Regulatory mechanisms underlying sexual differentiation in the honeybee

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Eidesstattliche Erklärung

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.

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(Oksana Netschitailo)

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Kapitel I

Allgemeine Einleitung

Sexualdimorphismus ist ein im Tierreich weit verbreitetes Phänomen, das sich in vielen Taxa findet. Männchen und Weibchen einer Art können sich in zahlreichen Merkmalen wie Farbgebung, Körpergröße und –form oder spezialisierten Körpermodifikationen unterscheiden. Morphologische Unterschiede zwischen den Geschlechtern können so weit reichen, dass eine Zuordnung beider Geschlechter zu einer Art auf Grundlage des Phänotyps allein erschwert werden kann. Die Anpassungen der Geschlechter dienen dabei der erfolgreichen Reproduktion und der Steigerung der eigenen Fitness. Beispielhaft dafür ist das auffällige, gemusterte Rad aus Schwanzfedern beim männlichen Pfau, bei dem Muster mit mehr Augenflecken von Weibchen während der Partnerwahl bevorzugt werden (Petrie et al., 1991). Ein weiteres Beispiel sind die Geweihe von Rothirschen, bei denen ein größeres Geweih in Rivalitätskämpfen zwischen Männchen von Vorteil ist und den Paarungserfolg steigert (Kruuk et al., 2002; Bartoš and Bahbouh, 2006).

Die Frage nach der Entstehung der Diversität zwischen männlichen und weiblichen Individuen der gleichen Art ist aufgrund der Komplexität ein bereits lange untersuchtes aber immer noch aktuelles Forschungsfeld in der Biologie. Bereits Darwin befasste sich mit dem Sexualdimorphismus, den er auf sexuelle Selektion von intrageschlechtlich variierenden Merkmalen zurückführte (Darwin, 1871). Lange war unbekannt, wie die ausgeprägten Merkmalsvariationen auf Basis der zum größten Teil identischen Erbinformation in Männchen und Weibchen realisiert werden.

Heute wissen wir, dass dimorphe Merkmale ihren Ursprung in der Geschlechtsdetermination haben. Die geschlechtliche Identität kann auf unterschiedliche Art vermittelt werden, resultiert aber immer in Expressionsunterschieden zwischen Männchen und Weibchen während der Entwicklung zum adulten Organismus. So reguliert die Geschlechtsbestimmung in Säugetieren lediglich das Geschlecht der Gonaden. Im weiteren Verlauf der Entwicklung wird dann die Ausbildung von sekundären Geschlechtsmerkmalen durch Hormone bestimmt, die in den männlichen bzw. weiblichen Gonaden gebildet werden (Lavranos et al., 2006). Bei vielen Insekten, wie der Fruchtfliege *Drosophila melanogaster* oder auch der Honigbiene *Apis mellifera*, erfolgt die Geschlechtsbestimmung zellautonom (Drescher and Rothenbuhler, 1964; Cline, 1993). Hier wird durch eine Geschlechtsdeterminationskaskade die geschlechtliche Identität für jede Zelle separat bestimmt. Bei Fehlern in der Kaskade kann es zu

sogenannten Gynandern, sexuellen Mosaiken, kommen. Dies sind Individuen, die sowohl männliche als auch weibliche Gewebe und deren Charakteristika vereinen (Nöthiger et al., 1977; Cline, 1984).

Sexualdimorphismus in der Honigbiene *Apis mellifera*

Honigbienen sind soziale Insekten, die in Kolonien mit bis zu 100.000 Individuen leben von denen die meisten sterile Weibchen, die Arbeiterinnen, sind. Neben den Arbeiterinnen, leben noch eine fertile Königin und einige hundert bis zweitausend saisonal vorkommende Männchen im Stock (Winston, 1987; Bourke, 1999). Im Gegensatz zu anderen Spezies existieren bei den Honigbienen drei unterschiedliche Morphen (Abbildung 1A). Die verschiedenen sekundären Geschlechtsmerkmale sind Anpassungen an die speziellen Aufgaben, wie die Futtersuche, Nestbau, Larvenaufzucht und Erhalt der Kolonie bei den Arbeiterinnen und die Reproduktion bei Drohnen und Königinnen (Eickwort and Ginsberg, 1980; Winston, 1987; Seeley, 1995).

Abbildung 1: Die männliche und weiblichen Morphen der Honigbiene. A: laterale Ansicht. B: frontale Ansicht von Drohn (links) und Arbeiterin (rechts).

Bei den Drohnen steht die Lokalisation der Königin während des Hochzeitsfluges und die Fähigkeit sich mit ihr in der Luft zu paaren im Vordergrund (Loper et al., 1992; Koeniger and Koeniger, 2004; Koeniger et al., 2005; Robert, 2005; Alcock et al., 2008). Drohnen haben eine stärker ausgebildete Flugmuskulatur als Arbeiterinnen, sodass

ihre Flugeigenschaften trotz ihrer höheren Körpermaße besser sind (Radloff et al., 2003). Des Weiteren weisen Männchen im Vergleich zu Weibchen ein besser ausgeprägtes optisches System für die Orientierung während des Hochzeitsfluges auf (Gries and Koeniger, 1996). Zur besseren Orientierung sind die Augen am Kopf dorsoventral positioniert und decken ein weiteres Sichtfeld ab als in Weibchen (Abbildung 1B). Die Fläche der Augen der Drohnen ist im Vergleich um das 3,9 fache vergrößert und auch die Anzahl der Ommatidien ist doppelt so hoch (Menzel et al., 1991; Streinzer et al., 2013). Neben der Anzahl ist auch die Verteilung der Ommatidien bei Drohnen anders als bei den Arbeiterinnen. Im dorso-frontalen Bereich des Facettenauges sind die Ommatidien der Drohnen größer und besser dazu geeignet kleinere Objekte im Flug zu entdecken (van Praagh et al., 1980; Ribi et al., 1989; Menzel et al., 1991; Vallet and Coles, 1993).

Die Wahrnehmung von Arbeiterinnen ist spezialisiert auf das Auffinden von Futterquellen. Ihr Blickfeld, die Ommatidienverteilung im Auge und die Spektralsensitivität der Rezeptoren sind adaptiert an diese Aufgabe (Seidl and Kaiser, 1981; Peitsch et al., 1992; Dafni et al., 1997).

Neben dem optischen ist auch das olfaktorische System der Drohnen besser ausgestattet, mit aus 13 Segmenten bestehenden Antennen und fünf Mal mehr sensorischen Zellen im Vergleich zu Arbeiterinnen, mit nur 12 Antennensegmenten (Snodgrass and Morse, 1956; Esslen and Kaissling, 1976). Spezialisierte Rezeptoren zum Beispiel für das Königinnenpheromon 9-oxo-2-decensäure (9-ODA) helfen den Männchen bei der Lokalisation der Königin während des Paarungsfluges (Gary, 1962; Brockmann et al., 1998; Ayasse et al., 2001; Wanner et al., 2007; Brandstaetter et al., 2014; Jain and Brockmann, 2020). Reize von den Antennen werden in die Antennalloben projiziert, dort verarbeitet und weitergeleitet. In den Antennalloben von Drohnen gibt es nur ca.100 Glomeruli für die Prozessierung von olfaktorischen Reizen, während es bei Arbeiterinnen ca. 150 sind. Jedoch besitzen Drohnen vier vergrößerte, sogenannte Makroglomeruli. Einer dieser Makroglomeruli ist für die Verarbeitung des Königinnenpheromons 9-ODA zuständig (Arnold et al., 1985; Brockmann et al., 1998; Sandoz, 2006; Sandoz et al., 2007).

Arbeiterinnen besitzen spezialisierte Strukturen an den Beinen und Mundwerkzeugen für das Eintragen von Futter, die den Drohnen fehlen. So finden sich für das Sammeln und den Transport von Pollen an den Hinterbeinen von Arbeiterinnen am Tarsus ein Pollenkörbchen und an der Tibia symmetrische Haarreihen, die den sogenannten

Pollenkamm bilden. Für das Sammeln von Nektar haben Arbeiterinnen einen längeren Proboscis und auch die Mundwerkzeuge unterscheiden sich zu denen von Männchen und Königinnen. Die Mandibeln von Arbeiterinnen haben ein glattes Ende im Vergleich zu einem gekerbten bei Königinnen und Drohnen. Zusätzlich sind die Mundwerkzeuge von Drohnen kleiner als bei den Weibchen (Michener, 1944; Snodgrass and Morse, 1956). Neben den Spezialisierungen der Arbeiterinnen am Kopf, finden sich auch Anpassungen am Abdomen, in Form von Wachsdrüsen für den Nestbau, die an der ventralen Seite des Abdomens lokalisiert sind (Blomquist et al., 1980; Winston, 1987; Hepburn et al., 1991; Cassier and Lensky, 1995). Ein weiterer Geschlechts-dimorphismus am Abdomen der Honigbiene ist die Zahl der Segmente des Abdomens. Drohnen besitzen sieben Segmente, während Weibchen nur sechs haben. Weibliche Honigbienen besitzen im Gegensatz zu Drohnen einen Stachel, den die Arbeiterinnen zur Verteidigung des Nestes einsetzen (Michener, 1944; Snodgrass and Morse, 1956).
In der Honigbiene gibt es neben dem Sexualdimorphismus zwischen den männlichen Drohnen und den Weibchen zusätzlich den Kastendimorphismus innerhalb der Weibchen zwischen Arbeiterinnen und Königinnen (Shuel and Dixon, 1960). Bei der Kastendifferenzierung handelt es sich um einen Polyphänismus, der durch die Nahrung im Larvenstadium ausgelöst wird. Königinnenlarven erhalten nach dem Schlupf im Bienenstock von den Arbeiterinnen spezielles Futter, das Gelee Royal (Haydak, 1970). Die spezielle Komposition des Futters und die größere Menge führen zu schnellerem Wachstum und zur vollen Ausbildung der Ovarien mit bis zu 200 Ovariolen pro Ovar (Eckert, 1934; Rembold and Dietz, 1966; Dietz and Lambremont, 1970; Evans and Wheeler, 2001; Buttstedt et al., 2016; Slater et al., 2020). Bei Arbeiterinnen sind die Ovarien verkümmert, mit durchschnittlich 3-5 Ovariolen, und die Spermathek ist nur rudimentär ausgebildet (Velthuis, 1970). Vergleicht man Königinnen und Arbeiterinnen, sind die Königinnen größer und schwerer (Linksvayer et al., 2011). Am verlängerten Abdomen der Königin zeigt sich der Kastendimorphismus zwischen Arbeiterinnen und Königinnen besonders deutlich (Abbildung 1A; Snodgrass and Morse, 1956).
Während die Kastendifferenzierung erst im Larvenstadium stattfinden und jedes Weibchen durch entsprechendes Futter zur Arbeiterin oder Königin werden kann, findet die primäre Geschlechtsbestimmung im Embryonalstadium über die Geschlechtsdeterminationskaskade statt.

Geschlechtsbestimmung in Insekten

Initiale Signale für die Geschlechtsbestimmung sind innerhalb der Insekten divers und folgen keinem einheitlichen Muster. Das initiale Signal kann entweder weibchenspezifisch über Z-Chromosomen ausgelöst werden wie bei vielen Schmetterlings und Mottenarten (*Lepidoptera;* Traut et al., 2007) oder männchenspezifisch über Y Chromosomen wie bei den Moskitos *Anopheles gambiae, Aedes aegypti* und der Mittelmeerfruchtfliege *Ceratitis capitata* (Abbildung 2C; Pane et al., 2002; Hall et al., 2015; Krzywinska et al., 2016; Meccariello et al., 2019). In Hymenoptera, zu denen auch die Bienen und Wespen zählen, wird das Geschlecht über den Mechanismus der Haplodiploidie bestimmt (Abbildung 2A). Aus Organismen mit einem haploiden Chromosomensatz entstehen dabei Männchen während sich bei einem diploidem Chromosomensatz Weibchen entwickeln (Bull, 1983). In der parasitoiden Wespe *Nasonia vitripennis* wird der initiale Faktor als mRNA bei der Befruchtung von der Mutter bereitgestellt (Verhulst et al., 2010a). In einigen Arten von *Sciara* entscheiden Umwelteinflüsse, wie die Temperatur, die auf das Weibchen einwirken über das Geschlecht ihrer Nachkommen, indem sie im Embryo dazu führen, dass Geschlechtschromosomen eliminiert werden (Sanchez, 2008; Sanchez, 2014). Bei *D.melanogaster* entscheidet das Verhältnis von Autosomensätzen zu X-Chromosomen über das Geschlecht. Ist das Verhältnis 1 entstehen Weibchen, bei einem Verhältnis von 0,5 entwickeln sich Männchen aus dem Embryo. Integriert wird dieses Signal über das Gen *Sex-lethal* (*Sxl*; Abbildung 2B; Cline, 1979; Cline and Meyer, 1996; Erickson and Quintero, 2007). Selbst zwischen nahe verwandten Arten kann es grundlegende Unterschiede geben wie bei unterschiedlichen Stämmen von *Musca domestica*. Hier wurden sowohl weibchenspezifische als auch männchenspezifische initiale Signale beschrieben die autosomal oder auch über Geschlechtschromosomen vererbt werden können (Hediger et al., 2010; Bopp, 2010; Hamm et al., 2015; Sharma et al., 2017).

Bei dieser Vielfalt an initialen Signalen ist es erstaunlich, dass noch innerhalb der initialen Kaskade ein konservierter Mechanismus in Form von Homologen *femenizer* (*fem*)/*transformer* (*tra*) vorhanden ist (Verhulst et al., 2010b). Die Transkripte des Gens *fem*/*tra* werden geschlechtsspezifisch gespleißt und resultieren in Weibchen in aktivem Protein, während in Männchen ein inaktives gebildet wird. In Spezies mit männchen-spezifischem primärem Signal wird die Bildung von funktionalem Protein in Männchen gestört, indem zusätzliche Sequenzinformation in die *fem/tra* mRNA integriert wird.

