCACCTTAAAATCGCACAAGAGGTA
CTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGT
G

#43 i2-18

>allele a, aa 32

ATGTATCGCGAAGAGAACGAGCAAAACAGACGCGGACTTGGCTCCCCAACAACCGAGTGG
TGTAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTC
CAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAA
TTGTGCACGATGTCTGAATCGTGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCA
AGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#58 i2-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#60 i2-18

>allele a, aa 35

ATGTATCGCGAAGAGAACGAGCAAAACAGATCTCCATCGGCTGGAGATCACCTTAAAATC
GCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAA
TCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#7 i3-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#29 i3-18

>allele a, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACCGCGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGATGTCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCA
AGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGAGTGCACGGAATTG
TGCACGATGTCTGAAGCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAA
GTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#30 i3-18

>allele a, aa 30

ATGTATCGCGAAGAGAACGAGCAAAACAGACTTGGCTCCCCAACAACCGAGTGGTGCAAA
CACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAAGAA
GGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGC
ACGATGTCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTG
TACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#44 i3-18

>allele a, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGAGTGCACGGAATTG
TGCACGATGTCTGAAGCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAA
GTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 36

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGTTGGAGATCGGCTGGAGATCACCTTAAA
ATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGC
CAATCGGCAGCAAGTG

#51 i3-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 36

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGTTGGAGATCGGCTGGAGATCACCTTAAA
ATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGC
CAATCGGCAGCAAGTG

#58 i3-18

>allele a, aa 19

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCCGATGCCATCGGCTGGAGATCACCTTAA
AATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTG
CCAATCGGCAGCAAGTG

>allele b, aa 15 (11.6%)

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#69 i3-18

>allele a/b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#1 i4-18

>allele a, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACCGCGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 91

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCGGACTTGGCTCCCCAACAACCGAGTGGT
GCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCC
AAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAAT
TGTGCACGATGTCTGAATAATTCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTA
CTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGT
G

#33 i4-18

>allele a, aa 30

ATGTATCGCGAAGAGAACGAGCAAAACAGACTTGGCTCCCCAACAACCGAGTGGTGCAAA
CACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAAGAA
GGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGC
ACGATGTCTGAAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACC
GTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#38 i4-18

>allele a, aa 72

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCGGACTTGGCTCCCCAACAACCGAGTGGT
GCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCC
AAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAAT
TGTGCACGATGTCTGAATCACGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCA
AGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#42 i4-18

>allele a/b, aa 64

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCGGACTGGCTCCCCAACAACCGAGTGGTG
CAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCA
AGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATT
GTGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGT
ACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCATCGGCAGCAAGTG

#49 i4-18

>allele a, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGAGTGCACGGAATTG
TGCACGATGTCTGAAGCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAA
GTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAACAATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#52 i4-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 67 deletion of ZnF domain

ATGTATCGCGAAGAGAACGAGCAAAACAGTC[- 168bp]CAGATCGGCTGGAGATCACC
TTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATC
ACTGCCAATCGGCAGCAAGTG

#53 i4-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 17

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCCATCGGCTGGAGATCACCTTAAAATCGC
ACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATC
GGCAGCAAGTG

#66 i4-18

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 35

ATGTATCGCGAAGAGAACGAGCAAAACAGATTCAGATCGGCTGGAGATCACCTTAAAATC
GCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAA
TCGGCAGCAAGTG

#17 i1-19

>allele a, aa 19

ATGTATCGCGAAGAGAACGAGCAAAACGTTGTGGAGATCATCGGCTGGAGATCACCTTAA
AATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTG
CCAATCGGCAGCAAGTG

>allele b, aa 91

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCGGACTTGGCTCCCCAACAACCGAGTGGT
GCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCC
AAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAAT
TGTGCACGATGTCTGAATCCATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTA
CTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGT
G

#43 i1-19

>allele a, aa 35

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGTCGATCGGCTGGAGATCACCTTAAAATC
GCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAA
TCGGCAGCAAGTG

>allele b, aa 34

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCATCGGCTGGAGATCACCTTAAAATCGCA
CAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCG
GCAGCAAGTG

#1 i2-19

>allele a, aa 33

ATGTATCGCGAAGAGAACGAGCAAAACAGATCGGCTGGAGATCACCTTAAAATCGCACAA
GAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCA
GCAAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#7 i2-19

>allele a, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 90

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCGGACTTGGCTCCCCAACAACCGAGTGGT
GCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCC
AAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAAT
TGTGCACGATGTCTGAATTCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTG
CAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#8 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 20

ATGTATCGCGAAGAGAACGAGCAAAACAGAGCTCGGCTCGTGATCGGCTGGAGATCACCT
TAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCA
CTGCCAATCGGCAGCAAGTG

#9 i2-19

>allele a, aa 17

ATGTATCGCGAAGAGAACGAGCAAAACAGATCCATCGGCTGGAGATCACCTTAAAATCGC
ACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATC
GGCAGCAAGTG

>allele b, aa 19

ATGTATCGCGAAGAGAACGAGCAAAACAGAGATCCAGCCATCGGCTGGAGATCACCTTAA
AATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTG
CCAATCGGCAGCAAGTG

#13 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 51 (49.9 %)

ATGTATCGCGAAGAGAACGAGCAAAACAGTTCGAGCGTTTGGAACATTCTCAGGATAGCA
AAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTC
CAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAAT
CGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCA
ATCGGCAGCAAGTG

#14 i2-19

>allele a/b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#18 i2-19

>allele a, aa 10

ATGTATCGCGAAGAGAACGAGCAAAACAGATAACGGACTAGCCTTATTTTAGCGGATTGG
CTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCA
AAAATGGGGACGATGGTCCCAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTC
CAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCGTGCTGGAGATCACCTTAAAA
TCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCC
AATCGGCAGCAAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCATCGTCACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGG
TACTGCAAGTACCGTACTTGTACCCGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAA
GTG

#19 i2-19

>allele a/b, aa 28

ATGTATCGCGAAGACAACGATAGAGGGACAAACAACCGTTCAAACATTTCATTGAGATTG
TTCTTTGTAATTATGAAAAGGCTGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGA
GCAAAACAGAGCAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT
TATTTTCTCCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGT
ACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGGAGAACGAGCAA
AACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA
CCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#22 i2-19

>allele a, aa 62

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGGCTCCCCAACAACCGAGTGGTGCAAACA
CGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGAAGG
TGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCAC
GATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTA
CTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGC

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGG

#23 i2-19

>allele a, aa 21

ATGTATCGCGAAGAGAACGAGCAAAACAGAGACGGAACGATGAACATCGGCTGGAGATCA
CCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGA
TCACTGCCAATCGGCAGCAAGTG

>allele b, aa 64

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGGACTTGGCTCCCCAACAACCGAGTGGTG
CAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTTCCA
AGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATT
GTGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGT
ACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATAGGCAGCAAGTGC

#31 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#33 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCTGCAAGATACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGT
AAGATCACTGCCAATCGGCAGCAAGTG

#37 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

#38 i2-19

>allele a, aa 19

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGATCTCCGATCGGCTGGAGATCACCTTAA
AATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTG
CCAATCGGCAGCAAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACCGCGGACTTGGCTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGC

#42 i2-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 31

ATGTATCGCGAAGAGAACGAGCAAAACAGAGGACTTGGGTCCCCAACAACCGAGTGGTGC
AAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA
GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTG
TGCACGATGTCTGAATCATTAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGT
ACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAG
TG

#40 i2-19

>allele a, aa 32

ATGTATCGCGAAGAGAACGAGCAAAACAGACTTGGACTTGGCTCCCCAACAACCGAGTGG
TGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCC
CAAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAA
TTGTGCACGATGTCTGAATTTTCGCGATCGGCTGGAGATCACCTTAAAATCGCACAAGAG
GTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCA
AGTG

>allele b, aa 67

ATGTATCGCGAAGAGAACGAGCAAAACAGAGACTTGGCTTGGACTTGGCTCCCCAACAAC
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ACTGCCAATCGGCAGCAAGTG

#50 i2-19

>allele a, aa 15

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AAGTG

>allele b, aa 31

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CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#176 i2-19

>allele a, aa 22

ATGTATCGCGAAGAGAACGAGCAAAACAGAGACAGGATCGGCTCCAGCATCGGCTGGAGA
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AGATCACTGCCAATCGGCAGCAAGTG

>allele b, aa 15

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GCAAGTG

#1 i3-19

>allele a, aa 15

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AAGTG

>allele b, aa 17

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GGCAGCAAGTG

#2 i3-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
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AAGTG

>allele b, aa 19

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ACAATCGGCAGCAAGTG

#10 i3-19

>allele a, aa 15

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AGTG

>allele b, aa 31

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#17 i3-19

>allele a, aa 15

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AAGTG

>allele b, aa 31

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#19 i3-19

>allele a, aa 15

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AAGTG

>allele b, aa 15

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AAGTG

#20 i3-19

>allele a, aa 33

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GCAAGTG

>allele b, aa 31

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TGCACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTA
CCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#26 i3-19

>allele a, aa 15

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AAGTG

>allele b, aa 33

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GCAAGTG

#27 i3-19

>allele a, aa 15

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AAGTG

>allele b, aa 30

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CACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAAGAA
GGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGC
ACGATGTCTGAATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCG
TACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTG

#41 i3-19

>allele a, aa 15

ATGTATCGCGAAGAGAACGAGCAAAACATCGGCTGGAGATCACCTTAAAATCGCACAAGA
GGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTGCCAATCGGCAGC
AAGTG

>allele b, aa 19

ATGTATCGCGAAGAGAACGAGCAAAACAGAGAACAAGACATCGGCTGGAGATCACCTTAA
AATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACCTGCGAGAAGTGTAAGATCACTG
CCAATCGGCAGCAAGTG

Table S4. Survival of the laboratory reared experimental worker bees.

Treatment	# of 2-day old larvae	# of eclosed adults	% survival to adult stage	Fisher´s exact test, *P*-value, df = 1
Injected	732	171	23.1 %	0.25
Control	907	235	25.9 %	

Table S5. Sizes of the standardized areas on the combs for each replicate.

SD = standard deviation.

replicate	brood (cm^2)	pollen (cm^2)	Sugar solution (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 S6. Detection rate of the bees.

SD = standard deviation.

Replicate	% detection rate	Maximal # of bees detected
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	459
SD +/-	0.07	6.1

Table S7. The rearing of the 151 larvae in each replicate.

SD = standard deviation.

Replicate	% of larvae
i2-2018	53.6
i3-2018	68.2
i4-2018	70.2
i2-2019	63.6
i3-2019	58.3
mean	62.8
SD +/-	6.2

Table S8. The rate of cell inspection behaviors for *dsx*$^{stop/stop}$ and wildtype (wt) worker bees.

SD = standard deviation. MWU = Mann-Whitney U-test. min: minutes

Cell inspection /min	***dsx*$^{stop/stop}$**				**wt control**				MWU	
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	z
All	42	0.49	0.63	± 0.53	45	0.87	1.05	± 0.85	0.006	2.74
Larvae	26	0.15	0.20	± 0.18	31	0.15	0.35	± 0.46	0.67	0.83
Food	25	0.09	0.18	± 0.23	33	0.17	0.27	± 0.29	0.04	2.04
Empty	41	0.24	0.41	± 0.47	44	0.48	0.63	± 0.59	0.04	2.03

Table S9. The rate of task behaviors.