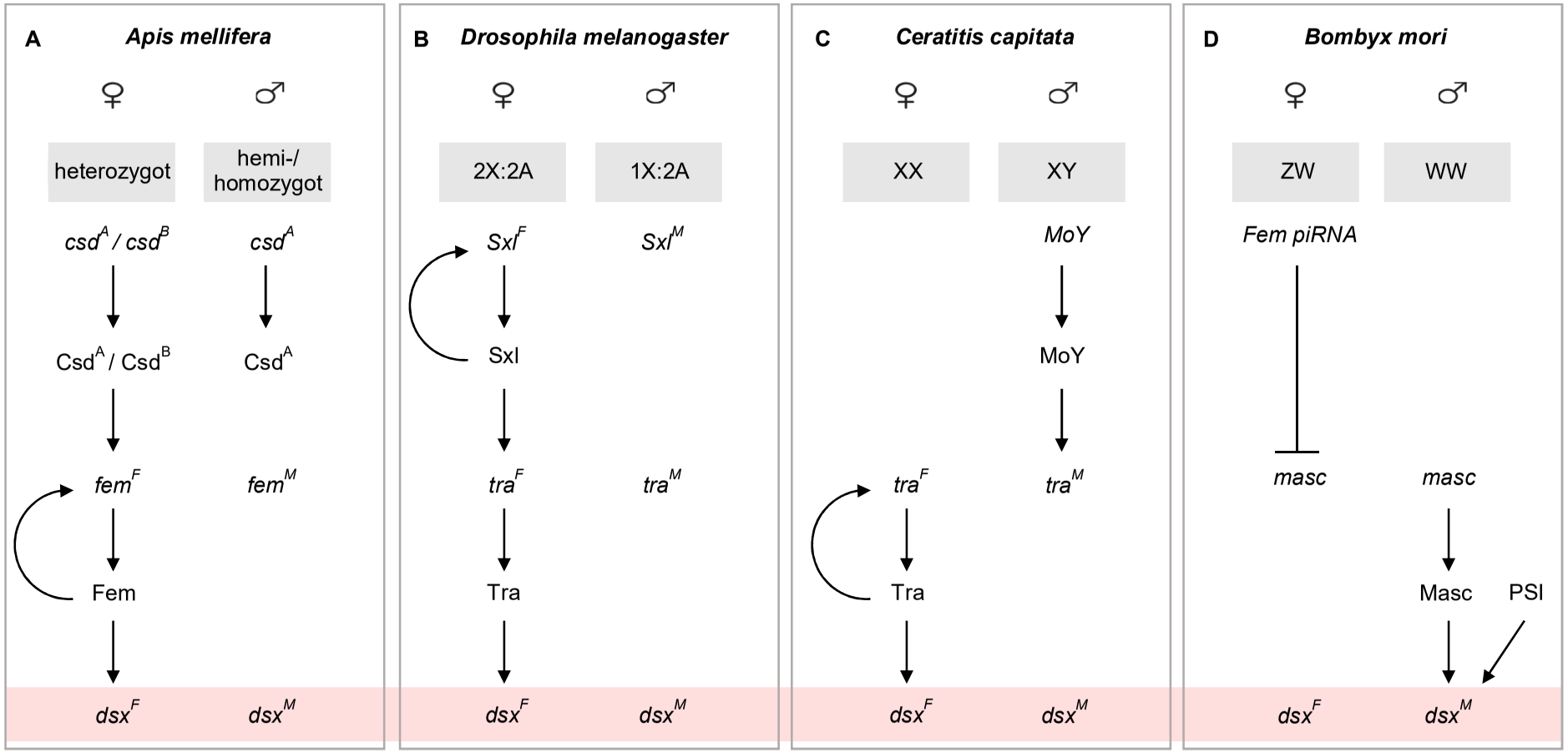

Abbildung 2: Darstellung unterschiedlicher Mechanismen zur Geschlechtsbestimmung bei Insekten mit diversen initialen Signalen. Bei *Apis mellifera* (A) entscheidet der allelische Zustand des *csd* Gens über das Geschlecht, bei *Drosophila melanogaster* (B) das Verhältnis von Autosomen- zu Geschlechtschromosomen, bei *Ceratitis capitata* (C) ist das initiale Signal auf dem Y-Chromosom lokalisiert und bei *Bombyx mori* (D) auf dem Z-Chromosom. Bei drei der vier gezeigten Beispiele (A-C) regulieren die initialen Signale das Spleißen von *fem/tra* homologen Genen. Fem/Tra bei *C.capitata* und *A. mellifera* bzw. Sxl bei *D.melanogaster* sind in der Lage den eigenen Spleißprozess über Autoregulation aufrecht zu erhalten. Weiterhin sorgen Fem/Tra Proteine in Weibchen für das geschlechtsspezifische Spleißen von dsx^F. Die Ausnahme findet sich beim Seidenspinner *B.mori,* wo es kein homolog von *fem/tra* gibt. Bei *B.mori* kontrolliert das initiale Signal in Form einer piRNA in Weibchen die Expression von *masc.* Im *B.mori* Männchen steuert Masc zusammen mit PSI das Spleißen von dsx^M. Abbildung modifiziert und ergänzt nach Gempe und Beye (2011).

Auf der anderen Seite wird in Spezies mit weibchenspezifischem primären Signal die Bildung des weibchenspezifischen Transkripts von *fem/tra* induziert (Gempe and Beye, 2011; Bopp et al., 2014).

Funktionales Fem/Tra ist ein SR-typ Protein und ein Spleißregulator, der das weibchenspezifische Spleißen von Transkripten des Gens *doublesex* (*dsx*) steuert (Tian and Maniatis, 1992; Lynch and Maniatis, 1996). Als Kofaktor, der die Bindung an zu spleißende RNA vermittelt, dient dabei das Transformer2 (Tra2) Protein (Hedley and Maniatis, 1991). Tra2 ist ein ubiquitärer Spleißfaktor, dessen Homologe auch in Säugern auftreten und dort an einer Vielzahl essentieller Prozesse wie zum Bespiel der neuronalen Entwicklung in Mäusen beteiligt sind (Tacke and Manley, 1999; Best et al., 2014; Roberts et al., 2014). In *D.melanogaster, N.vitripennis, C.capitata, Tribolium castaneum, M.domestica, Lucilia cuprina* und *A.mellifera* wurden Homologe von Fem/Tra und Tra2 bereits als entscheidend für das weibchenspezifische Spleißen der mRNA von *dsx* beschrieben (Burtis and Baker, 1989; Hedley and Maniatis, 1991; Inoue et al., 1992; Verhulst et al., 2010a; Geuverink et al., 2017; Pane et al., 2002; Salvemini et al., 2009; Shukla and Palli, 2012; Shukla and Palli, 2013; Hediger et al., 2010; Burghardt et al., 2005; Concha and Scott, 2009; Gempe et al., 2009; Nissen et al., 2012). Der Proteinkomplex aus Homologen von Fem/Tra und Tra2 reguliert in *C.capitata, T.castaneum, M.domestica, L.cuprina* und *A.mellifera* zusätzlich als ein Mechanismus zur Autoregulation, den Spleißprozess der *fem/tra* mRNA und erhält so den weiblichen Signalweg aufrecht (Hediger et al., 2010; Salvemini et al., 2009; Shukla and Palli, 2013; Concha and Scott, 2009; Nissen et al., 2012). In *D.melanogaster* wird das geschlechtsbestimmende Signal über die Autoregulation des Spleißprozesses von *Sxl* Transkripten durch Sxl^F Protein aufrecht erhalten (Bell et al., 1991).

Eine Ausnahme bei der Konvergenz der Kaskaden auf der Ebene des *fem*/*tra* Gens bildet der Seidenspinner *Bombyx mori,* bei dem bisher kein *fem*/*tra* Homolog identifiziert wurde (Abbildung 2D). Hier ist das initiale Signal eine piRNA, die in Weibchen die Degradation von *masculinizer (masc*) mRNA auslöst und die männliche Geschlechtsdeterminationskaskade unterbricht (Kiuchi et al., 2014; Katsuma et al., 2018). Funktionales Masc Protein wird für das Spleißen der *dsx* mRNA in *B.mori* Männchen benötigt (Yuzawa et al., 2020). Erst auf der untersten Ebene der initialen Geschlechtsdeterminationskaskade, der Bildung von Dsx^F in Weibchen und Dsx^M in Männchen, konvergieren auch hier die Kaskaden (Suzuki et al., 2001; Ohbayashi et al., 2001). Unabhängig von den Unterschieden innerhalb der hier beschriebenen

Kaskaden verschiedener Spezies innerhalb der Insekten, steht das Gen *dsx* am Ende der Geschlechtsdeterminationskaskade und kodiert in Männchen und Weibchen zwei sich C-terminal unterscheidende Transkriptionsfaktoren (Verhulst and van de Zande, 2015).

Geschlechtsbestimmung in *Apis mellifera*

Die Geschlechtsbestimmung in der Honigbiene ist eine spezielle Form der Haplodiploidie, die komplementäre Geschlechtsbestimmung (Whiting, 1933; Whiting, 1943; Cook, 1993). Befruchtete Eier können sich zu Weibchen entwickeln, falls der diploide Chromosomensatz zwei unterschiedliche Allele für das *complementary sex determiner* Gen (*csd*) trägt (Abbildung 2A). Haploide, unbefruchtete Embryos und diploide Eier mit zwei identischen Allelen für das Gen *csd* entwickeln sich zu Männchen (Beye et al., 2003). Diploide Männchen werden in der Natur von den Arbeiterinnen erkannt und im Larvenstadium aus dem Stock entfernt (Mackensen, 1951; Woyke, 1963). In einer natürlichen Population der westlichen Honigbiene kommen bis zu 53 verschiedene Allele für *csd* vor und es wird davon ausgegangen, dass es insgesamt weltweit mehr als 100 verschiedene Allele gibt (Lechner et al., 2014; Zareba et al., 2017). Die hohe Anzahl von unterschiedlichen Allelen und die damit möglichen Kombinationen senken die Wahrscheinlichkeit, dass diploide Drohnen entstehen.
Befruchtete und unbefruchtete Eier werden von der Bienenkönigin produziert, dem einzigen sich paarenden und reproduzierenden Weibchen in einer Bienenkolonie. Nach einem Hochzeitflug im Alter von 5-10 Tagen nach dem Schlupf der Imago und der Paarung mit mehreren Drohnen kann sie für mehrere Jahre das gesammelte Sperma lagern und befruchtete Eier legen (Estoup et al., 1994; Schlüns et al., 2005; Koeniger and Koeniger, 2007).
Das primäre Signal der Geschlechtsbestimmung das Gen *csd* führt in Weibchen dazu, dass die RNA des *fem* Gens, ein Ortholog des *tra* Gens in *D.melanogaster,* geschlechtsspezifisch gespleißt wird (Hasselmann et al., 2008; Gempe et al., 2009). Der genaue Mechanismus dafür ist bisher nicht bekannt, da Csd selbst keine RNA-Interaktionsdomäne besitzt und somit nicht direkt mit der *fem* pre-mRNA interagieren kann. Es wird angenommen, dass Csd mit einem bisher nicht bekannten Cofaktor interagiert, der die Bindung an die *fem* pre-mRNA vermittelt. Bei Csd handelt es sich um ein SR-Typ Protein, das eine Arginin-Serin reiche Domäne besitzt, wie sie auch in

bereits bekannten spleißregulatorisch aktiven Proteinen, wie dem Spleißfaktor Tra2, vorkommt (Fu, 1995; Beye et al., 2003). Durch das Csd Protein wird das weibchenspezifische Spleißprozess der *fem* mRNA induziert, bei dem ein Teil von Exon 3, sowie die Exons 4 und 5 entfernt werden. Das im männchenspezifischen Teil von Exon 3 enthaltene Stopcodon führt zu einem verkürzten Protein, während im Weibchen der Leserahmen im Exon 12 endet und für ein funktionales Protein kodiert (Hasselmann et al., 2008; Gempe et al., 2009).
In Weibchen kann das aktive Fem Protein das weibchenspezifische Spleißen der *fem* pre-mRNA und somit das weibliche Signal über eine Rückkopplungsschleife aufrechterhalten. Das Fem Protein, ebenfalls ein SR-Typ Protein, kann über den Cofaktor Tra2 eine RNA-Interaktion eingehen und das weibchenspezifische Spleißen der *dsx* pre-mRNA regulieren. Der männliche Entwicklungsweg resultiert ohne aktives Csd und Fem Protein in männchenspezifisch gespleißten *dsx* Transkripten (Gempe et al., 2009; Nissen et al., 2012). Beide geschlechtsspezifischen Transkripte von *dsx* kodieren für funktionale Proteine, Dsx^M und Dsx^F, mit unterschiedlichen C-terminalen Domänen (Cho et al., 2007).

Das Gen *doublesex* und seine Rolle als zentrales Element bei der Geschlechtsdifferenzierung in den Insekten

Homologe Gene von *dsx* sind in vielen Spezies und Ordnungen der Insekten neben der Realisierung von primären Geschlechtsmerkmalen auch an der Entwicklung von diversen sekundären Geschlechtsmerkmalen beteiligt. Beispielsweise steuert das *dsx* Gen innerhalb der Coleoptera bei den Blatthornkäfer *Onthophagus sagittarius* und *Trypoxylus dichotomus* die Ausbildung von überproportionierten Hörner in Männchen (Kijimoto et al., 2012; Ito et al., 2013). Die bei Männchen in *Cyclommatus metallifer* vergrößerten Mandibeln sowie die geschlechtstypische Pigmentierung sind weitere sekundäre Geschlechtsmerkmale, die durch das *dsx* Gen reguliert werden (Gotoh et al., 2016). Weiterhin wird in *Nilaparvata lugens* (Hemiptera) die geschlechtstypische Körpergröße vom *dsx* Gen beeinflusst (Zhuo et al., 2018). In den Lepidoptera *Agrotis ipsilon* und *Bombyx mori* konnte gezeigt werden, dass es bei Funktionsverlust des *dsx* Gens zur abnormalen Zahl von Abdominalsegmenten und Morphologie von externen Genitalien kommt (Xu et al., 2017; Chen et al., 2019). Im Moskito *Aedes aegypti*

(Diptera) reguliert das *dsx* Gen die Morphologie der Flügel, der Mundwerkzeuge und der Antennen in Weibchen (Mysore et al., 2015). Ähnlich, sind in der verwandten Stechmückenart *Anopheles gambiae* bei Mutationen im *dsx* Gen in Weibchen, neben der eingeschränkten Fertilität, Anomalien beim Proboscis und den Antennen zu beobachten (Kyrou et al., 2018).

Am besten untersucht ist die Funktion des *dsx* Gens innerhalb des Modelorganismus der Taufliege *D.melanogaster.* Hier konnte bereits nachgewiesen werden, dass das *dsx* Gen Einfluss auf die geschlechtstypische Pigmentierung (Kopp et al., 2000; Williams et al., 2008), die geschlechtsspezifischen Anpassungen der Segmente im Abdomen (Wang and Yoder, 2012; Foronda et al., 2012), die Borsten auf den Vorderbeinen der Männchen (Devi and Shyamala, 2013; Rice et al., 2019) und sogar das männliche Paarungsverhalten hat (Rideout et al., 2007; Kimura et al., 2008; Rideout et al., 2010; Rezaval et al., 2016).