SD = standard deviation. MWU = Mann-Whitney U-test. min: minutes

Task behavior /min	*dsx*$^{stop/stop}$				wt control				MWU	
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	z
All tasks	39	0.17	0.27	± 0.27	39	0.39	0.42	± 0.34	0.02	2.35
Larval feeding	20	0.09	0.13	± 0.11	22	0.21	0.25	± 0.21	0.054	1.93
Food handling	15	0.03	0.05	± 0.03	21	0.04	0.05	± 0.03	0.53	0.65
Cleaning empty cells	33	0.15	0.22	± 0.24	37	0.18	0.27	± 0.27	0.29	1.05

Table S10. The length of the task behaviors.

SD = standard deviation. MWU = Mann-Whitney U-test. sec: seconds

Length task behavior [sec]	*dsx*$^{stop/stop}$				wt control				MWU	
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	z
All	39	13.42	16.17	± 7.67	39	17.84	20.27	± 10.05	0.04	2.03
Larval feeding	20	16.69	21.12	± 12.99	22	16.57	19.72	± 11.12	0.82	0.23
Food handling	15	6.75	17.37	± 22.22	21	13.50	25.81	± 29.19	0.02	2.28
Cleaning empty cells	33	13.42	15.03	± 7.33	37	15.37	22.76	± 21.63	0.21	1.25

Table S11. Rate of trophallaxis related behaviors.

SD = standard deviation. MWU = Mann-Whitney U-test. min: minutes

Rate /min	*dsx*$^{stop/stop}$				wt control				MWU	
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	z
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

Table S12. Length of the trophallaxis behavior.

SD = standard deviation. MWU = Mann-Whitney U-test. sec: seconds

Length [sec]	*dsx*$^{stop/stop}$				wt control				MWU	
	n	Median	Mean	± SD	*n*	Median	Mean	± SD	*P*-value	z
Food exchange	15	5.50	5.2	± 2.83	21	11.25	13.49	± 9.11	0.001	3.23

Table S13. Walking distance of the *dsx*$^{stop/stop}$ and the wildtype (wt) worker bees on the comb.

SD = standard deviation. MWU = Mann-Whitney U-test. m: meter; h: hour

Walking behavior [m/h]	*dsx*$^{stop/stop}$ (*n* = 47)			wt control (*n* = 49)			MWU	
	Median	Mean	± SD	Median	Mean	± SD	*P*-value	z
Distance	0.59	0.64	± 0.25	0.53	0.62	± 0.33	0.21	1.26

Table S14. The number of visits the experimental bees make to the different areas of the comb.

SD = standard deviation. MWU = Mann-Whitney U-test.

Visits per hour [visits/h]	***dsx*$^{stop/stop}$ (*n* = 47)**			**wt control (*n* = 49)**			MWU	
	Median	Mean	± SD	Median	Mean	± SD	*P*-value	z
Brood	1.25	1.31	± 0.61	1.22	1.42	± 0.50	0.85	0.19
Food	2.96	3.77	± 3.10	3.21	4.88	± 2.34	0.17	1.36

Table S15. The time the *dsx*$^{stop/stop}$ and wildtype (wt) worker bees spent in the different areas of the comb.

SD = standard deviation. MWU = Mann-Whitney U-test.

Time spent in area [min/h]	***dsx*$^{stop/stop}$ (*n* = 47)**			**wt control (*n* = 49)**			MWU	
	Median	Mean	± SD	Median	Mean	± SD	*P*-value	z
Larvae	2.48	3.22	± 3.19	1.85	2.61	±1.75	0.33	0.97
Food	4.32	5.22	± 5.55	5.99	5.98	± 3.51	0.06	1.86
Empty	16.79	17.52	± 7.06	18.31	18.17	± 4.50	0.36	0.92

Table S16. The morphology and anatomy of the *dsx*$^{stop/stop}$ and wildtype (wt) worker bees.

Structure	# of *dsx*$^{stop/stop}$ with malformed structure (# examined)	# of wt worker bees with malformed structure (# examined)	Fisher's exact test *P*-value df = 1
Head	0 (29)	0 (26)	1
Body morphology	0 (17)	0 (11)	1
Abdominal segments [1)]	0 (17)	0 (11)	1
Antennal segments [2)]	0 (24)	0 (26)	1
Ovaries	6 (12)	0 (5)	0.10
Hypopharyngeal gland	0 (23)	0 (27)	1

[1)] wt worker bees have female-specific 6 abdominal segments.

[2)] wt worker bees have female-specific 13 antennal segments.

Table S17. The number of *dsx*$^{stop/stop}$ worker bees with in the brain.

Group	**Mutant**	**Wildtypic**	**Fisher's exact test, *P* value, df=1**
dsx$^{stop/stop}$	6	20	0.008
Wildtype control	0	29	

Table S18. The location of malformations in specific brain structures of the *dsx*$^{stop/stop}$ worker bees.

*(A: extra structure, A^M: multiple such structures, B: deformed structure, C: misplaced structure, l-CA: lateral Calyx, m-CA: medial Calyx, LH: lateral horn)

			Affected structure *						**Average size of additional structure [µm]**					
Bee ID	Replicate	Genotype allele 1/ allele 2	left l-CA	left m-CA	right m-CA	right l-CA	left LH	right LH	left l-CA	left m-CA	right m-CA	right l-CA	left LH	right LH
17	i1-19	stop/stop			A^M , B	A^M , B	A				27.3 to 40.3	21.8 to 71.5	47.6	
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			40.0	33.3		46.6		

Table S19. Number of the *dsx*$^{stop/stop}$, the wt laboratory and the hive reared worker bees in the different behavioral analyses and biological replicates.

Replicate	# of bees				
	Type	Assembled [1]	Moving [2]	Encounters [3]	Cell inspection/task [4]
I2 2018	*dsx*$^{stop/stop}$	8	5	5	5
	wt lab	50	8	8	6
	Wildtype	444	-	-	-
I3 2018	*dsx*$^{stop/stop}$	14	7	5	6
	wt lab	42	8	6	7
	Wildtype	439	-	-	-
I4 2018	*dsx*$^{stop/stop}$	10	10	8	7
	wt lab	18	8	8	7
	Wildtype	474	-	-	-
I2 2019	*dsx*$^{stop/stop}$	19	16	15	15
	wt lab	57	16	16	13
	Wildtype	417	-	-	-
I3 2019	*dsx*$^{stop/stop}$	12	9	9	9
	wt lab	36	9	9	12
	Wildtype	443	-	-	-

[1] Number at the begin of the tracking.

[2] Number detected and computer-based examined over 24 hours

[3] Number detected and computer-based annotated in the time frame examined (antennation, begging and trophallaxis behavior)

[4] Number detected and computer-based annotated in the time frame examined (cell inspection/task behaviors).

Supplementary movies

Movie S1.

A middle brain z-stack of a wt worker bee which is stained with phalloidin.

Movie S2.

A middle brain z-stack of a *dsx* ^{myrGFP} worker bee which is stained with phalloidin and anti-GFP. Only the phalloidin staining is shown.

Movie S3.

A middle brain z-stack of a *dsx* ^{myrGFP} worker bee which is stained with phalloidin and anti-GFP. Only the anti-GFP staining is shown.

Movie S4.

A middle brain z-stack of a *dsx* ^{myrGFP} queen bee which is stained with phalloidin and anti-GFP. Only the phalloidin staining is shown.

Movie S5.

A middle brain z-stack of a *dsx* ^{myrGFP} queen bee which is stained with Phalloidin and anti-GFP. Only the anti-GFP staining is shown.

Movie S6.

Example of a laboratory reared *dsx*$^{stop/stop}$ worker bee (highlighted with the ID # 60) showing cell inspection behavior. The head is in the cell and the antennae are not visible. Movie is at half speed.

Movie S7.

Example of a laboratory reared wt worker bee (highlighted with the ID # 99) showing two cell inspection behaviors. The head is in the cell and the antennae are not visible. Movie is at half speed.

Movie S8.

Example of a laboratory reared *dsx*$^{stop/stop}$ worker (highlighted with the ID # 13) bee showing task behavior (≥ 5 sec head in cell). The head is in the cell and the antennae are not visible. Movie is at half speed.

Movie S9.

Example of a laboratory reared wt worker bee (highlighted with the ID # 63) showing task behavior (≥ 5 sec head in cell). The head is in the cell and the antennae are not visible.

Movie S10.

Example of a colony reared wt worker bee showing antennation behavior (marked by a circle). The bee encounters another bee. They face each other and have repeated contact with their antennae.

Movie S11.

Example of a colony reared wt worker bee showing begging behavior (marked by a circle). The bee encounters another bee. The bee to the left display begging behavior. It moves the head toward the other bee and reaches with its forelegs the other bee.

Movie S12.

Example of a colony reared wt worker bee showing trophallaxis behavior. The bee has contact via its proboscis with another worker bee, which indicates food sharing. Occasionally, one bee contacts the other bee with its forelegs and antennae during this behavior.

Movie S13.

Example of a laboratory reared *dsx*$^{stop/stop}$ worker bee (ID # 176) showing antennation behavior with another wt worker bee (ID # 479). They face each other and have repeated contact with their antennae. Movie is at half speed.

Movie S14.

Example of wt a laboratory reared worker bee (ID # 78) showing antennation behavior with another wt worker bee (ID # 146). They face each other and have repeated contact with their antennae. Movie is at half speed.

Movie S15.

Example of a laboratory reared *dsx*$^{stop/stop}$ worker bee (ID # 29) showing begging behavior. Bees have repeated contacts with their antennae to other wt bees (ID # 160). The respective bee moves its head towards the other bee and touches it with its front legs. Movie is at half speed.

Movie S16.

Example of a laboratory reared wt worker bee (ID # 93) showing begging behavior. Bees have repeated contacts with their antennae to other wt bees (ID # 434). The respective bee moves its head towards the other bee and touches it with its front legs. Movie is at half speed.

Movie S17.

Example of a laboratory reared *dsx*$^{stop/stop}$ worker bee (ID # 42) showing food sharing behavior (trophallaxis). The bees have contact via its expanded proboscis. Occasionally, the one bee contacts the other bee with its forelegs during this behavior. Movie is at half speed.

Movie S18.

Example of a laboratory reared wt worker bee (ID # 63) showing food sharing behavior (trophallaxis). The bees have contact via its expanded proboscis. Occasionally, the one bee contacts the other bee with its forelegs during this behavior during this behavior. Movie is at half speed.

Movie S19.

The middle brain z-stack of *dsx*$^{stop/stop}$ worker bee ID #17. Multiple additional structures (marked by arrows) and deformed lip (marked by a circle) in the medial and lateral right calyx. Additional structure (marked by a circle) in the lateral left horn.

Movie S20.

The middle brain z-stack of *dsx*$^{stop/stop}$ worker bee ID #7. Additional structures (marked by arrows) in the medial and lateral left calyx.

Movie S21.

The middle brain z-stack of *dsx*$^{stop/stop}$ worker bee ID #9. Additional structure (marked by an arrow) in the lateral calyx.

Movie S22.

The middle brain z-stack of *dsx*$^{stop/stop}$ worker bee ID #31. Additional structure in the lateral left horn area.

Movie S23.