Die Regulation der Entwicklung von all diesen Merkmalen wird durch eine vom *dsx* Gen kodierte DM-Domäne ermöglicht, über die Dsx als Dimer mit der DNA interagieren und als Transkriptionsfaktor agieren kann. Die DM-Domäne ist benannt nach dem Gen *doublesex* aus *D.melanogaster* und dem Homolog *male-abnormal-3* aus *Caenorhabditis elegans,* in deren Proteinprodukten diese Domäne zuerst identifiziert wurde (Shen and Hodgkin, 1988; Burtis and Baker, 1989; Raymond et al., 1998). Neben ihren DNA Bindeeigenschaften, durch zwei verschlungene CCHC und HCCC Zinkfingerdomänen, enthält die DM-Domäne auch eine Oligomerisierungsdomäne (OD1). Die OD1 wird genutzt um die aktiven Protein-Dimere zu bilden. Da die DNA-Bindedomäne des Dsx Proteins in beiden Geschlechtern identisch ist, binden sowohl Dsx^M als auch Dsx^F identische DNA-Sequenzen (Erdman and Burtis, 1993; Erdman et al., 1996; An et al., 1996; Zhu et al., 2000; Clough et al., 2014). Es wird vermutet, dass die geschlechtsspezifische Interaktion mit der Transkriptionsmaschinerie über eine zweite Oligomerisierungsdomäne (OD2) im Dsx Protein, die C-terminal lokalisiert ist, vermittelt wird (Erdman et al., 1996; An et al., 1996). Der C-terminale Bereich des Dsx Proteins ist geschlechtsspezifisch, durch die unterschiedlich gespleißten Transkripte des *dsx* Gens. So können Dsx^M und Dsx^F zwar gleiche Gene regulieren, aber einen unterschiedlichen Effekt herbeiführen (Arbeitman et al., 2004; Bayrer et al., 2005; Yang et al., 2008; Lebo et al., 2009; Arbeitman et al., 2016). Eine Interaktion von Dsx Proteinen mit Enhancern der Gene *Yolk protein 1, bric-a brac* und *Flavin-containing monooxygenase-2* aus *D.melanogaster* ist bereits nachgewiesen worden. Die

Expression dieser Gene wird in Weibchen durch Dsx^F hochreguliert und in Männchen durch Dsx^M reprimiert (Burtis et al., 1991; Coschigano and Wensink, 1993; Williams et al., 2008).

Die Interaktion mit anderen Proteinen ermöglicht es Dsx geschlechtsspezifischen Einfluss auf weitere Zielgene zu nehmen. In der Taufliege *D.melanogaster* konnte gezeigt werden, dass für die regulatorische Funktion von Dsx^F in Weibchen die Interaktion mit dem Genprodukt von *intersex* notwendig ist (Waterbury et al., 1999; Garrett-Engele et al., 2002; Siegal and Baker, 2005). Für die Ausbildung geschlechtsspezifischer Gehirnmorphologie in *D.melanogaster* nutzen Dsx Proteine das Genprodukt des Hox-Gens *Abdominal-B* (*Abd-B*) als Cofaktor (Ghosh et al., 2019). Die geschlechtlich dimorphe Morphologie und Pigmentierung des Abdomens in *D.melanogaster* wird ebenfalls durch das Zusammenspiel der Gene *dsx* und *Abd-B* gesteuert (Williams et al., 2008; Wang and Yoder, 2012; Foronda et al., 2012).

Nicht alle Zellen in *D.melanogaster* exprimieren das *dsx* Gen, haben also eine geschlechtliche Identität. Das Gen *dsx* wird sowohl gewebespezifisch wie auch abhängig vom Entwicklungszeitpunkt exprimiert (Rideout et al., 2010; Robinett et al., 2010; Tanaka et al., 2011). Die Regulation nachgeschalteter Gene entsteht somit nicht nur direkt über die Funktion von Dsx als Transkriptionsfaktor, sondern auch indirekt durch die Anwesenheit oder Abwesenheit von Dsx Protein. Die Transkription des *dsx* Gens selbst wird durch eine Vielzahl von *cis*-regulatorischen Elementen gesteuert, die modular aufgebaut sind (Rice et al., 2019). So sind an der Entwicklung von Kämmen auf den ersten beiden Tarsengliedern der Vorderbeine von *D. melanogaster* Männchen drei Enhancer beteiligt um die notwendige zeitliche und räumliche Expression von *dsx* zu gewährleisten. Eine unterschiedliche Kombination von jeweils zweien dieser Enhancer wird benötigt, um die Ausbildung von Kämmen auf dem Vorderbein zu steuern und Cluster von chemosensorischen Borsten auszubilden. Weitere Enhancer steuern gewebespezifisch z.B. die Expression von *dsx* in Gonaden oder im Gehirn (Zhou et al., 2014; Rice et al., 2019).

Studien in der Gattung *Drosophila* zur Diversifikation von Merkmalen haben gezeigt, dass viele Unterschiede zwischen Geschlechtern und auch Arten auf die Evolution von *cis*-regulatorischen Elementen in Zielgenen von Dsx Proteinen zurückzuführen sind (Stern, 2000; Carroll, 2000). So resultieren Variationen in den *cis*-regulatorischen Elementen des *desat-F* Gens in unterschiedlichen *Drosophila* Spezies in einer Änderung der Kohlenwasserstoffe, die über die Cuticula abgesondert werden und als

Pheromone der Weibchen dienen (Shirangi et al., 2009; Luo and Baker, 2015). Konservierte Transkriptionsfaktoren wie Dsx können durch Modifikation anzestraler *cis*-regulatorische Elemente rekrutiert werden um Neuerungen zu erschaffen (Prud'homme et al., 2007; Williams and Carroll, 2009). Evolutionäre Schranken, die durch die Pleiotropie von Transkriptionsfaktoren entstehen, können so durch die Modifikation von regulatorischen statt kodierenden Sequenzen umgangen werden. Eine Mutation von kodierenden Sequenzen von *dsx*, die z.B. eine Änderung der Erkennungssequenz der DNA-Bindedomäne erzeugen würde, hätte potentiell schwere negative Folgen auf den Reproduktionserfolg und die Fitness (Carroll, 2008; Luo and Baker, 2015). Im Gegensatz dazu kann über die Mutation von *cis*-regulatorischen Elementen die Expression von Genen modelliert werden unabhängig von deren funktionalen Eigenschaften. Hierbei werden weder andere Gene, die Proteinfunktion oder bei modular aufgebauten regulatorischen Elementen, weitere *cis*-regulatorische Elemente im selben Gen, beeinflusst (Carroll, 2008).

Zielsetzung

Männchen und Weibchen der Honigbiene *Apis mellifera* zeigen einen ausgeprägten Sexualdimorphismus zahlreicher Merkmale, wie der Kopfmorphologie mit den Antennen, Augen und Mundwerkzeugen (Snodgrass and Morse, 1956; Winston, 1987). Welches genetische Programm diesen Merkmalsausprägungen zugrunde liegt und wie es reguliert wird, ist bisher ungeklärt. In anderen Insekten, wie der Fruchtfliege *D.melanogaster,* sind Homologe des Gens *dsx* für die Ausbildung von primären und sekundären Geschlechtsmerkmalen verantwortlich. Der Transkriptionsfaktor Dsx wird in *D.melanogaster* gewebespezifisch exprimiert und steuert über die Interaktion mit *cis*-regulatorische Elementen in der DNA eine Vielzahl von Prozessen (Christiansen et al. 2002; Verhulst and van de Zande, 2015).

Das Homolog von *dsx* in der Honigbiene wird durch die Geschlechts-determinationskaskade reguliert. Das initiale Signal der Geschlechtsdetermination ist dabei der allelische Zustand des *csd* Gens, das in befruchteten Eiern, die sich weiblich entwickeln, heterozygot und in unbefruchteten, männlichen Eiern hemizygot vorliegt (Beye et al., 2003). Aktives Csd Protein in Weibchen reguliert das weibchenspezifische Spleißen der *fem* mRNA, das in aktivem Fem Protein resultiert. Für das weibchenspezifische Spleißen von *dsx* mRNA wird in der Honigbiene aktives Fem

Protein benötigt (Gempe et al., 2009). In Männchen und Weibchen kodieren die geschlechtsspezifischen *dsx* Transkripte für die Transkriptionsfaktoren Dsx^M und Dsx^F (Cho et al., 2007). Es ist bislang ungeklärt, ob das *dsx* Gen allein die Ausbildung von Geschlechtsmerkmalen in der Honigbiene steuert oder ob weitere Gene dafür benötigt werden. Im letzteren Fall müssten auch diese Gene in Zusammenhang mit der Geschlechtsdetermination stehen.

Ziel dieser Arbeit ist es, die regulatorischen Mechanismen zu identifizieren, die an der Realisierung von geschlechtlich dimorphen Merkmalen in der Honigbiene beteiligt sind. Dafür soll zunächst untersucht werden, ob das *dsx* Gen in der Honigbiene die Entwicklung von primären und sekundären Merkmalen reguliert. Hierzu soll die geschlechtsspezifische Merkmalsausprägung in Weibchen betrachtet werden, die mit Hilfe der CRISPR/Cas9 Methode induzierte Mutationen im *dsx* Gen tragen und denen infolgedessen funktionales Dsx Protein fehlt.

In einem zweiten experimentellen Ansatz sollen mögliche weitere Gene identifiziert werden, die die Differenzierung von Männchen und Weibchen in der Honigbiene beeinflussen. Zu diesem Zweck sollen Expressionsunterschiede zwischen männlichen und weiblichen Embryonen erfasst werden. Differenziell exprimierte Gene und alternativ gespleißte Transkripte in drei unterschiedlichen embryonalen Stadien sollen Aufschluss über den Einfluss der Befruchtung und der Geschlechtsdetermination auf die Diversifizierung der Expression zwischen den Geschlechtern liefern. Ein frühes Stadium, 10-15 h nach der Eiablage, stellt den Zustand nach der Befruchtung und den Beginn der Geschlechtsbestimmung mit der einsetzenden Expression von *csd* 12 h nach Eiablage dar (Beye et al., 2003). Spätere Stadien sollen Aufschluss über Expressionsunterschiede liefern, die durch die Geschlechtsdetermination ausgelöst werden. Hierzu werden Embryonen im Alter von 25-40 h und 55-70 h nach Eiablage verwendet, in denen die geschlechtsspezifische Expression des *fem* Gens 33 h (Gempe et al., 2009) und des *dsx* Gens 57 h nach der Eiablage bereits begonnen hat (Tanja Gempe, persönliche Mitteilung).

Innerhalb der Gruppe von Gene, die Expressionsunterschiede zwischen den Geschlechtern zeigen, sollen Kandidatengene identifiziert und charakterisiert werden, die an der Entwicklung von sekundären Geschlechtsmerkmalen in der Honigbiene beteiligt wird. Da Unterschiede in der embryonalen Transkription entweder aus der Befruchtung oder aus frühen Signalen der Geschlechtsdetermination resultieren, soll zudem die Interaktion dieser Gene mit bekannten Komponenten der Geschlechts-

bestimmungskaskade untersucht werden. Hierzu sollen mit Hilfe des CRISPR/Cas9 Systems Mutationen im *csd* bzw. *fem* Gen induziert und Mutanten mit inaktivem Fem bzw. Csd Protein erzeugt werden. Der Einfluss der Mutation des *csd*/*fem* Gens auf die Expression der Kandidatengene soll untersucht werden. Potentielle Kandidaten sollen im weiteren Verlauf dieser Arbeit auf ihre Funktion getestet werden. Über das CRISPR/Cas9 System sollen nachfolgend Mutationen in den Kandidatengenen hervorgerufen werden, die zu einem Funktionsverlust führen. Morphologische Änderungen der G0 Generation von Mutanten, sollen Aufschluss über die mögliche Funktion geben. Ein Vergleich von Homologen der Kandidatengene in anderen Spezies soll Information über die evolutionären Ursprünge der Gene liefern.

Kapitel II: Manuskripte

Manuscript I

A genetic switch for worker nutrition-mediated traits in honeybees

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RESEARCH ARTICLE

A genetic switch for worker nutrition-mediated traits in honeybees

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Abstract

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

Author summary

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

Introduction

Highly social insects are characterized by caste dimorphism, with morphologically and physiologically distinct reproductive queens and more or less sterile workers [1–3]. In honeybees, the

Abbreviations: CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; csd, complementary sex determiner; dsx, doublesex; fem, feminizer; FL, fragment length; fru, fruitless; HEX, hexachlorofluorescein; IIS, insulin/IGF signaling; NGS, next-generation sequencing; ORF, open reading frame; PAM, Protospacer adjacent motif; RJ, royal jelly; RNAi, interference RNA; RT-PCR, reverse transcription PCR; sgRNA, single guide RNA; SR, serine arginine rich; TOR, target of rapamycin; WT, wild type; WJ, worker jelly.

development of two distinct phenotypes is controlled by different nutrition, and it is a prominent example of developmental plasticity and polyphenism [4, 5].

One major concern for the study of caste development involves explaining how a usually sterile worker and a queen that lays up to 2,000 eggs per day develop from different diet and feeding regimens [4, 6, 7]. Worker-destined larvae receive restricted amounts of a reduced sugar content diet (worker jelly [WJ]), while queen-destined larvae receive large quantities of a sugar-rich diet (royal jelly [RJ]) [8–11]. WJ and RJ drive the development of female larvae in two distinct morphs. Workers have a five-day longer developmental time, lower body mass, two small ovaries containing few ovarioles, and mid- and hind-leg structures adapted for pollen collection and transport. Queens have a five-day shorter developmental time, larger body mass, and two large ovaries that contain many more ovarioles, and they lack the pollen collection structures on the legs.

Two types of models have been proposed to explain how diets and feeding regimens mediate worker/queen development. The Nutrition/Growth model suggests that queen/worker development is driven by the amount of food and balance of nutrition [7, 11, 12], which modulate a developmental program. Queen-destined larvae have abundant nutrition, and organ growth is only limited by the intrinsic program. Worker-destined larvae have a shortage of nutrition that restricts growth and influences metabolic parameters accordingly. In contrast, the Instruction model proposes that the RJ has a compound (or compounds) that instruct the development of queens [13–15]. In support of the Instruction model, research over the past decades has attempted to identify a single compound from RJ [12, 14] that can determine queen development.

A recent study provided evidence that the protein royalactin has queen-determining activity [15]. However, follow-up experiments in another laboratory were unable to repeat these results [7], questioning the existence of a single determinant for queen development [4]. Gradually increasing the sugar levels of WJ and altering the composition of RJ-containing diets produced workers, intercastes, and eventually queens [9–11, 16], but it failed to rear only queens. The more continuous caste characteristics resulting from different feeding regimes [17] have been proposed in support of the Nutrition/Growth model. The RJ and the WJ produce different reaction norms of the general developmental program that determines the caste polyphenism. An alternative explanation is that the essential higher sugar levels for queen-destined larvae are a secondary effect and reflect the higher energy requirements for the faster and larger-growing queen organs of an otherwise instructed queen program. The rearing of larvae at day 5 in queenless colonies yielded bees with ovariole numbers that were discontinuous (either more worker or queen-like distributed), while other queen and worker traits were either absent or present in a noncorrelated fashion [18], suggesting two distinct states of the developmental program and the possible existence of regulatory switches [19].

One possible mechanism by which nutrients are sensed by bee larvae is the insulin/IGF signaling (IIS) and target of rapamycin (TOR) pathways, which link the abundance of nutrition with worker and queen differential gene expression [20–23]. Indeed, nutritional input can also influence growth and metabolic programs via the IIS and TOR pathways in mammals and other insects [24–26]. However, whether regulation of the IIS and TOR pathways drives caste differentiation or whether the regulation is a response to the activation of a queen developmental program is currently unknown. Consistent with the faster and larger growth of queens, gene expression studies have revealed the up-regulation of physiometabolic genes in queens, reflecting their higher metabolic rate [27, 28]. Chromatin modifications and DNA methylation analyses have indicated distinct epigenetic states in worker- and queen-destined larvae, suggesting another level of regulatory control associated with caste-specific gene expression [29–31].

Here, we explored whether nutrition is the only factor directing size polyphenism and whether further genetic instruction from the sex determination pathway is required. To do so, we introduced a method to screen mutations directly in worker bees using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technique.

Results

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

According to the Nutrition/Growth model, nutrition is the only driver of reduced reproductive organ size, the most prominent trait in caste development. Males, like queens, receive high amounts of sugar during larval development [32] and develop large reproductive organs unlike sterile worker bees. Gradually increasing the sugar levels of WJ produces intercaste development [9, 10, 16]. Hence, if a shortage of nutrition in the worker diet (and reduced sugar levels) is the only driving component, we would expect that this diet would also mediate the size reduction of reproductive organs in males.

We manually reared genetic females and males on worker nutrition [16, 33] and compared their phenotypes with those of workers and genetic males reared in the colony (Fig 1 and S1 and S2 Tables). The reproductive organs of genetic female bees raised on worker nutrition either inside the colony ($n = 14$) or manually outside ($n = 15$) were equivalent in size (Fisher's exact test, df = 1, $P = 1$). In both laboratory- and colony-reared genetic females, there were few ovarioles, and the size of each ovary was small compared with the size of the heads (Fig 1 and S1 Table). This contrasts with the large ovaries of the female larvae fed a queen diet in the hive (queens alone cannot be consistently reared under laboratory conditions [7]; see Fig 4A and 4B as an example of a queen phenotype). This result indicates that our manual feeding regime mirrors the effect of a worker diet in the hive [16, 33]. To examine whether only the balance and amount of nutrition (low amount of sugar) determine small reproductive organs, we

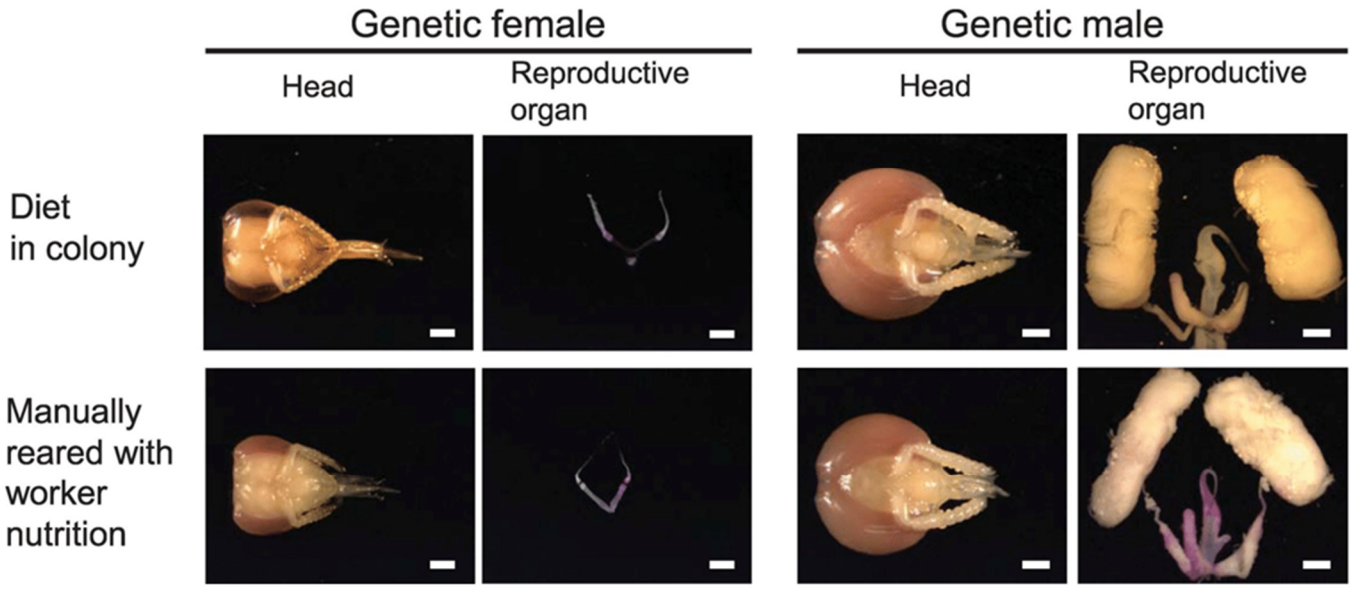

Fig 1. Reproductive organ and head phenotypes of females and males reared on worker nutrition in the laboratory and in the colony. Scale bar = 1 mm.

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reared genetic male larvae on worker nutrition in the laboratory and compared these with males that received high amounts of sugar in the colony [32]. Genetic males that were reared on the worker nutrition diet had large male reproductive organs (Fig 1 and S2 Table). They were equivalent in size ($n = 20$) to the males obtained from the colony ($n = 8$) that were reared on drone nutrition (Fisher's exact test, df = 1, $P = 1$). These results indicate that worker nutrition (and a shortage of sugar) is not the only requirement for the size polyphenism, suggesting input from the sex determination pathway.

Somatic mutational screening in reared bees

We next established a method that enables the mutational screening of sex-determining genes directly in worker bees using the CRISPR/Cas9 method [34–36]. Following traditional mutant approaches, we would need to produce mutant queens and drones that need to be crossed to generate double-mutant worker bees. If we could mutate all nuclei in the embryo, we would be able to directly rear mutated worker bees without maintaining colonies and performing crossings. To examine whether we could mutate worker bees entirely using the CRISPR/Cas9 method, we tested different embryonic injection conditions. To determine the robustness of this approach, we studied at least two sites for three genes, the *doublesex* (*dsx*), *fruitless* (*fru*), and *loc552773* genes (S1 Fig). Only the *dsx* gene was used later on for phenotyping. We injected into the anterior embryos of very young female embryos (0 to 1.5 hours after egg deposition) [37]. We tested a set of single guide RNAs (sgRNAs; S3 Table) at different concentrations and observed that we repeatedly mutated each injected embryo.

The fragment length (FL) and sequence analyses of the amplicons in larval stage 1 larvae revealed that up to 100% of the *fru* and *dsx* and 60% of the *loc552773* target embryos were mutated (Tables 1 and S4 and S5 and Fig 2). The wild-type (WT) allele was consistently not

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

Treatment	pg of Cas9 mRNA per embryo	pg of sgRNA per embryo	No. of surviving embryos 24 h after injection	No. (%) of hatched L1 larvae	No. of genotyped larvae	No. of larvae with length variant[1]	Efficiency of mutagenesis[2]
fru-sgRNA1	800	29.2	105	10 (10%)	8	2	20%
fru-sgRNA2	400	14.6	467	72 (15%)	7	6	86%
fru-sgRNA1	240	8.8	78	2 (3%)	2	2	100%
fru-sgRNA4	400	14.6	125	3 (2%)	3	3	100%
fru-sgRNA5	400	14.6	98	10 (10%)	10	10	100%
loc-sgRNA1[3]	400	14.6	93	7 (8%)	5	3	60%
loc-sgRNA2	400	14.6	102	32 (31%)	28	1	4%
dsx-sgRNA1	400	5.5	52	1 (2%)	1	1	100%
dsx-sgRNA1	400	3.7	93	5 (5%)	4	1	25%
dsx-sgRNA2	400	5.5	178	2 (1%)	2	2	100%
dsx-sgRNA2	400	3.7	89	5 (6%)	5	5	100%
dsx-sgRNA2	400	0.7	82	21 (26%)	19	3	16%
H_2O	-	-	48	27 (56%)	11	0	0%
Uninjected	-	-	65	55 (85%)	19	0	0%

[1]Fragments differed in length compared with fragments isolated from 7 nontreated (WT) larvae.

[2]Relative ratio of the number of mutant larvae to the number of all larvae.

[3]Targeted the gene *loc552773*.

Abbreviations: Cas9, CRISPR-associated protein 9; FL, fragment length; pg, picogram; sgRNA, single guide RNA; WT, wild type.

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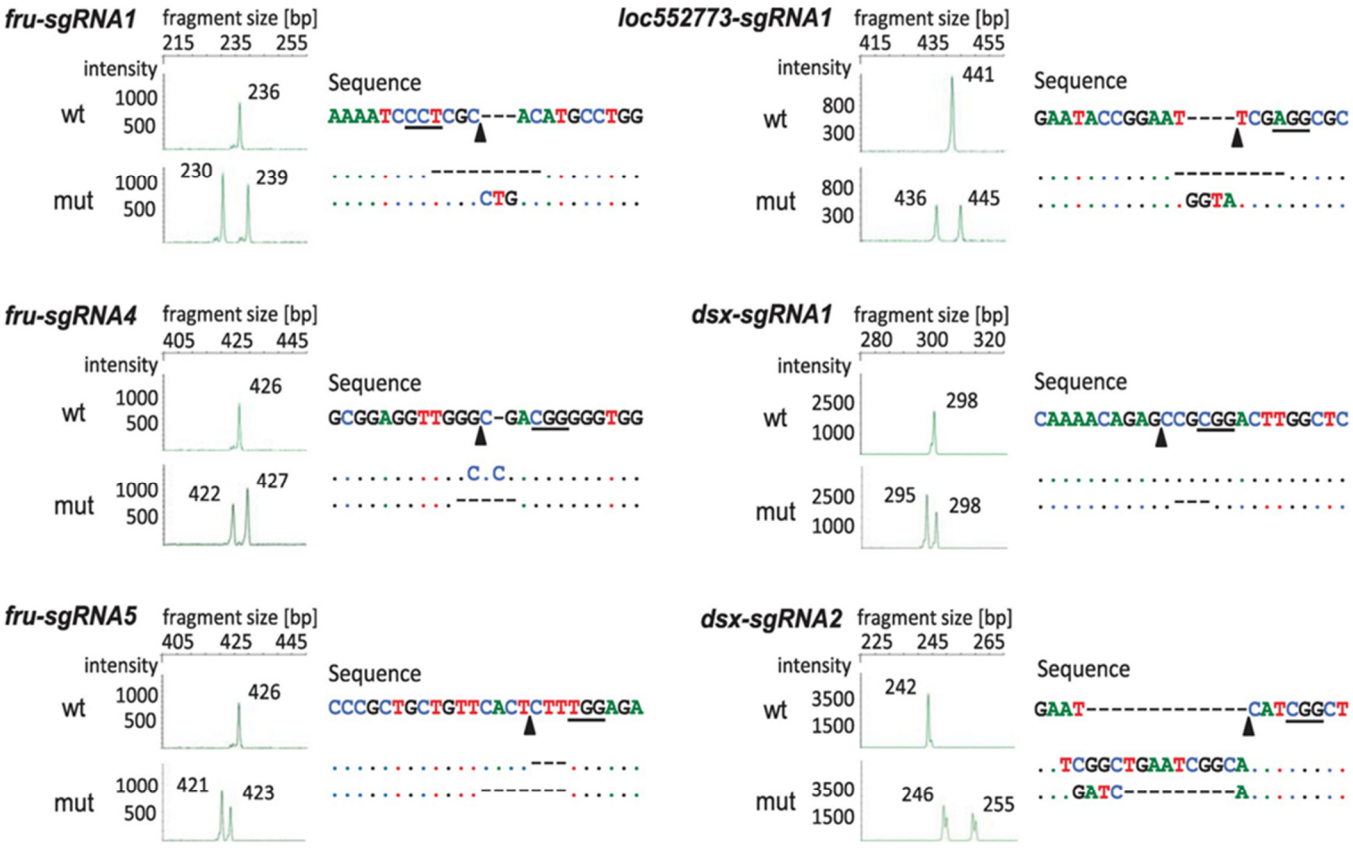

Fig 2. Examples of FL and nucleotide sequence analyses of the targeted genomic sites of single bees using the efficient CRISPR/Cas9 method. FL analysis is presented on the left, and the nucleotide sequences are presented on the right for single bees. Examples of WT alleles and mutated sequences are shown.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

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detected in 30 of the 39 mutated larvae (77%), suggesting that all nuclei (to the level of detection) and both alleles in the larvae were mutated (generating double mutants). More than two mutated sequence variants were detected in a single larva (3%), while singly mutated sequences together with the WT allele were detected in 8 larvae (20%) (S4 and S5 Tables). Indels occurred most frequently between the 5 bp to 1 bp range, with 44% of mutations being deletions and 20% resulting in insertions (S5 and S6 Tables). All mutations occurred at the designated target site. Therefore, our results on the adjustments demonstrate that nearly 80% of the injected embryos had mutations on both alleles (double mutants) affecting the bee entirely (absence of mosaicism). This high proportion enabled us to screen for mutant effects of the sex-determining genes directly in the injected bees.