The middle brain z-stack of *dsx*$^{stop/stop}$ worker bee ID #19. Additional structures (marked by arrows) in the lateral and medial left calyx.

Author's contribution: Manuscript II

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Author's contribution to Manuscript I

- Experimental design
- sgRNA synthesis
- Implementation of microinjections
- In-vitro rearing
- Genotyping of mutant honeybees
- Measurement of mutant brains and control brains
- Data analyses: sequencing data, tracking data, statistics
- Authoring the manuscript

Manuscript III

Conditional control of worker honeybee behaviors via *dsx* locus expressed chemogenetic tool

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Abstract

Honeybees (*Apis mellifera*) are highly socially organized. The colony organization results from sophisticated behavioral interactions, which are controlled by neuronal circuits that process a variety of sensory signals and control behavioral decisions and motor programs. The *dsx* gene specifies during development specifically initiation and sustainment aspects of worker behaviors and is highly spatially expressed in the nervous system. However, tools that specifically inhibit dedicated neural circuitry are so far lacking in honeybees and other social insects which would be a key tool to understand the mechanism underlying the control of the sophisticated social worker behaviors. Here, we show that the expression of a chemogenetic system from the *dsx* gene locus established a tool to conditional control worker behaviors. We knocked in the hM4Di receptor gene into the *dsx* locus, conditionally silenced the *dsx*-expressing cells by feeding the drug C21 leading to activation of the receptor and hyperpolarization and examined behavioral locomotion responses of worker bees to the queen mandibular pheromone QMP. We found that the $dsx^{hM4Di/+}$ worker bees specifically changed their QMP behavioral responses under C21 versus no such condition. The drug C21 treatment alone cannot explain the QMP response because the wildtype worker bees did not show such change in behavior under drug versus no drug condition. The results suggest that the drug C21 induced a silencing of *dsx* expression cells via the hM4Di receptor that influenced the worker-specific behaviors. Introducing a chemogenetic method in *A. mellifera* now offers the great prospect to functionally dissect the circuitry underlying of the sophisticated control of behaviors in worker bees.

Introduction

The Western honeybee (*Apis mellifera*) exhibits extraordinary behavior that relates to its social organization. A honeybee colony contains thousands of altruistic behaving worker bees, hundreds of drones, and a queen. While the drones and the queen take care of the reproduction of the colony, the workers exhibit a variety of behaviors to maintain the colony such as the collective rearing of brood and nest construction (Johnson, 2008; Johnson, 2010; Rösch, 1925; Seeley, 1982, 1995; von Frisch, 1967). The social organization is characterized by a remarkable ability of the worker bee caste to communicate. For example, worker bees communicate during their waggle dance behaviors the location of a food source to other worker bees in the hive. The queen uses a variety of pheromones to communicate with worker bees. The pheromones either induces immediate changes in behavior (a releaser function) or have other long-term effects on physiology and behavior (a primer effect) (Keeling et al., 2003; Le Conte & Hefetz, 2008; Mumoki & Crewe, 2021; Slessor et al., 2005). The queen mandibular pheromone (QMP) has both a releaser and primer function. Primer function involves in the inhibition of ovary development in worker bees (Hoover et al., 2003; Le Conte & Hefetz, 2008; Mumoki & Crewe, 2021; Slessor et al., 2005) and the rearing of new queens (Melathopoulos et al., 1996). QMP regulates the transition between middle aged bees and foragers by for example modulation of the dopamine level (Beggs et al., 2007; Beggs & Mercer, 2009; Pankiw et al., 1998). Releaser function involves the worker's retinue behavior of the queen (Slessor et al., 2005) and a reduction of locomotion (Beggs et al., 2007). Some bees lick the QMP from the cuticle of the queen and distribute it throughout the colony which ensures rapid dispersal (Naumann et al., 1991; Seeley, 1979).

The worker-specific behaviors including locomotion response to QMP are innate and are inherited, which requires that the capacities of these behaviors are developmentally programed into the nervous system of a worker bee. Worker caste developmental requires the combination of sex determination signals (the female Feminizer Fem^F protein) and worker/queen nutrition-derived signals, the latter is mediated by differential feeding of the larvae (Asencot & Lensky, 1988; Buttstedt et al., 2016; Gempe et al., 2009; Haydak, 1970; Kucharski et al., 2008; Otte et al., 2023; Roth et al., 2019; Seiler & Beye, 2024). The downstream components of the sex determination pathway includes the sex-specific activated transcription factor gene

doublesex (*dsx*) together with the worker nutrition-derived signals that determine worker developmental characteristics (Figure 1A) (Netschitailo et al., 2023; Roth et al., 2019).

Behavioral studies of *dsx* knockout worker mutants found dysfunction in initiation and sustainment aspects of otherwise stereotypic motor program patterns of behavioral task inside the colony (Sommer et al., 2024). General sensorimotor functions (responses to odor, light and honey and locomotion behavior) were unaffected in these mutants, suggesting that specifically the worker-specific behaviors were affected. Expression of the *dsx* gene is prominent in neural populations of the mushroom body (MB; class I and II Kenyon Cells (KCs)) of the brain but also involves the antennal nerve, cortical regions and olfactory glomeruli. The neurons in the MBs were repeatedly miswired in *dsx* knockout workers suggesting a link of neural circuitry with dysfunction of worker-specific behaviors. However, which function such developmentally hardwired circuitry has in specifying worker-specific behaviors is unknown and requires tools that inhibit such circuitry.

Powerful new tools combining genetics and pharmacology to examine the role of specific neural circuits in behaviors have recently been developed for mammalian and *Drosophila* systems. These protein-based methods when selectively activated by synthetic ligands can inhibit or enhance neuronal activity and can control circuitry function. Such system has been termed designer receptors exclusively activated by a designer drug (DREADDs) and make use of modified G protein coupled receptors (GPCRs) (Armbruster et al., 2007; Coward et al., 1998; Roth, 2016). The current set of DREADDs, which have been created through directed evolution, largely consist of mammalian muscarinic acetylcholine receptors that no longer have affinity for, and therefore have no response to their natural ligand, acetylcholine. However, the receptors are fully activated by the synthetic ligand clozapine-N-oxide (CNO) or compound 21 (C21) (Bonaventura et al., 2019; Chen et al., 2015; Jendryka et al., 2019). C21 cannot be metabolized into clozapine, a metabolite that is problematic in some animal systems (Roth, 2016). DREADDs have thus produced the possibility to conditionally control behaviors through the inhibitory or excitability effect on neurons via effector pathways (Sasaki et al., 2011; Zhu & Roth, 2014).

The DREADD hM4Di receptor is a neuronal silencer (Armbruster et al., 2007; Atasoy & Sternson, 2018; Roth, 2016). Binding of ligand C21 results in a conformational

change of the receptor, which causes the release of the three subunits of the coupled Gi protein (α, β and γ) (Atasoy & Sternson, 2018; Lee et al., 2023; Sandhu et al., 2019). The βγ complex acts as an allosteric modulator and increases the affinity of GIRK (G protein-coupled inwardly rectifying potassium) channels for their natural ligand, leading to increased potassium efflux and hyperpolarization of the neuron, while the α subunit of the Gi protein inhibits adenylate cyclase (AC) (Coward et al., 1998; Shan et al., 2022; Simonds, 1999). The resulting reduction in cAMP production leads to reduced activation of protein kinase A (PKA; Figure 1B) (Atasoy & Sternson, 2018; Majeed et al., 2013). Since PKA is involved in numerous cellular processes, including phosphorylation and activation of L-type calcium channels, the reduced PKA activity contributes to further inhibition of neuronal activity. Inhibition of calcium channels reduces calcium influx, resulting in decreased neurotransmitter release and overall decreased excitability of the neuron (Atasoy & Sternson, 2018; Kamp & Hell, 2000). As a result, the role of this silenced circuitry on behavior can be examined.

We recently showed that site-specific integration of coding sequences into genes is efficiently feasible in the honeybee that uses endogenous gene promoter for expression (Sommer et al., 2024; Wagner et al., 2022). Here, we translated DREADD technology into the honeybee system. We introduced the hM4Di receptor coding sequence into the *dsx* locus and administered drug C21 via feeding to the adult worker bee. We showed that the DREADD receptor hM4Di selectively and conditionally altered the worker bee behaviors in response to QMP. We thereby demonstrate the utility of DREADD technology for conditional control of behaviors by combining genetic manipulation and pharmacological approaches. The presented method offers the prospect of dissecting the neural circuitry control of worker bee behaviors.

Material and Methods

Gene manipulation and honeybee handling

To introduce the *hM4Di* nucleotide coding sequence 31 bp downstream of the start codon of *dsx* we used *dsx*-sgRNA1 (Roth, 2019; Sommer et al., 2024; Wagner et al., 2022) and Cas9 protein (New England Biolabs, Ipswich, MA) that were co-injected with the DNA donor fragment DREADD-hM4Di (Table S1). We injected 400 pl per egg of a solution that consisted of 500 ng/µl Cas9 protein, 46.25 ng/µl sgRNA1, and 75 ng/µl DREADD-hM4Di donor DNA (Wagner et al., 2022). The donor (DREADD-hM4Di) DNA encoded hM4Di protein sequence (Addgene plasmid # 83896), a GSG linker, the P2A endopeptidase sequence (Szymczak-Workman et al., 2012; Wagner et al., 2022) and 5 c-Myc-tag repeats (Table S1), which we codon-optimized for the honeybee (Otte et al., 2018). The left and right arms that were homologous to the integration site we approximately 250 bp long in the DREADD-hM4Di donor fragment (NCBI; gene ID: 725126; Reference Sequence: NC_037642; Annotation: Amel_HAv3.1) (Fig. 1C, Table S1). The DREADD-hM4Di donor fragment was synthesized, cloned, and sequenced (standard gene, Eurofins, Ebersberg, Germany) and amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) before injections. Eggs were collected from queens caged in Jenter egg collecting cages (Jenter Queen rearing Kit, Karl Jenter GmbH, Frickenhausen, Germany). Eggs were collected every 1.5 hours (Schulte et al., 2014). Queens were reared as described (Otte et al., 2018; Schulte et al., 2014) and the homozygous queens ($dsx^{hM4Di/\ hM4Di}$) were inseminated with wildtype (wt) drones (dsx^{+}). The queens were introduced into small colonies (nucs) together with worker bees. and maintained in containments. Eclosed adult worker bees were day by day labeled to obtain age standardized worker bees for the behavioral examinations.

Genotyping and sequencing

To genotype the queens, we isolated genomic DNA from a midleg using the innuPrep Mini Kit (Analytic Jena, Jena, Germany). PCR_1 amplified the sequence of the upstream integration site (5’- GATTCGTAATAATTCCTGTGC-3’, 5’- CACCACAGTCA CCAGGCTCAGAG -3’; Eurofins, Ebersberg, Germany). PCR_2 amplified the sequence of the downstream integration site (5’- GCCAATATAGAAATATCGGTACT

GCC-3'; 5'- CTTCCGCTACTCTTACTTTGAC -3'). PCR_3 amplified the wt sequence (5'- GATTCGTAATAATTCCTGTGC-3'; 5'- CTTCCGCTACTCTTACTTTGAC -3').