The *feminizer* gene is required for small size polyphenism

To examine whether the *feminizer* (*fem*) gene is required for small size polyphenism, we mutated the gene in genetic females and reared them with worker nutrition. The *fem* gene instructs female development and maintains the female signal during development, as revealed from *fem* interference RNA (RNAi) knockdown and mosaic studies using a non–worker-

specific diet for bee rearing [19, 38]. The Fem protein is encoded by female-specific spliced *fem* transcripts but not the male spliced variant, which harbors an early stop codon [19] (Fig 3A). The female splicing of *fem* is directed by the *complementary sex determiner* (*csd*) gene when the genotype is heterozygous (Fig 3A) [39]. If the *fem* gene is required for small size polyphenism, we would expect that worker nutrition cannot drive size reduction when *fem* is inactive. If the *fem* gene is dispensable, worker nutrition would drive size reduction even when the *fem* gene is inactive. We induced mutations at two target sites in the first half of the female open reading frame (ORF) of the *fem* gene with *fem-sgRNA1* and *fem-sgRNA2* (S1 and S2 Figs) and reared genetic females with worker nutrition to larval stage 5. Fifteen percent of the reared

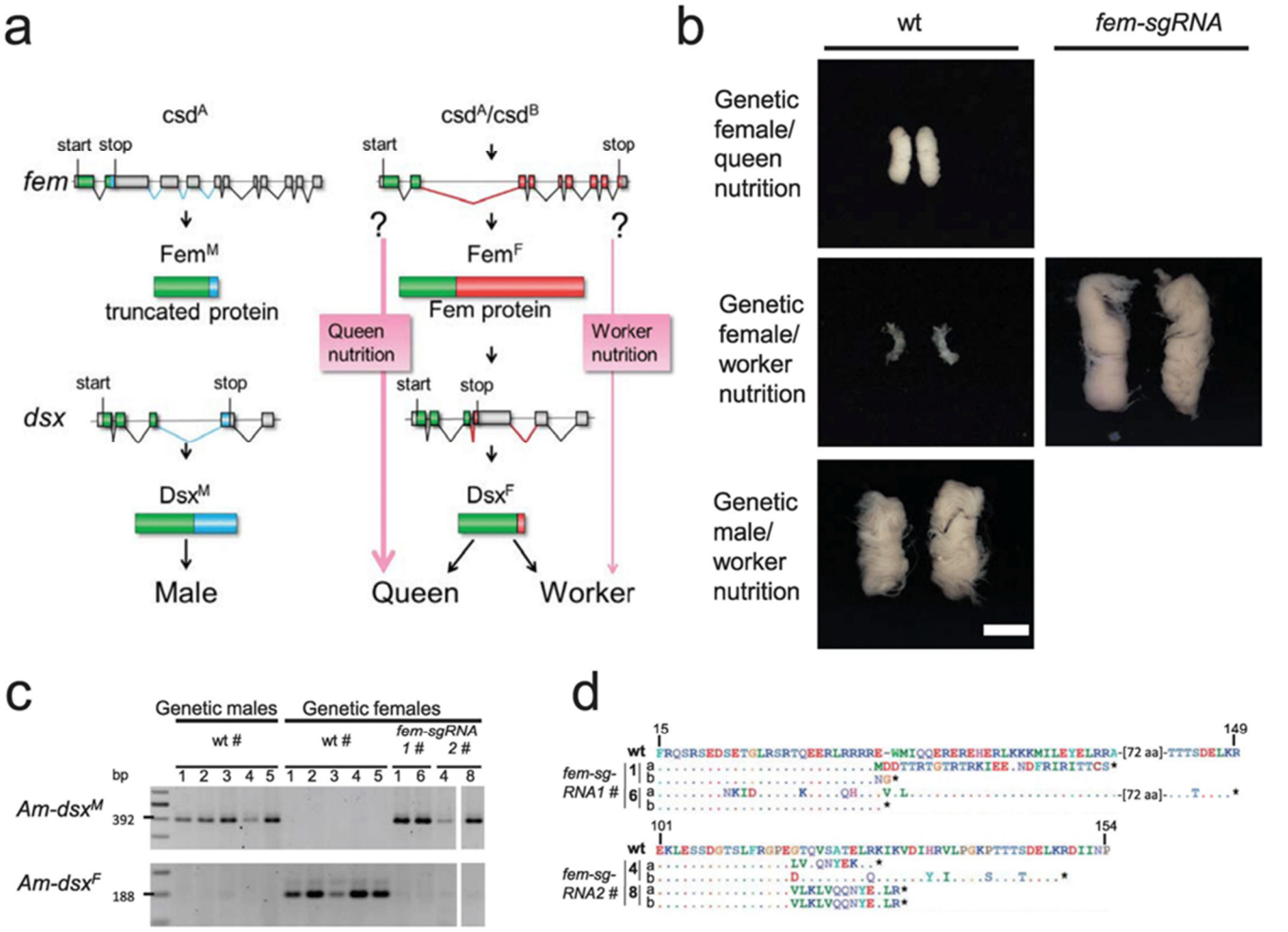

Fig 3. Size polyphenism of gonads in genetic females at larval stage 5 that were double mutants for the *fem* gene. (a) Model of the known components of the sex-determining pathway in honeybees with nutritional differences in females. (b) Gonad development at larval stage 5. (Right) A pair of large gonads (male type) from *fem sgRNA2*-treated genetic females reared on worker nutrition. The gonads display densely packed layers of folded testioles, similar to those observed in haploid males (WT males). (Left) Pairs of small gonads (female type) from WT workers and genetic female bees reared on worker nutrition. A WT large queen ovary from a queen reared in a colony on queen nutrition. A large WT testis of a haploid male manually reared on worker nutrition. (c) Male *dsx* (dsx^M) and female *dsx* (dsx^F) transcripts in mutated genetic females with male phenotypes (*fem-sgRNA1* or *fem-sgRNA2*). Male and female transcripts were separately amplified by RT-PCR [64], and the male and female fragments of each single bee were resolved via agarose gel electrophoresis. Numbers indicate different control and mutated bees. (d) Deduced amino acid sequences from sequenced amplicons of the *fem* gene at the designated CRISPR/Cas9 cleavage sites for the four worker nutrition-reared genetic female larvae with large gonads of the male type. Stars indicate premature translation stop codons. Numbers indicate different mutated bees. Scale bars, 1 mm. CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; dsx^F, female *dsx*; dsx^M, male *dsx*; RT-PCR, reverse transcription PCR; sgRNA, single guide RNA; WT, wild type.

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

dsx is dispensable for small size polyphenism

To examine the role of female *dsx* on size polyphenism of the reproductive organ, we mutated the *dsx* gene in genetic females and reared them on worker nutrition. If *dsx* is dispensable, we would expect small size polyphenism even when *dsx* activity is compromised. In *Drosophila melanogaster*, the *dsx* gene essentially controls, beside the reproductive organs, all aspects of somatic sexual differentiation [40, 41], and it controls at least reproductive organ development in other insects that belong to different insect orders, including hymenopteran insects [42–45]. The *dsx* transcripts in honeybees are sex-specifically spliced by the presence of the Fem protein in females and the absence of the Fem protein in males [19] (Fig 3A). The sexual splice variants encode a transcription factor with an intertwined zinc-containing DNA binding (DM) domain and male- and female-specific termini at the carboxyl end [46–50]. We mutated the *dsx* gene at two target sites in the non–sex-specific expressed N-terminal portion. *dsx-sgRNA2* targeted the DM domain, whereas *dsx-sgRNA6* targeted a downstream region in exon 3 (S1 Fig). The treated genetic females were reared on worker nutrition and were examined for morphological changes of the reproductive organ and head. Genotyping of the mutated bees with morphological changes via next-generation sequencing (NGS) of the amplicons revealed that they were regularly double mutants with an approximate ratio of 1:1, suggesting that the mutations belong to the two chromosomes of the diploid set. If we detected more than two sequence variants per bee, we excluded these bees from further phenotype analysis as they were genetic mosaics (e.g., a mosaic of differently mutated cells). Eleven (17%) of the adult or pupal bees had intersex morphology in the reproductive organs compared with the WT genetic females (S10 Table). No effect was observed for the heads. The following mutations were the most common ones in the genetic females: (i) different nonsense mutations that introduced new stop codons at various positions in exons 2 and 3, (ii) deletions of amino acids in the DM domain mainly the histidine codon at amino acid position 68 (ΔH68), and (iii) deletion of the alanine codon (ΔA191) at amino acid position 191 (Fig 4 with the deduced amino acid sequences and S3 Fig with the detected nucleotide sequences). The ΔH68 mutation removes a histidine of the DM domain that is essential for the zinc binding and DM domain functions [47, 51] and that is conserved between vertebrates and invertebrates (S4 Fig). The intersex reproductive organs were all of the same small size ($n = 11$) as the worker reproductive organs in WT genetic females that were manually reared on worker nutrition ($n = 17$, Table 2, Fisher's exact test, df = 1, $P = 1$). The small intersex reproductive organs displayed either male gonads with poorly or non–sex-specifically differentiated duct systems ($n = 4$), as observed in stop200/stop202 and ΔH68/stop91 genetic females (arrows in Figs 4 and S5). The potentially earlier developmental stage of some of these mutant bees cannot explain why these male-like gonads are so small because the distinct size differences of male and worker gonads are also present at

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Induced mutations in honeybees uncover genetic requirements for nutrition-driven caste development

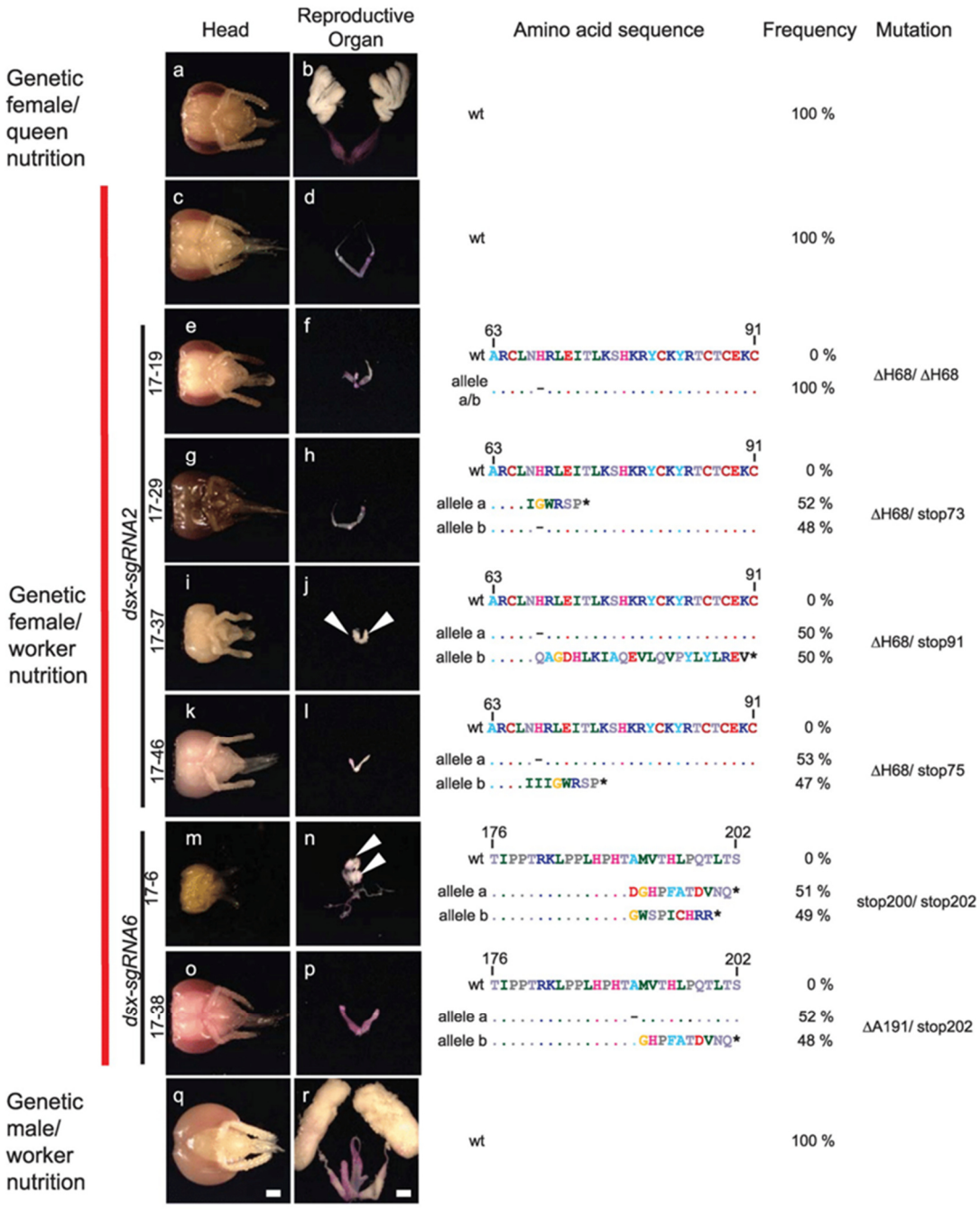

Fig 4. Size polyphenism of the reproductive organs in genetic female double mutants for the *dsx* gene. Pictures of the head and internal reproductive organs of mutated and WT control bees are shown on the left, while the genotypes at the *dsx* locus with the deduced amino acid sequences are displayed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

are marked with arrows. Scale bar, 1 mm. Dashes in the sequence indicate deletions, and stars illustrate early translational stop codons. RJ, royal jelly; WT, wild type.

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earlier pupal stages (S6 Fig). In other cases, the reproductive organs were underdeveloped (n = 7), and the oviducts were consistently misshaped while the ovarioles were repeatedly missing, as identified in ΔH68/ΔH68, ΔH68/stop73, ΔH68/stop75, and ΔA191/stop202 genetic females (Figs 4 and S5). The heads of the mutant genetic females with intersex reproductive organs were all of worker type (n = 11, Fig 4 and S10 Table), suggesting that *dsx* is not required for sexual development of the head. The results of the consistently small, intersex reproductive organs with varying degrees of masculinization suggest that *dsx* is not required for size polyphenism.

Discussion

Caste polyphenism in honeybees is determined by different nutrition with the size of the reproductive organ as an important trait. Most studies suggest that the balance and amount of nutrition (Nutrition/Growth model) drive the size polyphenism between queens and workers. Our genetic and rearing results now suggest that the response to nutrition relies on a genetic program that is switched on by the *fem* gene. The genetic females with a mutant *fem* gene show large size reproductive organ (large polyphenism), while WT genetic females (Fig 5A) reared on the same worker nutrition have only small reproductive organs (small polyphenism). Genetic females that have a mutated *dsx* gene (operating downstream of *fem*) do show small reproductive organs (small size polyphenism; Fig 5A). *dsx* mutants produce intersex reproductive organs and male-like gonads that are all of small size, demonstrating that small size does not rely on female development of the tissue. The small size polyphenism also did not result from *dsx* malfunction because (i) small phenotypes were consistently observed irrespective of the different degrees of *dsx* malfunctions we introduced by missense and nonsense mutations (Fig 4) and (ii) *dsx* mutations in other insects did not influence the size of the reproductive organs [42, 52, 53]. Thus, the results together suggest that the *fem* gene is required for the small size polyphenism. We conclude that the *fem* gene must be switched "ON" so that size polyphenism can be executed (Fig 5B). The essential role of the *fem* gene in small size polyphenism assigns a further key function to the *fem* gene. Previous studies demonstrated that the *fem* gene is also required to (i) induce entire female development in response to the primary signal *csd* [19, 38] and to (ii) maintain the female signal during development via a positive

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

Sex	Nutrition	Genotype	Reproductive organ	Numbers	Size of reproductive organ[a]	
					<2.5 mm; <0.7 times the size of the head width	>6 mm; >1.2 times the size of the head width
Genetic female	Manually reared on worker nutrition	*dsx* double mutants	Intersex	11	11 (100%)	0 (0%)
		WT	Worker	17	17 (100%)	0 (0%)
	Queen diet in colony	WT	Queen	3	0 (0%)	3 (100%)
Genetic male	Manually reared on worker nutrition	WT	Male	16	0 (0%)	16 (100%)

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

Abbreviation: WT, wild type.

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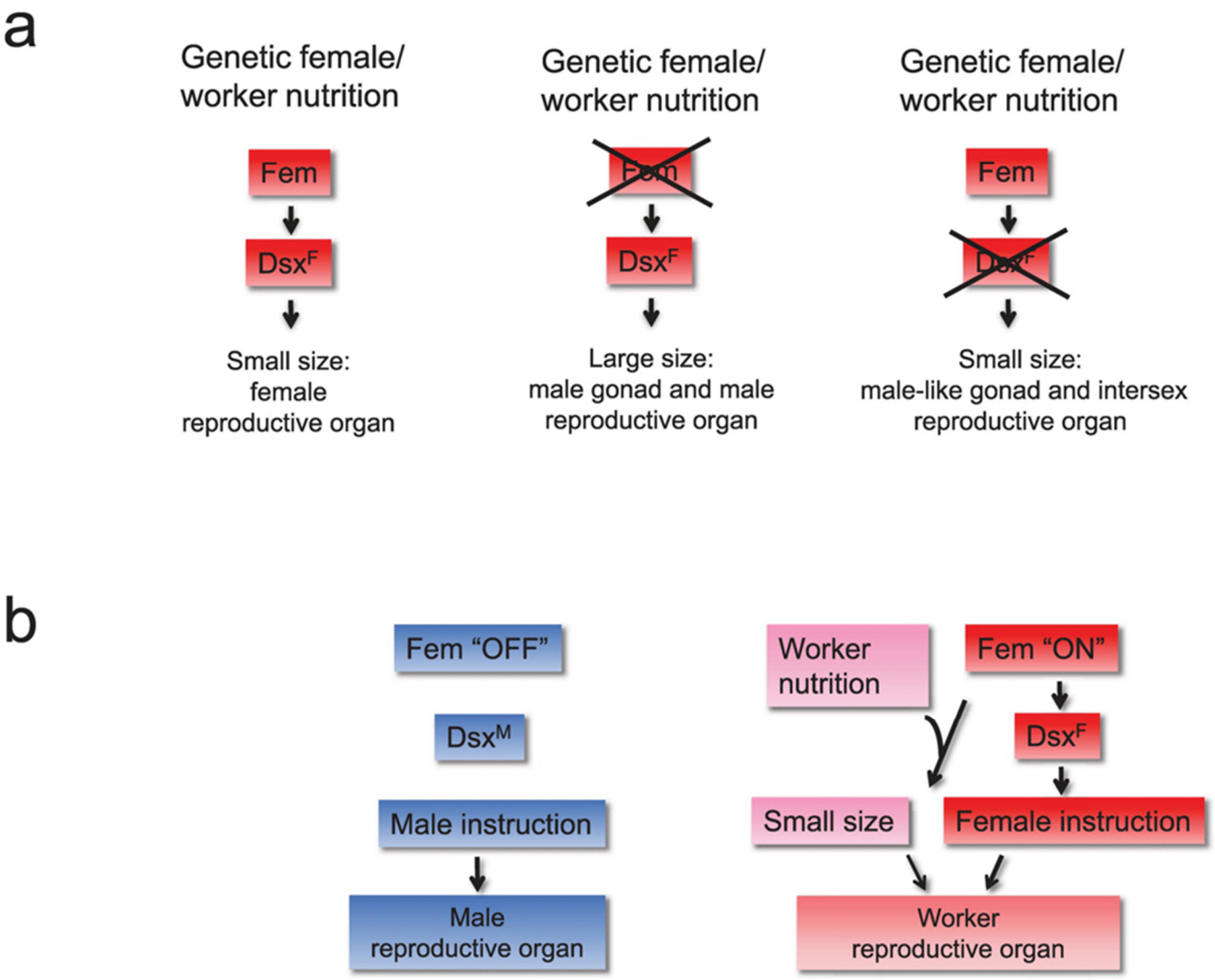

Fig 5. The role of the sex-determining genes *fem* and *dsx* in size polyphenism. (a) Schematic presentation of the mutant effects of *fem* and *dsx* gene on size polyphenism. Genetic female bees reared on worker nutrition produce only small reproductive organs. Genetic females with a mutant *fem* gene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

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regulatory feedback loop [19]. Whether *fem* also instructs the large size polyphenism of queens needs further functional testing once a queen-only rearing protocol has been developed for the laboratory [7].

The genetic instruction via the *fem* gene provides an entry point to dissect nutrition-mediated control. Our results suggest that the *fem* gene switches "ON" the machinery that is required for sensing the worker nutrition and for implementing the size polyphenism. Because the *fem* gene encodes a serine arginine rich (SR)-type protein, the direct targets of the *fem* gene

involved in size polyphenism may also be activated by sexual splicing. The *fem*-controlled candidate genes can be functionally tested by determining whether they affect the size polyphenism. The function will be directly tested in mutated genetic females as demonstrated in this study.

Our mutant analysis further demonstrate that *dsx* controls female differentiation of the reproductive organs. The mutant honeybee phenotypes of the reproductive organs in honeybees yielded similar phenotypes as in female *D. melanogaster*. Female *dsx*-mutant fruit flies have reproductive organs of varying intersex phenotypes. The organs are often underdeveloped with occasionally developed ovaries, but are frequently of the "male type" [52, 54, 55]. The internal duct system can develop into a mixture of female/male or single poorly differentiated ducts [52]. RNAi-mediated knockdown studies on the beetle *Tribolium molitor*, housefly *Musca domestica*, and sawfly *Athalia rosae*, as well as conditional expression and CRISPR/Cas9 experiments on the silkworm *Bombyx mori*, have revealed sex-related effects on internal reproductive organ development [42–46, 53]. Our results support a conserved role for *dsx* in the sexual development of the reproductive organ. However, in honeybees there is a nutrition-driven size control of reproductive organ development that operates upstream of or in parallel with *dsx*-regulated sexual development.

The first CRISPR/Cas9-induced morphological mutants in honeybees introduced a new genetic screening method for worker bees. We efficiently induced mutations in injected embryos using the CRISPR/Cas9 method [34, 35] and directly screened for somatic mutations in the reared honeybees (somatic mutation approach). Up to 100% of the embryos were mutated, and mosaicism among the mutated embryos was rare (<10%). The previous studies in honeybees using CRISPR/Cas9-induced mutations report on 1 out of 2 queens with only 12% and 2 out of 4 queens with only 5% and 10% mutant drone offspring, suggesting that the previously published method has a substantial lower rate and produced strong mosaicism in the queens [36, 56]. These previous studies generated no worker bees that would require further crossing experiments. With very early embryonic injections [37] and a selection step to identify the most efficient sgRNAs and Cas9 concentrations, we generated mutation rates of up to 100% and no mosaicism in worker bees directly. The rearing of the mutated embryos to worker bees was performed under controlled conditions in the laboratory [16, 33]. This required no rearing of queens and drones and crossing experiments. The procedure was demonstrated for mutations at two target sites for two genes and their morphological changes (Figs 3 and 4). The absence of mosaicism and completeness of mutagenesis of this procedure were shown by the results that most mutated bees lost the WT allele (they were double mutants; Figs 2, 3D and 4) and that double *fem* nonsense mutations produced an entire female to male switch, including *dsx* splice products (Fig 3C). This somatic mutation approach does not require further crossing experiments and laborious maintenance of hundreds of colonies and therefore offers the prospect of larger genetic screens in honeybees. In other insects in which somatic mutation approaches have been applied [57, 58], the adults were genetic mosaics in which parts of the butterfly wing were WT while other parts were mutated. Enhancing the efficiency of mutagenesis can thus provide an opportunity for somatically testing gene functions in insects that are not yet genetically trackable.

Methods

sgRNA and mRNA syntheses

Cas9 mRNA was synthesized from the *Cas9* gene [59] (Vector MLM3613, ID #42251, Addgene, Cambridge, MA) using a linearized plasmid via the T7 promoter and the mMESSAGE mMACHINE Kit (Ambion, Darmstadt, Germany). mRNAs were polyadenylated using

the Poly(A) Tailing Kit (Ambion). Target sites for the sgRNAs were identified via Optimal Target Finder software (http://tools.flycrispr.molbio.wisc.edu/targetFinder/). sgRNAs were 20 nt long with a G nucleotide at the 5′ end. sgRNAs with no off-target effects or with at least three nucleotide mismatches to alternative target sites were selected. sgRNAs were generated via PCR without a template using two overlapping oligonucleotide sequences containing the sequence of the T7 RNA polymerase transcription start site, the gene-specific target site and the Cas9 protein-binding site. sgRNAs were synthesized using a RiboMax Kit (Promega, Madison, WI) according to the manufacturer's instructions. RNAs were purified using the MEGAclear Kit (Ambion).

Microinjection and rearing

Embryos were microinjected 0 to 1.5 hours after egg deposition [19, 37, 60] using 53-mm injection pipettes (Hilgenberg, Malsfeld, Germany). Cas9 mRNA or protein (New England Biolabs, Ipswich, MA) was applied at 400 to 2,000 ng/μl and mixed with sgRNAs using a molar ratio of 1:2 to 1:0.75. The number of injected embryos that hatch can vary greatly between experiments and sgRNAs (5% to 40%). Rearing was performed using a mass rearing technique for the worker bees [16, 33]. Freshly hatched larvae were provisioned only once with the worker larval diet (50%–53% RJ, 4% glucose, 8% fructose, 1% yeast extract, and 30%–34% water), approximately 120 to 170 mg of which was consumed [16, 33]. The larvae were incubated at 34˚C and 90% humidity until the larval stage 5 or to adults. For pupal rearing we also used a slightly different diet for larvae at stage 5 (50 mg diet 2 [50% RJ, 12% fructose, 6% glucose, 2% yeast extract, and 30% water]).

DNA preparation, RNA isolation, and cDNA synthesis

For genotyping, genomic DNA was isolated from freshly hatched L1 or L5 larvae [61] using the peqGOLD Tissue DNA Mini Kit (VWR, Darmstadt, Germany). RNA was isolated using the TRIZOL method (Thermo Scientific, Braunschweig, Germany), and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Second-strand cDNA synthesis was performed by adding 10 μl of 10× DNA Polymerase Buffer, 40 U DNA Polymerase I, 0.8 U Ribonuclease H, and 65.68 μl of dH_2O to 20 μl of the cDNA first-strand synthesis product. Double-stranded cDNA was purified using the EZNA Cycle Pure kit (Omega Bio-Tek Inc., Norcross, GA).

PCR, sequencing, and FL analysis

All mutant bees were genotyped by sequencing the amplicons of the targeted site. PCR amplifications were performed using standard conditions [62] and GoTaq polymerase (Promega). Oligonucleotide sequences were synthesized at Eurofins (Ebersberg, Germany). Amplicons were either cloned and sequenced (Sanger sequencing [Eurofins]) or sequenced via NGS. NGS index PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA), and purification of the Index PCR products was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). NGS was performed on an Illumina MiSeq system using the MiSeq Reagent Kit version 2 (500 cycles; Illumina), generating 800,000 paired-end reads with a read length of 2 × 250 bp, resulting in approximately 15,000 paired-end reads per sample. We removed contamination by removing sequences that were less frequent than 5%. The FLs of hexachlorofluorescein (HEX)-labeled amplicons were determined using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and Peak Scanner software (Thermo Scientific). For the *fem* mutants, we conducted fragment and sequence analysis on

the amplicons of the cDNAs to ensure that the many *fem*-related sequences observed at the genomic *fem* locus (derived from duplication events) [63] were not amplified.

Supporting information

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

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

S3 Fig. Genotypes of *dsx*-mutated females of Fig 4 as obtained from NGS analyses. The *dsx* WT nucleotide sequences are represented as a reference sequence. NGS, next-generation sequencing; WT, wild type.
(PDF)

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

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

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

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

S2 Table. The genetic male bees reared in colony and manually on worker nutrition.
(PDF)

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

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

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

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

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

S8 Table. Large gonads of the male type in genetic females double mutant for *fem*.
(PDF)