To check the expression of the DREADD hM4Di receptor and the Dsx protein, RNA was isolated from the progeny of the *dsx*$^{hM4Di/\ hM4Di}$ queen Q21 (L1 larvae) using the innuPREP DNA / RNA Mini-Kit (Analytic Jena, Jena, Germany). First-strand cDNA was synthesized with Oligo(dT)18 primer and RevertAid Reverse Transcriptase (Thermo Fisher Scientific). PCRs were run under standard conditions using GoTaq G2 Polymerase (Promega, Madison, WI) and sequencing region was divided into four overlapping PCRs: PCR_1 amplified the upstream integration site (primer sequences: 5'-GCCGGTTATCTATGTATTGC-3', 5'-GCCAGTAACCTTTGATGATG-3'; Eurofins), PCR_2 the first middle part (primer sequences: 5'-GTAGCTCGGGTAATCAATCTG-3'; 5'-GACGCTTTGTTTCATCAATG-3'), PCR_3 the second middle part (primer sequences: 5'-CAGTAGAAGCCGAGTTCATAAAC-3'; 5'-CTTCTGGCAGTACCGATATTTC-3') and PCR_4 the downstream integration site (primer sequences: 5'-GTTACTGGCTCTGCTACGTC-3'; 5'-CTTCTAGGTGGTTGAGGGAC-3'), PCR amplicons were cloned into pGEM-T Easy Vector (Promega) for Sanger sequencing (Mix2Seq Kit, Eurofins). 4 to 5 clones per PCR were sequenced.

Behavioral examinations

For behavioral testing, 8- to 11-day-old *dsx*$^{hM4Di/+}$ or wt worker bees were collected the evening before the experiment. Each bee was harnessed in a small plastic tube, with its head protruding, and its antennae, mandibles, and proboscis free to move (Bitterman et al., 1983). The bees were fed to satiation with 0.88 M sucrose (Felsenberg et al., 2011) and kept harnessed at room temperature with high humidity overnight . Two hours prior to the experiment, bees received 2x 4 µl 0.88 M sucrose solution. To test the QMP response we used small glass cylinder (pseudo-queens) (Kaminski et al., 1990; Okosun et al., 2019). The control pseudo-queen contained 100 % isopropanol and the QMP pseudo-queen contained 0.001 queen equivalent (Qeq; Slessor et al., 1988). Both pseudo-queens were prepared 35 minutes before testing by applying 10 µl of the test chemical components to them and incubated in the fridge, which allows that the solvent isopropanol can evaporate (Kaminski et al., 1990). Experimental groups of 5 to 12 genetically identical bees were fed 2 µl of 0.88 M

sucrose solution with or without 56.6 µM (≈ 0.4 mg/kg) C21 30 minutes before the experiment and were placed in a small cage containing pollen paste and water. After 15 minutes the group of bees were introduced to the center of a 14 cm diameter polystyrene petri dish arena (VWR, International GmbH, Darmstadt, Germany) with 8 ventilation holes in the side walls and 3 in the lid. The arena was placed on a gridded paper with 1.5 cm² squares After 3 minutes, the control pseudo-queen was presented for 3 minutes, followed by a one-minute break and the QMP pseudo-queen was presented for 3 minutes. To document the worker bee behavior, we counted line crossings (LC) during both pseudo-queen presentations. Only bees crossing at least seven grid lines during the first 3 minutes were included in the analysis. Arenas and the paper with the grid were replaced by a new one, once another bee was examined. Behaviors were video recorded (60 fps, Full HD, 44100 Hz; Casio Exilim Pro EX-F1) and analyzed using VSDC Free Video Editor (Multilab LLC, https://www.videosoftdev.com/). The samples were blinded in respect to the treatment and the observer had no knowledge about the genotype of the bee.

Data analysis and statistics

Statistical analysis was performed using IBM SPSS Statistics 27 software (IBM, Armonk, USA). Mann Whitney U test was used for pairwise comparison.

Results

We integrated the *hM4Di* receptor nucleotide coding sequence upstream of the translational start codon of the *dsx* gene into the honeybee genome using the CRISPR/Cas9 method (Figure 1C). To do so, we injected the *dsx*-sgRNA1, the DNA fragment and the Cas9 protein into embryos, reared queens and produced $dsx^{hM4Di/+}$ worker bees (Figure 1D) (Roth et al., 2019; Sommer et al., 2024; Wagner et al., 2022). The *dsx* gene is spatially restricted expressed in the nervous system establishing a test case to locally express the *hM4Di* receptor protein in the nervous system via the endogenous *dsx* promoter (Sommer et al., 2024). The endopeptidase P2A (Figure 1C) which is also encoded in the integrated sequence directs the separation into hM4Di receptor and the Dsx^F protein (Szymczak-Workman et al., 2012).

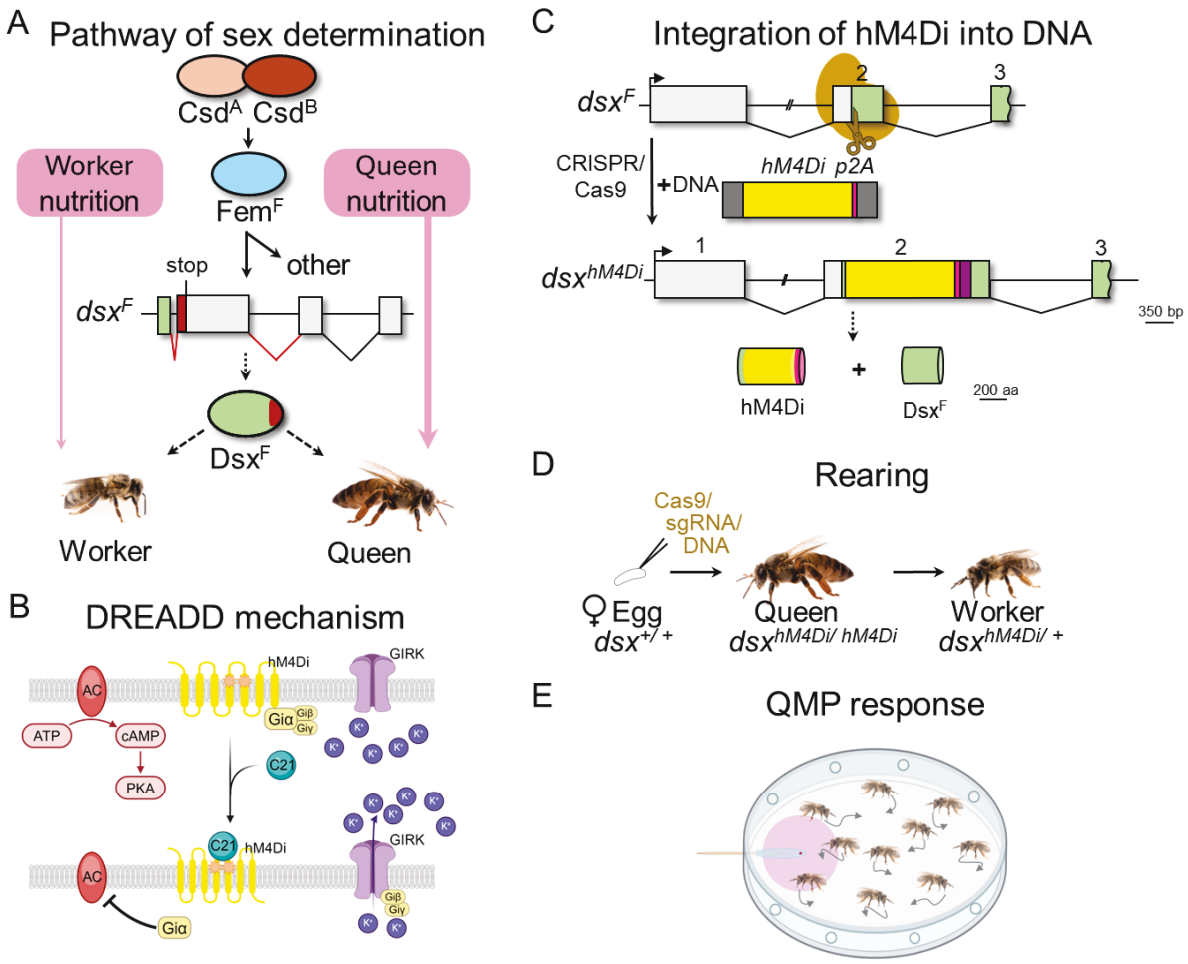


Figure 1. The generation and behavioral examination of $dsx^{hM4Di/+}$ worker bees. A. Caste-determining pathway of honeybee females: Heterozygous Csd and worker nutrition determine worker characteristics, including the worker-specific ovary and behavior. ^{F} and red represent female-specific regulated products. **B.** Scheme on the hM4Di-mediated neuronal inhibition. C21 ligand binding leads to conformational change and activation of the Gi protein. Gi-β/γ complex

activates GIRK channels (potassium ion outflux) and Giα inhibits adenylate cyclase (reduction of cAMP and PKA activity). This results in hyperpolarisation of the cell and reduced neuronal activity. **C.** Targeted integration of the *hM4Di* nucleotide coding sequence into the *dsx* locus. **D.** The generation of $dsx^{hM4Di/+}$ worker bees. Female embryos were injected and reared to queens. Biallic $dsx^{hM4Di/hM4Di}$ queens were inseminated with wildtype drones to produce heterozygous $dsx^{hM4Di/+}$ worker bees. **E.** Locomotion response of worker bees to QMP mimicking blend (QMP*; pink) in the behavioral assay.

PCR-based genotyping showed that of the 63 reared queens 12 (19 %) were homozygous ($dsx^{hM4Di/hM4Di}$), 16 (25 %) were hemizygous ($dsx^{hM4Di/wt}$) for the integration, whereas 35 (56 %) were wildtype ($dsx^{wt/wt}$) suggesting the general feasibility of this integration approach (Table 1). We isolated the mRNA from the $dsx^{hM4Di/+}$ worker bees and determined the nucleotide sequence of the transcript that derived from the integrated sequence.

Table 1. The integration rates of the hM4Di coding DNA fragment into the *dsx* gene.

	No. of queens with		
Genotype	$dsx^{hM4Di/hM4Di}$	$dsx^{hM4Di/wt}$	$dsx^{wt/wt}$
Frequency % (n)	19,0 % (12/63)	25,4 % (16/63)	55,6 % (35/63)

We found the sequence matched the designed sequence and integration site and the anticipated splicing establishing in frame co-expression of hM4Di protein and Dsx protein (Figure S2). We found a natural polymorphism in the non-coding region of exon 2 suggesting that we have sequenced both dsx^{hM4Di} alleles. In the sequence we further found two alternative splice variants that employ an alternative splice donor site (upstream of 18 bp leading to a shorter transcript in *dsx* exon 2 and one alternative splice acceptor site (upstream of 70 bp leading to a longer transcript) in *dsx* exon 3 (Figure S2). These results suggest that our integration of hM4Di receptor sequence into the *dsx* locus produced a functional mRNA encoding both a full length hM4Di and Dsx^F protein controlled from the endogenous *dsx* promoter.

Dsx^F protein is highly spatially restricted expressed in the nervous system and is required for the selection and maintenance of worker-specific behaviors (Sommer et

al., 2024). We next examined whether the C21 drug-based activation of hM4Di receptor protein in the *dsx* expressing cells affects the worker bee responses to QMP.

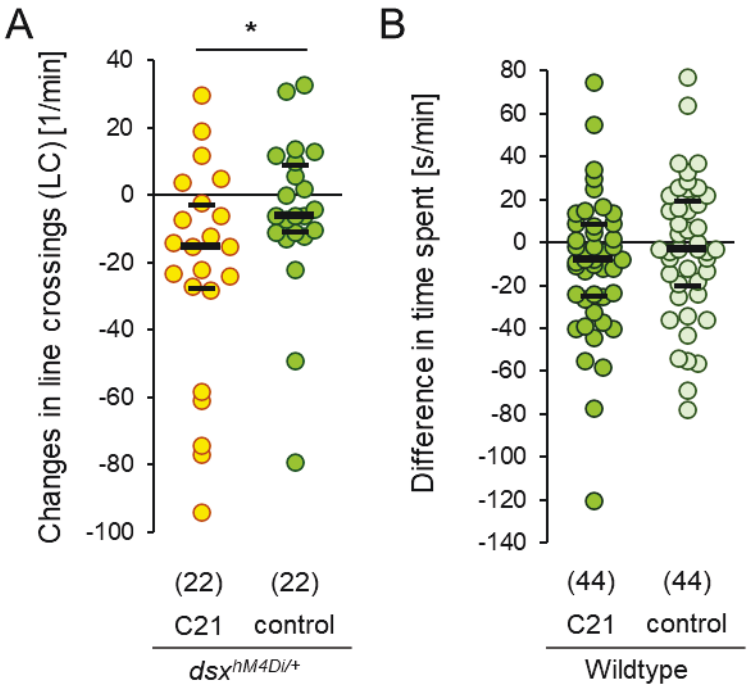


Figure 3. Queen mandibular pheromone (QMP)-induced changes in worker bee locomotion activity. Changes in line crossing (LC) in response to QMP* (* stands for a blend which provides main components of the queen's QMP) relative to solvent isopropanol response. **A.** Comparison of $dsx^{hM4Di/+}$ workers with (median: -15 LC) versus without C21 treatment (median: -5 LC; P = 0.026, z = -2.219; Mann-Whitney-U-test (MWU test). **B.** Comparison of wildtype worker bees with (median: -8 LC) versus without C21 (median: -3 LC; P = 0.563, z = -0.573; MWU test).

Worker bees usually change their locomotion activity in response to QMP* (* stands for a blend which provides main components of the queen's QMP) (Beggs et al., 2007; Keeling et al., 2003; Le Conte & Hefetz, 2008). Indeed, previous work showed that QMP* stimuli provided via a QMP strip, which are used by beekeepers to replace queens, elicit a reduction of locomotion behavior (Beggs et al., 2007). We measured locomotion in response to QMP* in groups of ≥ 7 of worker bees in terms of lines that were crossed (LC). By sampling (n= 2 to 7) worker bees across the ≥ 6 biological replicates we determined the locomotion differences before and after providing QMP* which is the locomotion response to QMP* (Figure 3). One group of $dsx^{hM4Di/+}$ worker bees were fed 2 µl sucrose solution with the drug (C21 condition) and the other one only sucrose solution (no drug control condition) and this 30 minutes before the behavioral experiment. We observed that the $dsx^{hM4Di/+}$ worker bees under drug C21 condition significantly changed their behaviors in response to QMP* as compared to

control *dsx*$^{hM4Di/+}$ worker bees that have not obtained the drug (P = 0.026, z = -2.219, MWU-test). The locomotion was thereby reduced from -5 LC to -15 upon C21 treatment. To exclude that the C21 treatment alone induced this change in QMP* response, we next examined the behaviors of the wt worker bees under drug C21 and no such (control) condition. We observed no locomotion differences of the wt worker bee under the C21 drug and control condition (P = 0.874, z = -0.159, MWU test, Figure 3B, Table S4) suggesting that the drug C21 alone has no such effect on behavior in our QMP* response assay. Collectively, these results showed that specifically the combination of C21 and *dsx*$^{hM4Di/+}$ worker bees directed a further behavioral change in response to QMP* which is not found in the wt worker bees/ drug and *dsx*$^{hM4Di/+}$ worker bees/ no drug condition. We conclude that the chemogenetic system, which requires the combination of the drug C21 and the hM4Di receptor, can conditionally control behaviors in the worker bees.

Discussion

Worker bee behaviors can selectively and conditionally be controlled by chemogenetic tools

The worker bee's nervous system controls a rich and remarkable behavioral repertoire that is a hallmark of the honeybees' sophisticated social organization. However, manipulation tools that can functionally examine the role of specific neural circuitries in the control of the worker bee behaviors are so far lacking. Here, we expressed the hM4Di receptor under control of the endogenous *dsx* promoter. We showed that hM4Di receptor was knocked in into the *dsx* locus in front of the *dsx* coding sequence and produced a functional mRNA with a single open reading frame suggesting expression under the *dsx* promoter. These knock-in alleles produced in homozygous condition no mutant phenotype in queens and are thus leave the *dsx* locus functional because homozygous *dsx* mutants show an intersexual phenotype in the reproductive system, which would not lead to a production of eggs (Roth et al., 2019; Sommer et al., 2024). Unfortunately, we were not able to detect hM4Di receptor protein expression via Anti-hM4Di immunostainings possibly because the protein shows a low abundance in these cells. Previous work showed that the *dsx* gene is highly spatially restricted expressed in the adult nervous system defining circuitries that at least include KCs of the MB (those that appear as an uppermost layer (class I) and a small layer in the ventral part (class II) in the vertical lobe (VL)), the sensory tract 1 (T1) of the antennal nerve and the cortical regions of the olfactory glomeruli in the antennal lobe (AL) (Sommer et al., 2024).

The drug C21 we fed to the worker bees should change the conformation of the hM4Di receptor and thereby activate the Gi-protein coupled signal transduction. This signaling mediates a decreases Ca^{2+} entry and increases outward K^+ currents which results in hyperpolarization of neuronal cells and the silencing of their activity (Atasoy & Sternson, 2018). We found that specifically under this neuronal silencing/drug C21 condition the $dsx^{hM4Di/+}$ worker bees changed their response to QMP* stimulus suggesting that the behaviors were specifically changed if the *dsx* expressing cells were silenced. This change was specific to the hyperpolarizing/silencing condition because this behavioral change was not found in either in the absence of the drug C21 in $dsx^{hM4Di/+}$ worker bees or the presence of drug C21 in wt bees that lacked the expression of the hM4Di receptor. Hence, we conclude that worker bee behaviors can

be selectively and conditionally examined by manipulating subset of cells, in which the chemogenetic system is spatially expressed and can be activated. In the case of QMP* behavioral response of C21/*dsx*$^{hM4Di/+}$ worker bees it appears that the silencing of *dsx* expressing cells enhanced the locomotion response to QMP not seen in wt worker bees (Beggs et al., 2007)., suggesting a possible enhancing function of the dsx circuitry inhibition on QMP response.

The high rate of integration of the coding sequence into the *dsx* locus (40% of queens carried the transgene) highlights the general feasibility of the technique suggesting that the chemogenetic system can be applied more generally to other genes and circuitries. Driving spatially restricted expressions of the chemogenetic system from other known cell type-specific marker genes (Kuwabara et al., 2023; Suenami et al., 2016; Zhang et al., 2022) or developmental genes (Sommer et al., 2024) has the great potential to examine the role of defined neuronal populations in the control of worker-specific behaviors including those involving learning and memory (Bitterman et al., 1983; Giurfa, 2003; Komischke et al., 2005; Kuwabara et al., 2023; Scheiner et al., 2013)

Conditional silencing via the chemogenetic system by feeding the drug C21 has the great advantage that is enable the examination of neural circuitry function in the context of the colony (Blut et al., 2017; Sommer et al., 2024), a condition that is hardly accessible to optogenetic tools (Duebel et al., 2015; Pulver et al., 2011). Also in the context of single bee behavioral assays that include learning processes (Bitterman et al., 1983; Giurfa, 2013; Menzel, 2012; Scheiner et al., 2013), optogenetic tools may have their limitations due to the requirement that light has to penetrate the cuticula and tissues (Ehmann & Pauls, 2020; Kohsaka & Nose, 2021). The repertoire of well-established other receptor variants expands the possibility to not only silencing but also activating specific circuitries (Armbruster et al., 2007; Becnel et al., 2013; Roth, 2016). When combined with different concentrations of the drug C21, the circuitry can gradually be silenced or activated which further expands the repertoire of functional tests that can be employed. This broad perspective suggests that we now have methods in our hand to functionally dissect the control of remarkable bee behaviors at the level of neural circuitry.

Acknowledgments

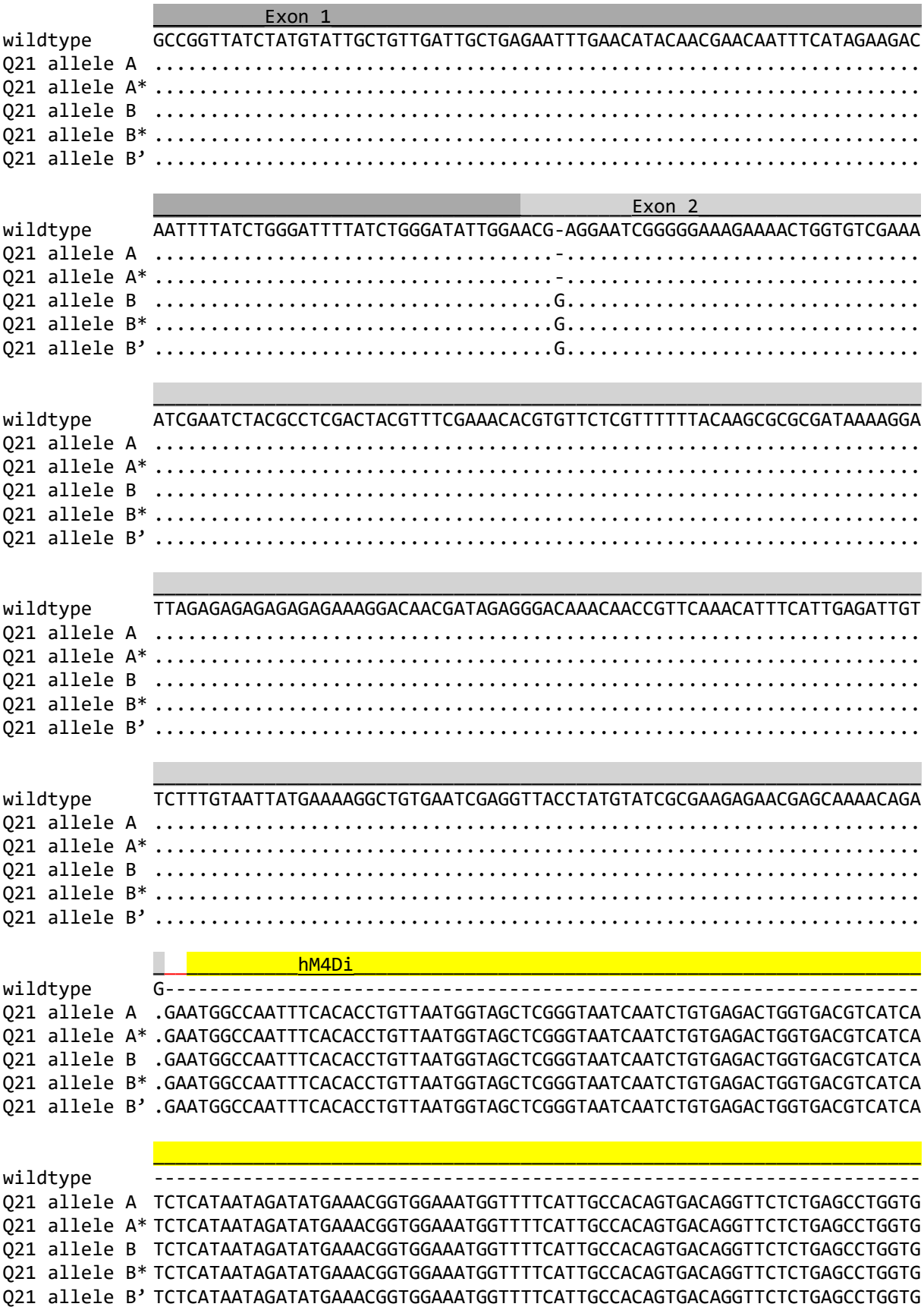
We thank Marion Müller-Borg for her assistance with bee handling and analysis support. We thank Michael Griese for providing bee colonies. We thank Pia Hebar and Sina Becker for supporting the laboratory work.