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

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

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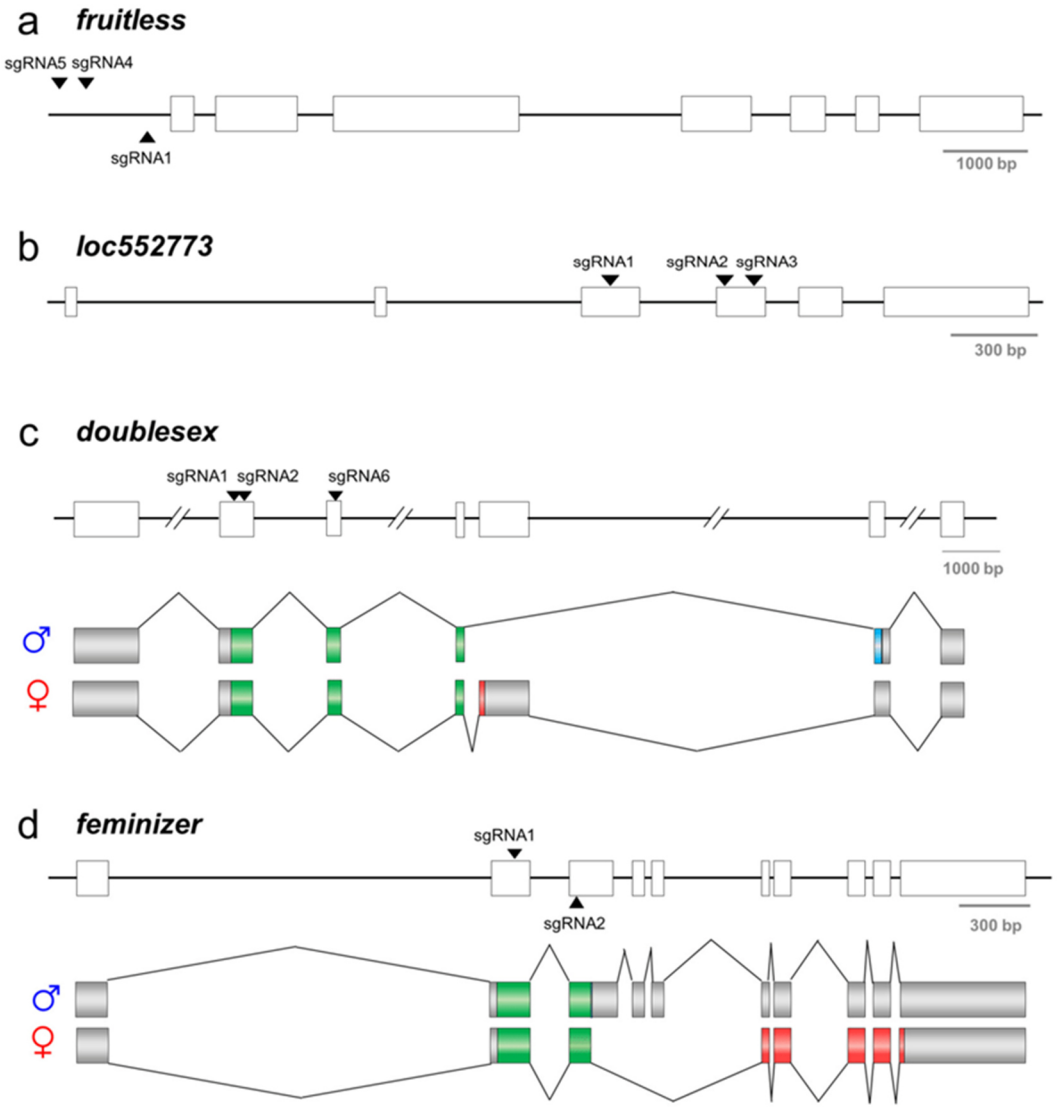
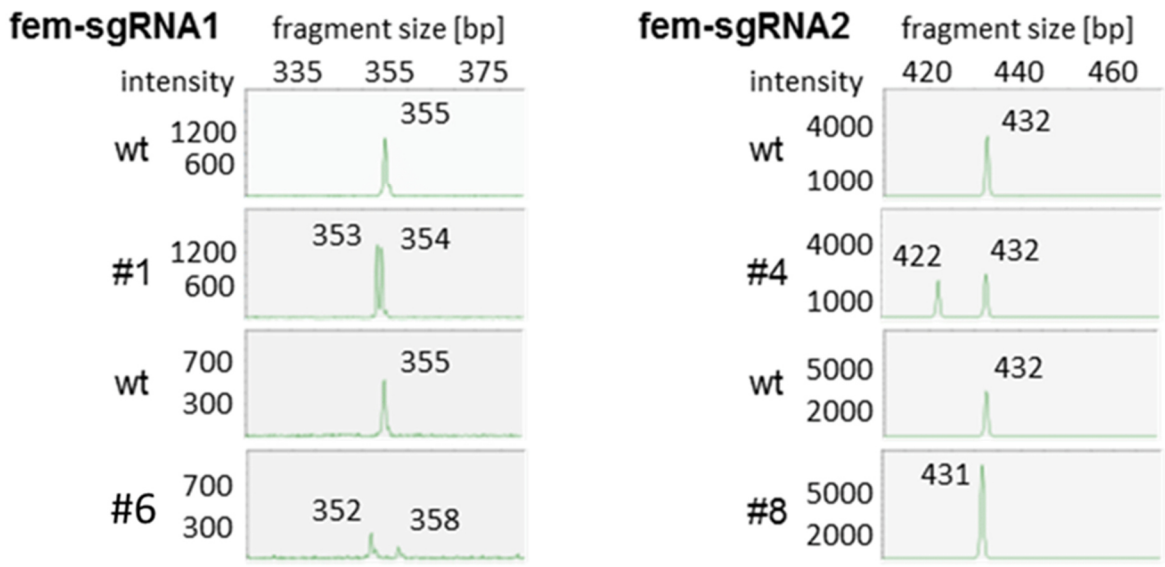
Supplementary data



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

a



b

Injected sgRNA	Larva No.	Alignment of *fem* sequences
fem-sgRNA1	1	Wildtype AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT Allele a AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT Allele b AAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAACGATT ACGACGTAGACGCGAATGGATGATACAACAAGAACGGGAACGAGAACACGAA ACGACGTAGACG--AATGGATGATACAACAAGAACGGGAACGAGAACACGAA ACGACGTAGACG-GAATGGATGATACAACAAGAACGGGAACGAGAACACGAA
fem-sgRNA1	6	Wildtype TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG Allele a TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG Allele b TGAAACGGAATACAACAAATCATTCGCATCATGATGAGAGATTTAG ACAATCACGCAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAA ACAATCACGCAGTGAAGATAGCGAAACTGGTCTGCGTTCAAGAACACAAGAAGAA ACAATCACGCAGTGAAGATAACAAAATTGATCTGCGTTCAAGAACAAAAGAAGAA CGATTACGACGTAGACGCGAA---TGGATGATACAACAAGAACGGGAACGAGAAC CGATTACGACGTAGACGCGAA---TAGAT---ACAACAAGAACGGGAACGAAAAT CGATTACAACATAGACGCGAAGTGTGGTTGATACAACAAGAACGGGAACGAGAAC ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA ACGAAAGATTGAAGAAAAAAATGATTTTAGAATACGAATTACGACGTGCTCGTGA GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGT GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAGT GAAAAAATTATCGAAAAGAAGTAAAAGTAGATCCCCAGAAAGCCGAGGTAGAAAT AATGCATCAAACACGTCTAAAACATTTATATTATCTGAAAAATTAGAATCTTCAG AATGCATCAAACATATCTAAAACATTTATATTATCCGAAAAATTAGAATCTTCAG AATGCATCAAATATATCTAAAACATTTATATTATCCGAAAAATTAGAATCTTCAG ATGGTACATCTTTATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACT ATGGTACATCTTTATTTAGAGGACCAGAAGGTACTCAAGTTAGTGCAACAGAACT ATGGTACATTTTTATTTAGAGGACCAGAAGATACTCAAGTTAGTGCAACAGAATT ACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAACAACAACA

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

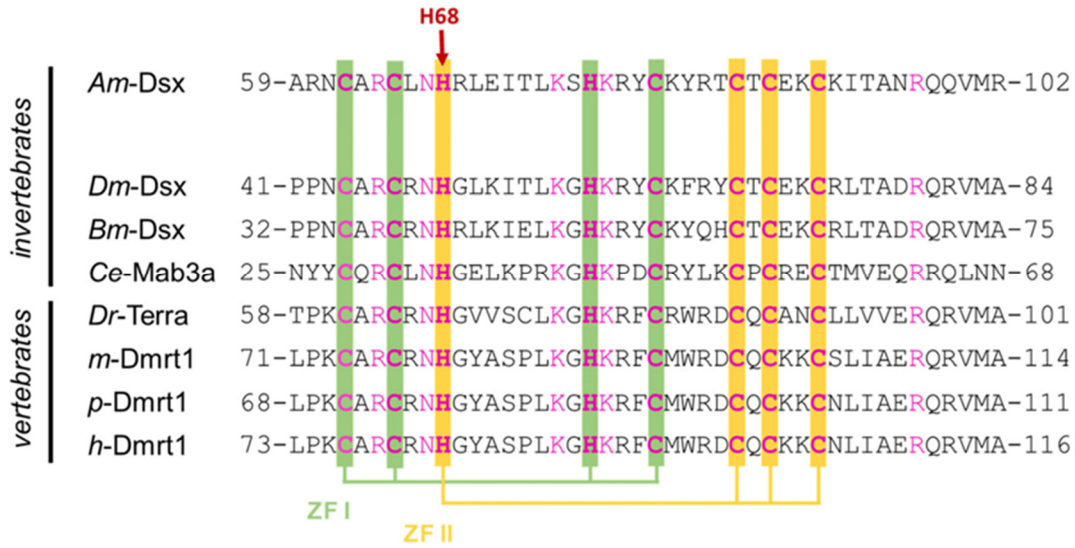
		Allele b GGTACATCTTTATTTAGAGGACCAGAAG-TACTCAAGTTAGTGCAA CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC CAGAACTACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAAC

S2 Fig. The nucleotide sequences of the fem-mutated genetic females that were reared on worker nutrition and that have large-sized gonads of the male type. (a) Diagrams of the FL analysis for each of the 4 individuals and WT worker bee examples. (b) The nucleotide sequences. We conducted fragment and sequence analysis on amplicons of cDNA to ensure that the many *fem*-related sequences observed at the *fem* locus (derived from duplication events) (Koch et al., 2014) were not amplified. The designated binding sites of the sgRNAs are underlined. Sequence b in larvae #4 resulted from fusion of exon 3 with exon 5. The sequences in larvae #4 resulted from fusion between exon 3 and other *fem*-related sequences (Koch et al., 2014). The WT sequences were obtained from a sample of 5 WT worker larvae (5 clones each). cDNA, complementary DNA; FL, fragment length; WT, wild type.

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

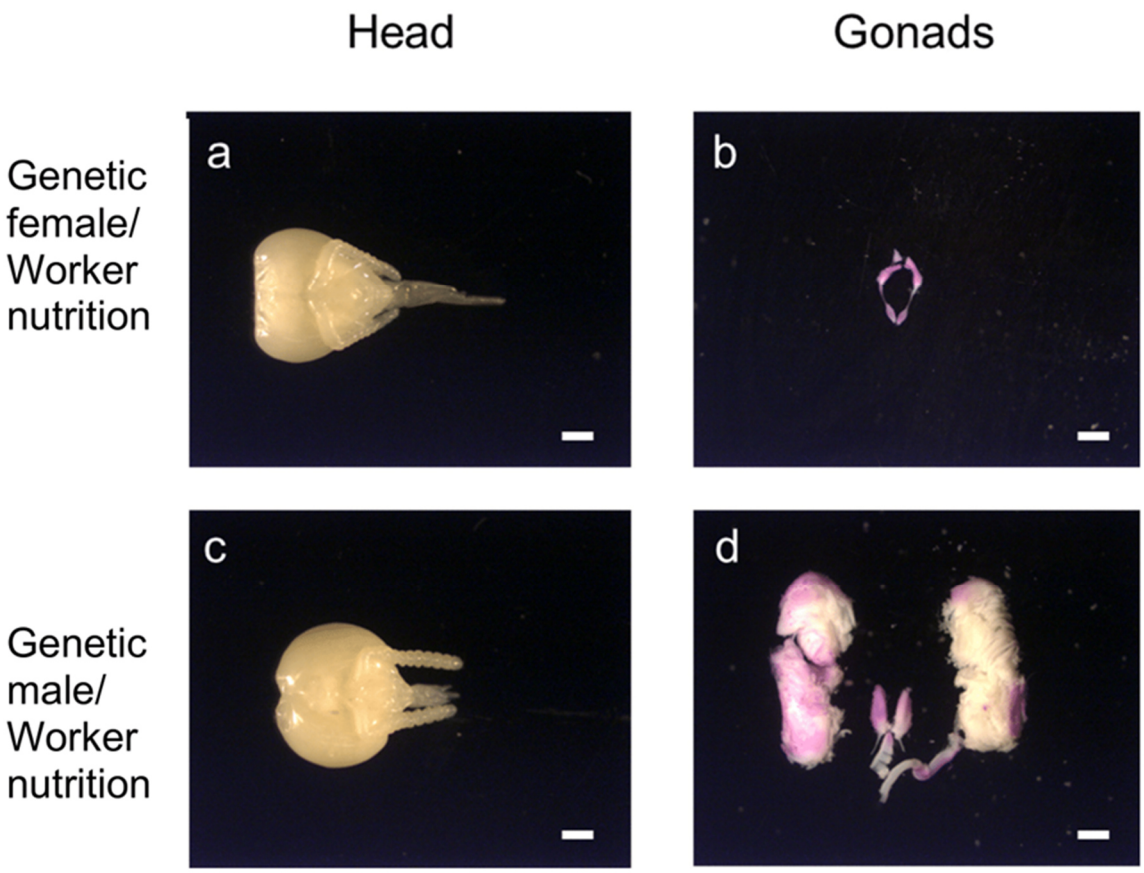
		Allele a .....-----..... Allele b .....TCATGATCCTGC.....
dsx-sgRNA6	17-6	Wildtype GCATCCTCACACTGCGATGGTCACCCATTTG Allele a .....--..... Allele b .....-.....
dsx-sgRNA6	17-38	Wildtype GCATCCTCACACTGCGATGGTCACCCATTTG Allele a .....---..... Allele b .....-----.....

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



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

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



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

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

		Numbers	Worker phenotypes		
			Head [1] (triangular shaped; upper part straight between compound eyes)	Size of the female reproductive organ [2] (length < 2,5 mm; < 0.7 times the size of the head width)	Ovariole numbers [3] (< 25)
Genetic female	Worker diet in colony	14	14 (100%)	14 (100%)	14 (100%)
	Manually reared on worker nutrition	15	15 (100%)	15 (100%)	15 (100%)

1) Frontal view of head. In contrast to workers, queens have a roundish shaped head; the upper part is curved between compound eyes (see Fig. 4a and b in the main text).

2) Length between the fused left and right part of the reproductive organ to its end in the sagittal plane. The length in queens is > 6 mm and > 1.2 times the size of the head width.