Supporting data

Table S1. Nucleotide sequence of the *dsx*-sgRNA1 and DREADD-hM4Di DNA fragment.

dsx-sgRNA1 1)	**GAACGAGCAAAACAGAGCCG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAU CAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
DREADD-hM4Di DNA fragment 2)	GTTGCAGAACGAGGAATCGGGGGAAAGAAAACTGGTGTCGAAAATCGAATCTACGCCTCGACTACG TTTCGAAACACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGATTAGAGAGAGAGAGAGAAAG GACAACGATAGAGGGACAAACAACCGTTCAAACATTTCATTGAGATTGTTCTTTGTAATTATGAAA AGGCTGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACAGAGGAATGGCCAATTT CACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCATCTCATAATAG ATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTGACTGTGGT GGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAATTACTT CCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTGTA CATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGT GGTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAA ACCTCTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGT ACTGTCTTTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGT GCCAGACAATCAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGC TGGTTTCTACCTGCCTGTGGTGATCATGACGGTGCTGTACATCCATATCTCTCTGGCCAGTAGAAG CCGAGTTCATAAACATAGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAA AAGCCCATTGATGAAACAAAGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAG AAATGGTAAACTGGAAGAAGCCCCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAA AGACACTTCTAATGAATCTAGCTCAGGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGA ACTGTCTACCACAGAAGCCACCACGCCAGCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAA TCCAGCCTCTAGATGGTCTAAAATCCAAATTGTGACGAAACAAACAGGTAATGAATGTGTGACAGC CATTGAAATTGTGCCTGCCACGCCGGCTGGTATGAGACCTGCGGCCAATGTGGCCAGAAAATTCGC CAGCATCGCTAGAAATCAAGTGAGAAAAAAAAGACAAATGGCGGCCAGAGAAAGAAAAGTGACACG AACGATCTTTGCCATTCTGCTGGCCTTCATCCTCACCTGGACGCCATACAATGTGATGGTTCTGGT GAATACCTTCTGCCAAAGCTGCATCCCTGACACGGTGTGGTCTATTGGTTACTGGCTCTGCTACGT CAATAGCACCATCAATCCTGCCTGCTATGCTCTGTGCAATGCCACCTTTAAAAAAACCTTCAGACA TCTGCTGCTGTGCCAATATAGAAATATCGGTACTGCCAGAAGATCTGGAGATAATGGATCTGGTGC TACAAATTTCTCTTTGTTAAAACAAGCGGGAGATGTGGAAGAAAATCCAGGTCCTGAACAAAAACT CATCTCGGAGGAGGATCTGGAGCAAAAGTTGATATCCGAGGAAGACCTCGAACAAAAGCTGATTTC GGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATCTCCGAAGA GGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAGCCGCGGACTTGGCTCCCCAACAACCGAG TGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCCAA GAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACG ATGTCTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTG TACCTGCGAGAAGTGTAAGATCA

1) bold letters indicate the target site in the genome.
2) *dsx* gene homologous sequence are shown in gray boxes, hM4Di coding sequence in yellow box, 2A peptide (P2A) coding sequence in pink box, 5x Myc tag in purple box and the GSG linker in blue box. The first two start codons are underlined. To maintain the open reading frame and the *dsx* gene, nucleotides were inserted (red letters). Codon usages were optimized for the honeybee.


Exon 1
wildtype GCCGGTTATCTATGTATTGCTGTTGATTGCTGAGAATTTGAACATACAACGAACAATTTCATAGAAGAC
Q21 allele A .....
Q21 allele A* .....
Q21 allele B .....
Q21 allele B* .....
Q21 allele B' .....
Exon 2
wildtype AATTTTATCTGGGATTTTATCTGGGATATTGGAACG-AGGAATCGGGGGAAAGAAAACTGGTGTCGAAA
Q21 allele A .....-.....
Q21 allele A* .....-.....
Q21 allele B .....G.....
Q21 allele B* .....G.....
Q21 allele B' .....G.....
wildtype ATCGAATCTACGCCTCGACTACGTTTCGAAACACGTGTTCTCGTTTTTTACAAGCGCGCGATAAAAGGA
Q21 allele A .....
Q21 allele A* .....
Q21 allele B .....
Q21 allele B* .....
Q21 allele B' .....
wildtype TTAGAGAGAGAGAGAGAAAGGACAACGATAGAGGGACAAACAACCGTTCAAACATTTCATTGAGATTGT
Q21 allele A .....
Q21 allele A* .....
Q21 allele B .....
Q21 allele B* .....
Q21 allele B' .....
wildtype TCTTTGTAATTATGAAAAGGCTGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACAGA
Q21 allele A .....
Q21 allele A* .....
Q21 allele B .....
Q21 allele B* .....
Q21 allele B' .....
hM4Di
wildtype G----------
Q21 allele A .GAATGGCCAATTTCACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCA
Q21 allele A* .GAATGGCCAATTTCACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCA
Q21 allele B .GAATGGCCAATTTCACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCA
Q21 allele B* .GAATGGCCAATTTCACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCA
Q21 allele B' .GAATGGCCAATTTCACACCTGTTAATGGTAGCTCGGGTAATCAATCTGTGAGACTGGTGACGTCATCA
wildtype ----------
Q21 allele A TCTCATAATAGATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTG
Q21 allele A* TCTCATAATAGATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTG
Q21 allele B TCTCATAATAGATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTG
Q21 allele B* TCTCATAATAGATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTG
Q21 allele B' TCTCATAATAGATATGAAACGGTGGAAATGGTTTTCATTGCCACAGTGACAGGTTCTCTGAGCCTGGTG

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                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    ACTGTGGTGGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAAT
Q21 allele A*   ACTGTGGTGGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAAT
Q21 allele B    ACTGTGGTGGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAAT
Q21 allele B*   ACTGTGGTGGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAAT
Q21 allele B'   ACTGTGGTGGGTAATATCCTGGTGATGCTGTCTATCAAAGTTAATAGACAACTGCAAACAGTGAATAAT

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    TACTTCCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTG
Q21 allele A*   TACTTCCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTG
Q21 allele B    TACTTCCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTG
Q21 allele B*   TACTTCCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTG
Q21 allele B'   TACTTCCTCTTCAGCCTGGCGTGTGCTGATCTCATCATAGGTGCCTTCTCTATGAATCTCTACACCGTG

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    TACATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGTG
Q21 allele A*   TACATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGTG
Q21 allele B    TACATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGTG
Q21 allele B*   TACATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGTG
Q21 allele B'   TACATCATCAAAGGTTACTGGCCACTGGGTGCCGTGGTTTGCGACCTGTGGCTGGCCCTGGACTGCGTG

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    GTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAAACCT
Q21 allele A*   GTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAAACCT
Q21 allele B    GTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAAACCT
Q21 allele B*   GTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAAACCT
Q21 allele B'   GTGAGCAATGCCTCTGTGATGAATCTTCTCATCATCAGCTTTGACAGATACTTCTGCGTTACCAAACCT

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    CTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGTACTGTCT
Q21 allele A*   CTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGTACTGTCT
Q21 allele B    CTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGTACTGTCT
Q21 allele B*   CTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGTACTGTCT
Q21 allele B'   CTCACCTACCCTGCCAGAAGAACCACCAAAATGGCAGGTCTCATGATTGCTGCTGCCTGGGTACTGTCT

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    TTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGTGCCAGACAAT
Q21 allele A*   TTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGTGCCAGACAAT
Q21 allele B    TTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGTGCCAGACAAT
Q21 allele B*   TTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGTGCCAGACAAT
Q21 allele B'   TTCGTGCTCTGGGCGCCTGCCATCTTGTTCTGGCAATTTGTGGTGGGTAAAAGAACGGTGCCAGACAAT

                ____________________________________________________________
wildtype        ------------------------------------------------------------
Q21 allele A    CAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGCTGGTTTCTACCTG
Q21 allele A*   CAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGCTGGTTTCTACCTG
Q21 allele B    CAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGCTGGTTTCTACCTG
Q21 allele B*   CAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGCTGGTTTCTACCTG
Q21 allele B'   CAATGCTTCATCCAATTCCTGTCTAATCCAGCAGTGACCTTTGGTACAGCCATTGCTGGTTTCTACCTG
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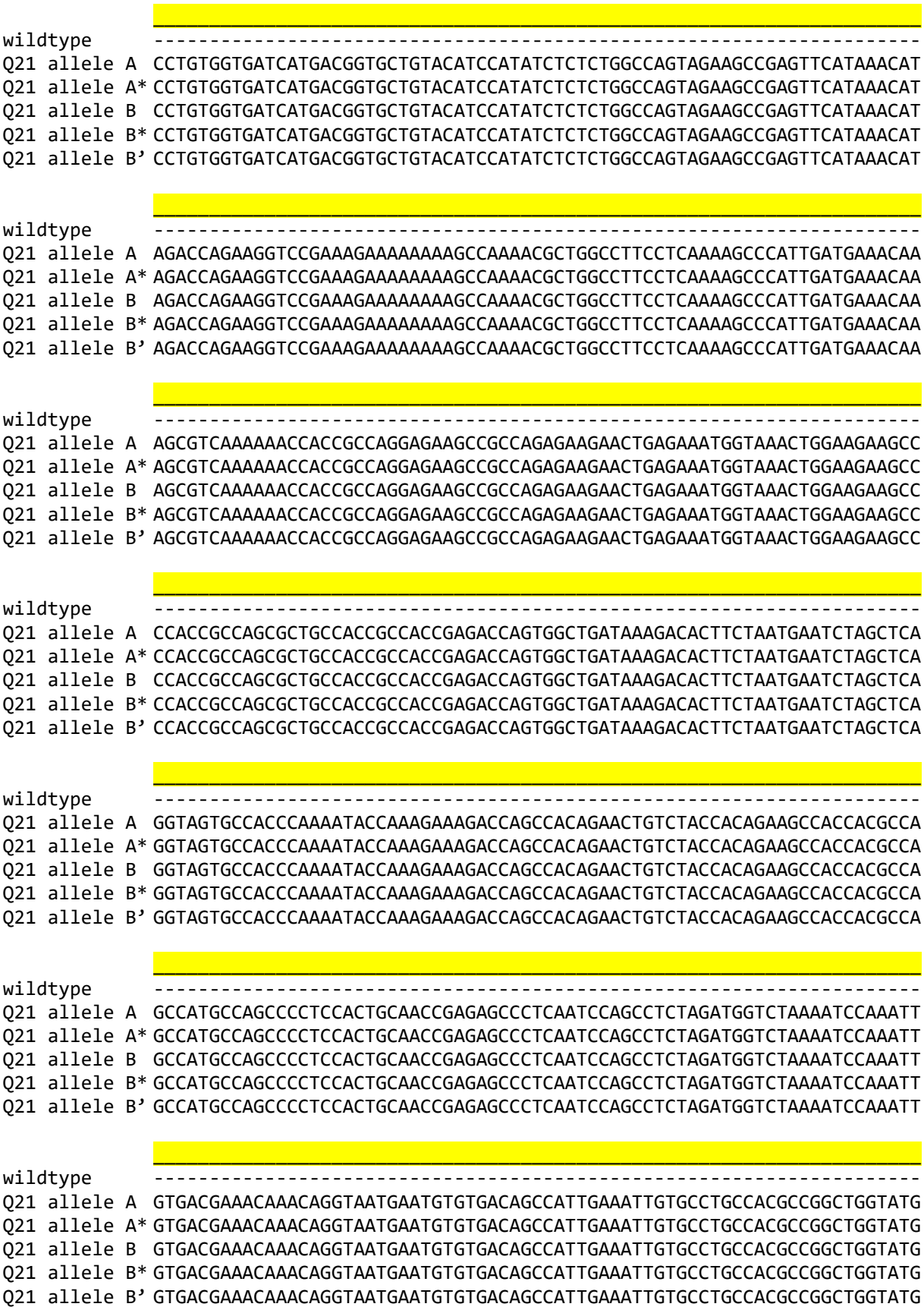
wildtype ----------
Q21 allele A CCTGTGGTGATCATGACGGTGCTGTACATCCATATCTCTCTGGCCAGTAGAAGCCGAGTTCATAAACAT
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Q21 allele B CCTGTGGTGATCATGACGGTGCTGTACATCCATATCTCTCTGGCCAGTAGAAGCCGAGTTCATAAACAT
Q21 allele B* CCTGTGGTGATCATGACGGTGCTGTACATCCATATCTCTCTGGCCAGTAGAAGCCGAGTTCATAAACAT
Q21 allele B' CCTGTGGTGATCATGACGGTGCTGTACATCCATATCTCTCTGGCCAGTAGAAGCCGAGTTCATAAACAT
wildtype ----------
Q21 allele A AGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAAAAGCCCATTGATGAAACAA
Q21 allele A* AGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAAAAGCCCATTGATGAAACAA
Q21 allele B AGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAAAAGCCCATTGATGAAACAA
Q21 allele B* AGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAAAAGCCCATTGATGAAACAA
Q21 allele B' AGACCAGAAGGTCCGAAAGAAAAAAAAGCCAAAACGCTGGCCTTCCTCAAAAGCCCATTGATGAAACAA
wildtype ----------
Q21 allele A AGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAGAAATGGTAAACTGGAAGAAGCC
Q21 allele A* AGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAGAAATGGTAAACTGGAAGAAGCC
Q21 allele B AGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAGAAATGGTAAACTGGAAGAAGCC
Q21 allele B* AGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAGAAATGGTAAACTGGAAGAAGCC
Q21 allele B' AGCGTCAAAAAACCACCGCCAGGAGAAGCCGCCAGAGAAGAACTGAGAAATGGTAAACTGGAAGAAGCC
wildtype ----------
Q21 allele A CCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAAAGACACTTCTAATGAATCTAGCTCA
Q21 allele A* CCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAAAGACACTTCTAATGAATCTAGCTCA
Q21 allele B CCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAAAGACACTTCTAATGAATCTAGCTCA
Q21 allele B* CCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAAAGACACTTCTAATGAATCTAGCTCA
Q21 allele B' CCACCGCCAGCGCTGCCACCGCCACCGAGACCAGTGGCTGATAAAGACACTTCTAATGAATCTAGCTCA
wildtype ----------
Q21 allele A GGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGAACTGTCTACCACAGAAGCCACCACGCCA
Q21 allele A* GGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGAACTGTCTACCACAGAAGCCACCACGCCA
Q21 allele B GGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGAACTGTCTACCACAGAAGCCACCACGCCA
Q21 allele B* GGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGAACTGTCTACCACAGAAGCCACCACGCCA
Q21 allele B' GGTAGTGCCACCCAAAATACCAAAGAAAGACCAGCCACAGAACTGTCTACCACAGAAGCCACCACGCCA
wildtype ----------
Q21 allele A GCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAATCCAGCCTCTAGATGGTCTAAAATCCAAATT
Q21 allele A* GCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAATCCAGCCTCTAGATGGTCTAAAATCCAAATT
Q21 allele B GCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAATCCAGCCTCTAGATGGTCTAAAATCCAAATT
Q21 allele B* GCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAATCCAGCCTCTAGATGGTCTAAAATCCAAATT
Q21 allele B' GCCATGCCAGCCCCTCCACTGCAACCGAGAGCCCTCAATCCAGCCTCTAGATGGTCTAAAATCCAAATT
wildtype ----------
Q21 allele A GTGACGAAACAAACAGGTAATGAATGTGTGACAGCCATTGAAATTGTGCCTGCCACGCCGGCTGGTATG
Q21 allele A* GTGACGAAACAAACAGGTAATGAATGTGTGACAGCCATTGAAATTGTGCCTGCCACGCCGGCTGGTATG
Q21 allele B GTGACGAAACAAACAGGTAATGAATGTGTGACAGCCATTGAAATTGTGCCTGCCACGCCGGCTGGTATG
Q21 allele B* GTGACGAAACAAACAGGTAATGAATGTGTGACAGCCATTGAAATTGTGCCTGCCACGCCGGCTGGTATG
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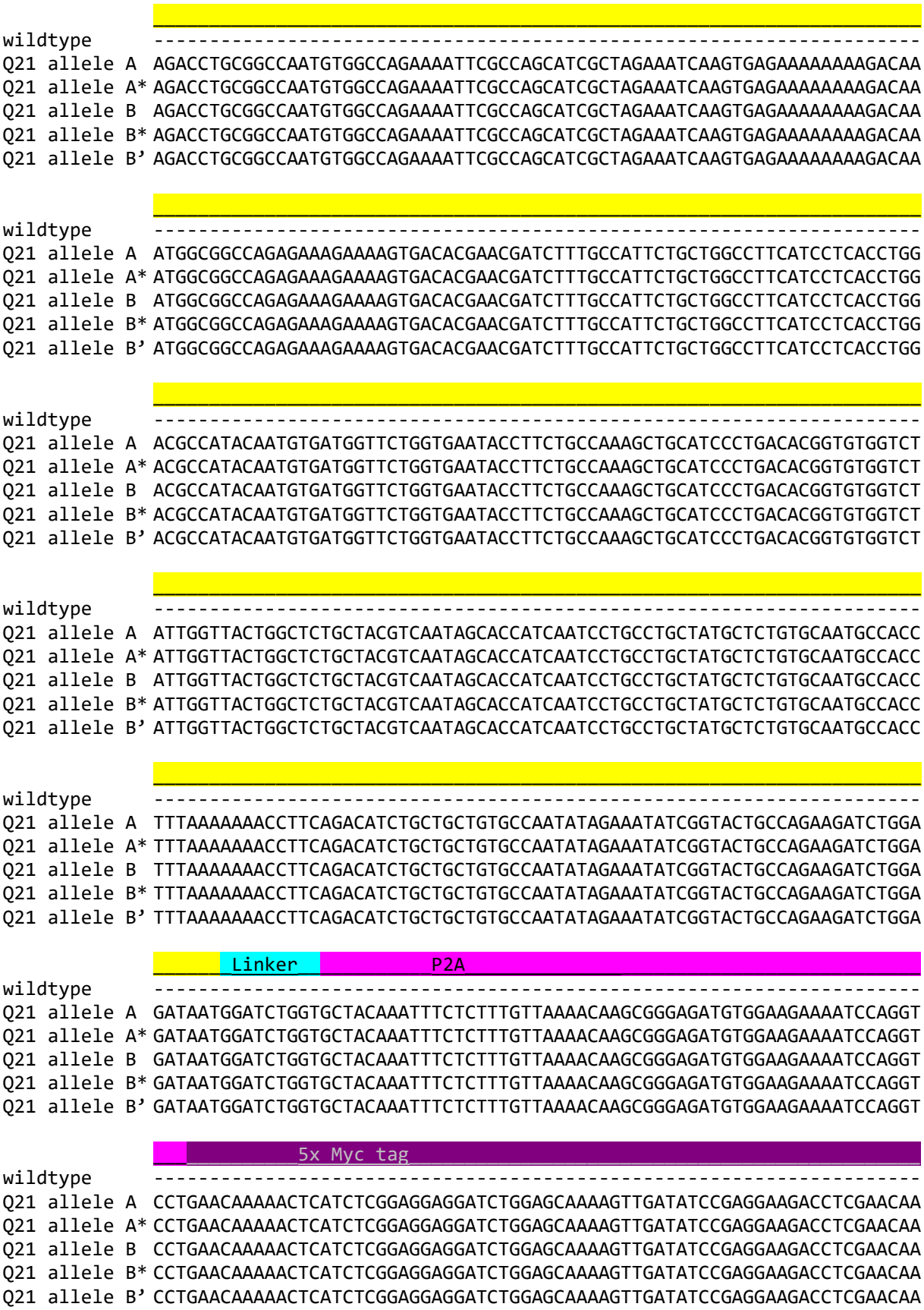
wildtype
Q21 allele A
Q21 allele A*
Q21 allele B
Q21 allele B*
Q21 allele B'
Linker
P2A
5x Myc tag

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wildtype      ---------------------------------------------------------------------
Q21 allele A  AAGCTGATTTCGGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATC
Q21 allele A* AAGCTGATTTCGGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATC
Q21 allele B  AAGCTGATTTCGGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATC
Q21 allele B* AAGCTGATTTCGGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATC
Q21 allele B’ AAGCTGATTTCGGAAGAAGATTTGGAGCAAAAATTGATCAGCGAGGAGGATCTCGAGCAAAAACTGATC

                             Maintenance: dsx gene                 Exon 2
wildtype      ----------------------------------------------CCGCGGACTTGGCTCCCCAACAA
Q21 allele A  TCCGAAGAGGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAG.......................
Q21 allele A* TCCGAAGAGGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAG.......................
Q21 allele B  TCCGAAGAGGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAG.......................
Q21 allele B* TCCGAAGAGGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAG.......................
Q21 allele B’ TCCGAAGAGGACTTGATGTACAGAGAGGAAAATGAACAGAATCGAG.......................

wildtype      CCGAGTGGTGCAAACACGTTCGAGCGTTTGGAACATTCTCAGGATAGCAAAAATGGGGACGATGGTCCC
Q21 allele A  .....................................................................
Q21 allele A* .....................................................................
Q21 allele B  .....................................................................
Q21 allele B* .....................................................................
Q21 allele B’ .....................................................................

wildtype      AAGAAGGTGCAAACAGACGCTTCCTCTTCGACTAATACTCCAAAGCCGCGTGCACGGAATTGTGCACGA
Q21 allele A  .....................................................................
Q21 allele A* .....................................................................
Q21 allele B  .....................................................................
Q21 allele B* .....................................................................
Q21 allele B’ .....................................................................

wildtype      TGTCTGAATCATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTACC
Q21 allele A  .....................................................................
Q21 allele A* .....................................................................
Q21 allele B  .....................................................................
Q21 allele B* .....................................................................
Q21 allele B’ .....................................................................

wildtype      TGCGAGAAGTGTAAGATCACTGCCAATCGGCAGCAAGTGATGCGGCAGAATATGAAGCTGAAAAGACAC
Q21 allele A  .....................................................................
Q21 allele A* .....................................................................
Q21 allele B  .....................................................................
Q21 allele B* .....................................................................
Q21 allele B’ .....................................................................

wildtype      CTGGCACAGGATAAAGTCAAAGTAAGAGTAGCGGAAGAG------------------------------
Q21 allele A  .......................................------------------------------
Q21 allele A* .....................------------------------------------------------
Q21 allele B  .......................................------------------------------
Q21 allele B* .....................------------------------------------------------
Q21 allele B’ .......................................GTTAGTCTTTTCCTTGTTTCATAATGGTTT
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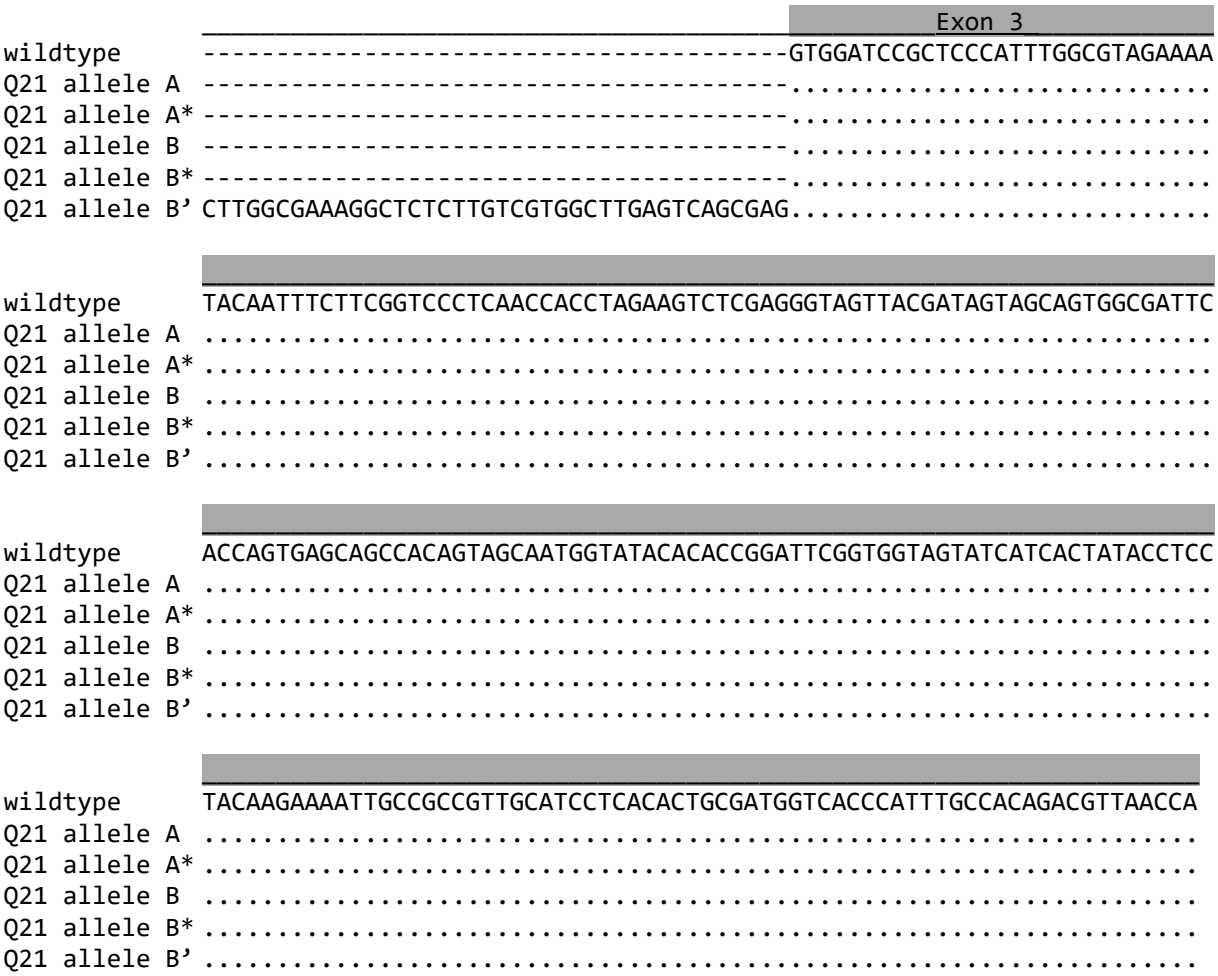

Figure S2: dsx locus with integrated hM4Di DNA. Sequencing of the queen allele of the dsx$^{hM4Di/hM4Di}$ mutant queen Q21 using cDNA from offspring. 2 different queen alleles are identified (A and B): one with an insertion of a guanine (G) in the 5' UTR at the beginning of exon 2 (B). All offspring have an additional splice donor (18 bp upstream; A* and B*). One other polymorphism was identified in allele B: insertion of 70 bp between exon 2 and 3 (B'). *dsx* exon sequence are shown in gray boxes, hM4Di coding sequence in yellow box, 2A peptide (P2A) coding sequence in pink box, 5x Myc tag in purple box and the GSG linker in blue box. To maintain the open reading frame and the *dsx* gene, nucleotides were inserted (red). Dot means some nucleotide and minus means deletion.

Table S3. The time by the *dsx*^{hM4Di} hive reared worker bees with C21 (+) and without C21 (-) spent in the 4 cm radius of the pseudo-queen.

SD = standard deviation. MWU = Mann-Whitney-U-test.

	dsx$^{hM4Di/+}$ + C21 (n = 22)			*dsx*$^{hM4Di/+}$ - C21 (n = 22)			MWU	
	Median	Mean	± SD	Median	Mean	± SD	p-value	z
Time spent [s/min]	-15.00	- 22.23	± 31.54	- 6.00	- 5.23	± 23.41	0.026	- 2.219

Table S4. The time by the wildtype (wt) hive reared worker bees with C21 (+) and without C21 (-) spent in the 4 cm radius of the pseudo-queen.

SD = standard deviation. MWU = Mann-Whitney-U-test.

	Wt + C21 (n = 44)			Wt - C21 (n = 44)			MWU	
	Median	Mean	± SD	Median	Mean	± SD	p-value	z
Time spent [s/min]	- 8.00	- 10.36	± 32.86	- 3.00	- 20.25	± 32.52	0.563	- 0.573

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Author's contribution: Manuscript III

Conditional control worker honeybee behaviors via *dsx* locus expressed chemogenetic tool

1st author

Author's contribution to Manuscript I

- Concept of study
- Experimental design
- sgRNA synthesis
- Implementation of microinjections
- Queen rearing and bee handling
- Genotyping of mutant honeybees
- Sensorimotor assays
- Data analyses: sequencing data, statistics
- Authoring the manuscript

Kapitel III

Zusammenfassung

Eine Entwicklung von einfacher Aggregation zu hochkomplexen Systemen mit kooperativem Verhalten resultiert in einer bemerkenswerten Vielfalt an sozialen Strukturen innerhalb des Tierreichs. Eine extreme Form dieses Verhaltens ist die sogenannte Eusozialität, die sich durch die Ausführung sämtlicher Aktivitäten zum Wohle des Kollektivs auszeichnet. Hierzu zählen unter anderem die Verteidigung gegen Fressfeinde und die Aufzucht der Nachkommen. Die Honigbiene *Apis mellifera* ist ein Beispiel für eine solche eusoziale Art, da sie sich durch eine Arbeitsteilung, ein reiches Verhaltensrepertoire und ausgefeilte Kommunikationsfähigkeiten der Arbeiterinnen auszeichnet. Die Frage, wie solche angeborenen Verhaltensweisen im Gehirn der Arbeiterinnen während ihrer Entwicklung festgelegt werden, ist bisher ungeklärt. Für diese Entwicklungsspezifikation sind Transkriptionsfaktoren von großer Bedeutung, da sie aufgrund ihrer Affinität zur Bindung an DNA die Aktivität einer Vielzahl nachfolgender Gene beeinflussen können, die für die finale Differenzierung von essentieller Bedeutung sind. In *Drosophila melanogaster* hat sich das Gen *doublesex* (*dsx*) als ein solcher herausgestellt. In dieser Arbeit wird berichtet, welche Rolle dieser hochkonservierte Transkriptionsfaktor in Bezug auf soziales Verhalten in der Honigbiene spielt. Mittels des CRISPR/Cas9-Systems konnte die funktionale Zinkfingerdomäne des Gens *dsx* eliminiert werden. Anschließend wurde das Verhalten von Arbeiterinnen mit dieser Mutation in der sozialen Umgebung eines kleinen Bienenvolkes mithilfe computergestützter Verfolgung analysiert. Dabei wurde festgestellt, dass *dsx* essenziell für die Programmierung von gruppenunterstützenden Verhaltensweisen bei Arbeiterbienen ist. Es beeinflusst die Rate und Dauer von Aufgaben wie Zellinspektionen, Larvenfütterung und Futteraustausch, ohne die allgemeinen sensorischen oder motorischen Funktionen zu beeinträchtigen. Zudem zeigten anatomische Untersuchungen der Gehirne dieser Arbeiterinnen, dass das *dsx*-Gen die neuronale Entwicklung, insbesondere im Bereich der Pilzkörper, die die multisensorische Verarbeitung und Entscheidungsfindung ermöglichen, reguliert. Mittels der in dieser Arbeit etablierten CRISPR/Cas9-HDR-Methode war es möglich, die *dsx*-exprimierenden Neuronen mit dem grünfluoreszierenden Protein GFP zu markieren. Dabei wurde festgestellt, dass das *dsx*-Gen spezifisch in den neuronalen Populationen der Pilzkörper aktiv ist und eine anatomisch und funktionell differenzierte Struktur in Arbeiterbienen fördert, die mit sozialen Verhaltensweisen in Zusammenhang stehen. Die Etablierung eines chemogenetischen Systems, wie des

hM4Di-Rezeptors in *dsx*-exprimierende Neuronen und dessen Aktivierung mit dem Liganden C21, ermöglichte eine gezielte Kontrolle neuronaler Aktivität, was zu einer Veränderung der Reaktionen auf das Königinnenmandibularpheromon (QMP) führt. Es konnte somit eine Beteiligung dieser Neuronen bei der Verarbeitung sozialer Signale nachgewiesen werden. Zusammenfassend lässt sich sagen, dass das *dsx*-Gen, welches ursprünglich für sexuelle Verhaltensweisen spezifiziert hat, in der Evolution der Honigbienen eine neue Rolle zuteilwurde, um soziale Verhaltensweisen zu steuern. Dies verdeutlicht, wie Gene für individuelle Verhaltensweisen evolutionär kooperatives Verhalten in eusozialen Gesellschaften fördern können.

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