3) Ovariole number in queens is > 100 [11]

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

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

1) Frontal view of head

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

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

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

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

		No. of length-modified sequences [1]			Sum
		1	2	3	
No. of larvae	Without wt allele (%)	10 (26%)	20 (51%)	1 (3%)	31 (79%)
	With wt allele (%)	8 (21%)	0 (0%)	0 (0%)	8 (21%)

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

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

Injected sgRNA	Larva No.	Alignment of the nucleotide sequence at the target sites
fru-sgRNA4	1	Wildtype GAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGCGACGGGGGTGGCG Allele a GAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGC---------- Allele b GAGGGGACGGGTGGAAGCTGGCGGAGG---------- GCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTGG --CGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTGG ----------CTAAAGGGGAAAGGGGGGTGG
fru-sgRNA4	2	Wildtype GGAGGGGACGGGTGGAAGCTGGCGGAGGTTGGGCGACGGGGGTGGC Allele a GGAGGGGACGGGTGGAAGCTG----------ACGGGGGTGGC Allele b GGAGGGGACGG---------- GGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTG GGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAGGCTAAAGGGGAAAGGGGGGTG ---------- GCGGGAGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG GCGGGAGTGGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG ----------CGAACATAAAATCCCTCG CACAT CACAT CACAT
fru-sgRNA4	3	Wildtype AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele a AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele b AGTGGCGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA GGTTGGGC-GACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTGGCCCGACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTG-----ACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG
fru-sgRNA5	2	Wildtype GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACTC Allele a GGCTTCAACGCGGCTCGGTTGGGTGGTGACCCGCTGCTATTCCCT- Allele b GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACT- TTTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA
fru-sgRNA5	4	Wildtype GTTGGGTGGTGGCCCCGCTGCTGTTCACTCTTTGGAGAGGAAAGGGT Allele a GTTGGGTGGTGGCCCCGCTGCTGTTC---CTTTGGAGAGGAAAGGGT Allele b GTTGGGTGGTGGCCCCGCTGCTGTTCA--CTTTGGAGAGGAAAGGGT

		TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAG---GAGGA GGTGGGGGGAGAGTGG GGTGGGGGGAGAGTGG GGTGGGGGGAGAGTGG
fru-sgRNA5	7	Wiltdype GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACTC Allele a GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACT- Allele b GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTT----- TTTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA --TGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA --TGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA
dsx-sgRNA1	12	Wildtype TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele a TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele b TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGC---GGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT
dsx-sgRNA1	16	Wildtype TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele a TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA Allele b TGTGAATCGAGGTTACCTATGTATCGCGAAGAGAACGAGCAAAACA GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGCCGCGGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT GAGT---GGACTTGGCTCCCCAACAACCGAGTGGTGCAAACACGTTCGAGCGTTT
dsx-sgRNA2	9	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAT--- Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGATCGGC Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGATC ----------CATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA TGAATCGGCACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA ---------ACATCGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGTGTA
dsx-sgRNA2	11	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGCTG Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAAGCTG CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA TCGT--GAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA TCGT--GAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA
dsx-sgRNA2	68	Wildtype TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele a TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCAT Allele b TACTCCAAAGCCGCGTGCACGGAATTGTGCACGATGTCTGAATCGT CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA CGGCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA --GCTGGAGATCACCTTAAAATCGCACAAGAGGTACTGCAAGTACCGTACTTGTA
loc552773-sgRNA1	3	Wildtype CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAT- Allele a CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG Allele b CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGCTGG ----------TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC CGCCACCCATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC AATA------TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCC
loc552773-sgRNA1	6	Wildtype CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAT- Allele a CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG Allele b CGATCGATCAGCTTCGTGACAAATTATCGGCTGGAATACCGGAAGG ---TCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG TATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG TATTCGAGGCGCCACCCATCGAGCCCCTATACCTCAAGCAAATTCGCCTGTCCAG

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

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

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

S7 Table. The heterozygous, female genotype of thecsdgene in the femdouble nonsense mutants.

Injected sgRNA	Larva No.	Alignment of the hypervariable region of the *csd* alleles
fem-sgRNA1	1	*csd* allele 1 GAACCTAAAATAATTTCATCTTTATCGAACAATTACAATTAT *csd* allele 2 GAACCTAAAATAATTTCATCTTTATCGAGCAATTACAATTCT AATAATAATAATTATAATAATTATAATAATTATAATAATTATAATAATAATTATA AACAATTATAATAATTATAGTACTAATTAT---------- ATAATAATTATAATAAAAAATTA------TATTACAATATTAATTATATTGAACA --------------AAACAATTACAATATTGTTACAATATTAATTATATTGAACA AATTCCTGTTCCTGTT AATTCCTATTCCTGTT

fem-sgRNA1	6	*csd* allele 3 GAACCTAAAATAATTTCATCTTTATCGAATAAGACAATACAT *csd* allele 4 GAACCTAAAATAACTTCATCTTTATCGAACAATTACAATTCT AATAATAATAATTAT----------AAAAAATTATATT AATAATTATAATAATTATAATAAATATAATTATAATAATTCTAAAAAATTATATT ACAATATTAATTATATTGAACAAATTCCTATTCCTGTT ACAATATTAATTATATTGAACAAATTCCTGTTCCTATT
fem-sgRNA2	4	*csd* allele 5 GAACCTAAAATAATTTCATCTTTATCGAACAAGACAATACAT *csd* allele 6 GAACCTAAAATAATTTCATCTTTATCGAACAATTACAATTAC AATAATAATAAATATAATTATAATAATAATTATAATAATAATTGTAAAAAATTAT AACAATTATAATAATAATTAT----------AAACCATTAT ATTACAATATTAATTATATTGAACAAATTCCTATTCCTGTT ATTACAATATTAATTATATTGAACAAATTCCTGTTCCTGTT
fem-sgRNA2	8	*csd* allele 8 GAACCTAAAATAATTTCATCTTTATCGAATAATACAATACAT *csd* allele 9 GAACCTAAAATAATTTCATCTTTATCGAACAAGACAATACAT AATAATAATTATAAATATAATTATAATAATAATTATAATAA---TTATAAAAAAT AATAATAAT---AAATATAATTATAATAATAATTATAATAATAATTGTAAAAAAT TATATTACAATATTAATTATATTGAACAAATTCCCGTTCCTGTT TATATTACAATATTAATTATATTGAACAAATTCCTATTCCTGTT

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

Treatment	Number	Number of bees with large gonads (male type) and double nonsense mutations
fem-sgRNA (sgRNA1/sgRNA2)[1)]	27 (11/16)	4 (15%) (2 /2)
Untreated	38	0 (0%)

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

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

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

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

Treatment	Experiment	Numbers of bees at larval stage 1	Numbers of bees at stage of phenotyping[1)]	Bees with worker head [2)]	Bees with intersex reproductive organ	% of intersexes with *dsx* double mutations
dsx-sgRNA2	1	125	41	41 (100%)	4 (10%)	100%
	2	362	11	11 (100%)	5 (45%)	100%
dsx-sgRNA6		39	11	11 (100%)	2 (18%)	100%
No treatment		82	34	34 (100%)	0%[3)]	0%

1) Genetic mosaics were excluded

2) Frontal view: triangular shaped; upper part straight between compound eyes

3) 17 out of 34 were dissected

Author's Contribution: Manuscript I

A genetic switch for worker nutrition-mediated traits in honeybees

Journal: PLOS Biology

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3rd author

Author's contribution:

- Dissections of individuals
- analysis of phenotype

Manuscript II

Diversification of splice regulation during sexual development in honeybees (*Apis mellifera*), a haplodiploid system

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Abstract

The honeybee is a haplodiploid species in which sexual development is determined by the *complementary sex determiner* (*csd*) gene and is realized by sex-specific splice processes involving the *feminizer* (*fem*) gene. We used next generation transcriptome sequencing (RNA-Seq) to characterize transcriptional differences between the sexes caused by the fertilization and the sex determination process in honeybee (*Apis mellifera*) embryos. We identified 758, 372 and 43 differentially expressed genes (DEGs) and 58, 176 and 233 differentially spliced genes (DSGs) in 10-15 h old, 25-40 h old and 55-70 h old female and male embryos, respectively. The early difference in male and female embryos in response to the fertilisation and non-fertilization process resulted mainly in differentially expressed genes (758 DEGs versus 58 DSGs). In the latest sampled embryonic stage the transcriptional differences between the sexes were dominated by alternative splicing of transcripts (43 DEGs versus 233 DSGs). Interestingly, differentially spliced transcripts that encode RNA-binding properties were overrepresented in 55-70 h old embryos indicating a more diverse regulation via alternative splicing than previous work on the sex determination pathway suggested. The stage and sex-specific transcriptome data in honeybee embryos provide a comprehensive source to examine the role of fertilization and sex determination on developmental programming in a haplodiploid system.

Introduction

The regulation of sexual development is one of the major questions in biology. Despite general principles of female and male development, different species show a diversity of primary sex determination signals (Cline, 1979; Cline and Meyer, 1996; Beye et al., 2003; Sanchez, 2010; Hall et al., 2015; Krzywinska et al., 2016; Sharma et al., 2017; Meccariello et al., 2019). A common theme is that the diverse primary signals regulate directly or indirectly the sex-specific expression of the *doublesex* (*dsx*) gene repeatedly via *transformer* (*tra*) homologous genes (Tian and Maniatis, 1993; Pane et al., 2002; Ruiz et al., 2007; Lagos et al., 2007; Concha and Scott, 2009; Gempe et al., 2009; Hediger et al., 2010; Verhulst et al., 2010; Shukla and Palli, 2012; Schetelig et al., 2012; Li et al., 2013; Morrow et al., 2014; Suzuki et al., 2015; Liu et al., 2015; Jia et al., 2016; Gotoh et al., 2016; Luo et al., 2017; Geuverink et al., 2018a; Geuverink et al., 2018b; Petrella et al., 2019; Morita et al., 2019).

Honeybees are haplodiploid organisms in which females derive from fertilized and males from unfertilized eggs, a principle which is found in about 20% of animal species (Dzierzon, 1845; Bull, 1983; Cook, 1993). The sex determination process in honeybees relies on the *complementary sex determiner* gene (*csd*), which controls sexual fate via its heterozygous and homozygous/hemizygous genotype. In heterozygous females the Csd proteins direct the sex-specific splicing of the *tra* homolog in *Apis mellifera feminizer* (*fem*; Beye et al., 2003; Hasselmann et al., 2008; Gempe et al., 2009). The *fem* gene regulates female-specific splicing of *dsx* transcripts. In the absence of Fem proteins *dsx* is spliced by default (Gempe et al., 2009). Dimorphic spliced transcripts of *dsx* encode the transcription factors Dsx^F and Dsx^M that differ in the C-terminal end (Erdman et al., 1996; An et al., 1996; Cho et al., 2007). In female honeybees, the *dsx* gene does not control external sexual dimorphic traits. Disruption of the zinc finger domain via CRISPR/Cas9 mediated mutations had no effect on head differentiation of female workers, while the development of reproductive organs was masculinized (Roth et al., 2019), suggesting that at least one other gene beside *dsx* is required to control the development of sexual morphology. This study aims to examine the patterns of gene regulation that associate with haplodiploidy and sex determination. To do so, we deeply sequenced the transcriptomes of male and female embryos using RNA-Seq at different developmental stages. We studied expression differences in haploid and diploid embryos at 10-15 h after egg laying (AEL) to identify genes that are regulated

by the fertilization process. This time window comprises the blastoderm stage (at 10 h AEL; Nelson, 1915; Schnetter, 1935; Fleig and Sander, 1985) and the initial phase of *csd* expression (12 h AEL; Beye et al., 2003). We also examined sexual regulated transcripts of embryos 25-40 h AEL to identify genes that are controlled by the *csd* gene. At this developmental stage (during the gastrulation 33-40 h AEL; Fleig and Sander, 1985; Fleig and Sander, 1986) female specific splicing of *fem* is induced by the *csd* gene (33 h AEL; Gempe et al., 2009). Moreover, we compared male and female transcriptomes of embryos 55-70 h AEL to identify expression differences that are controlled by the *fem* gene. Female specific splicing of *dsx* transcripts, which is controlled by the Fem protein, is present 60 h AEL (Tanja Gempe, personal communication; Gempe et al., 2009). This time window represents the stage in which the larval body is completed (from 55 h AEL on; Fleig and Sander, 1986).

Results

Number of sex-biased spliced genes increases with the progression of embryonic development

For each time window 10-15 h, 25-40 h and 55-70 h AEL we sequenced two replicates of pooled embryos with an average of 72 million 100 bp reads per sample (Table 1). On average, 69 % of the reads could be mapped to the honeybee genome Assembly v.4.5. We obtained FPKM values (fragments per kilobase of exon per million reads mapped) using the software Cuffdiff (Trapnell et al., 2012). We found that FPKM values between replicates were highly correlated (Spearman's ρ 0.92-0.95; Supplementary table 1). These correlation coefficients were all well above 0.9 and in the range of correlations observed in previous studies on the honeybee transcriptome (Vleurinck et al., 2016; He et al., 2019) indicating a reliable quality of our sequencing approach.

FPKM comparisons revealed that in embryos 10-15 h AEL 758 genes were differentially expressed (DEGs) in the two sexes ($p < 0.001$, Table 2). We found in embryos 25-40 h AEL 372 genes and in embryos 55-70 h AEL 43 genes were sexually differentially expressed. These results demonstrate that the number of sexually dimorphic expressed genes decreases with the progression of embryonic development.

When we analysed splice junctions using the software Spanki (Sturgill et al., 2013) we found 58 differentially spliced genes (DSGs) in males and females in 10-15 h, 176

genes in 25-40 h and 233 genes in 55-70 h old embryos. This result shows that the number of sex-specific spliced genes increases with the maturing age of the embryo and progression of development.

Table 1: Number of sequenced and mapped reads.

Condition and replicate	Number of sequenced reads	Number of mapped reads	Proportion of mapped reads
M1-a	74761874	51516899	68.9 %
M1-b	88100392	63812966	72.4 %
M2-a	71080138	47779051	67.2 %
M2-b	63404944	42994429	67.8 %
M3-a	64624747	46872807	72.5 %
M3-b	69666200	54207595	77.8 %
F1-a	73545785	48017220	65.3 %
F1-b	69219183	46402985	67.0 %
F2-a	86089063	58358971	67.8 %
F2-b	70893558	47295742	66.7 %
F3-a	68941625	45300195	65.7 %
F3-b	69103047	46149672	66.8 %
Mean	72452546	49892378	68.8 %

* F: female, M: male
1: 10-15 h old embryos; 2: 25-40 h old embryos; 3: 55-70 h old embryos
a and b replicates of the same condition

Table 2: Differentially expressed and spliced genes in male and female honeybee embryos

		10-15 h after egg deposition	25-40 h after egg deposition	55-70 h after egg deposition
differentially expressed*	total	758	372	43
	protein coding	739	346	39
	ncRNA	18	36	4
	pseudogenes	1	-	-
differentially spliced	total	58	176	233
	protein coding	56	167	220
	ncRNA	2	9	13

*cut off value $p < 0.001$

Next, we studied if the same genes are sex-biased expressed in more than one of the developmental time windows. For the alternatively spliced genes in males and females, we found the largest overlap of 19 genes that were consistently sex-biased spliced in embryos 25-40 h AEL and 55-70 h AEL (Figure 1). We observed an overlap of 55 DEGs in 10-15 h and 25-40 h old embryos which was the highest number of consistently differentially expressed transcripts in two analysed time windows. Only

one gene was differentially expressed in all three sampled stages while no gene was consistently alternatively spliced in male and female embryos. These results indicate, that there is consistent usage for a set of sexually DEGs in the first half of honeybee embryogenesis (15-40 h AEL) which is lost during later stages. In contrast, differences in gene regulation in later stages (25-75 h AEL) rely on sets of alternatively spliced genes.

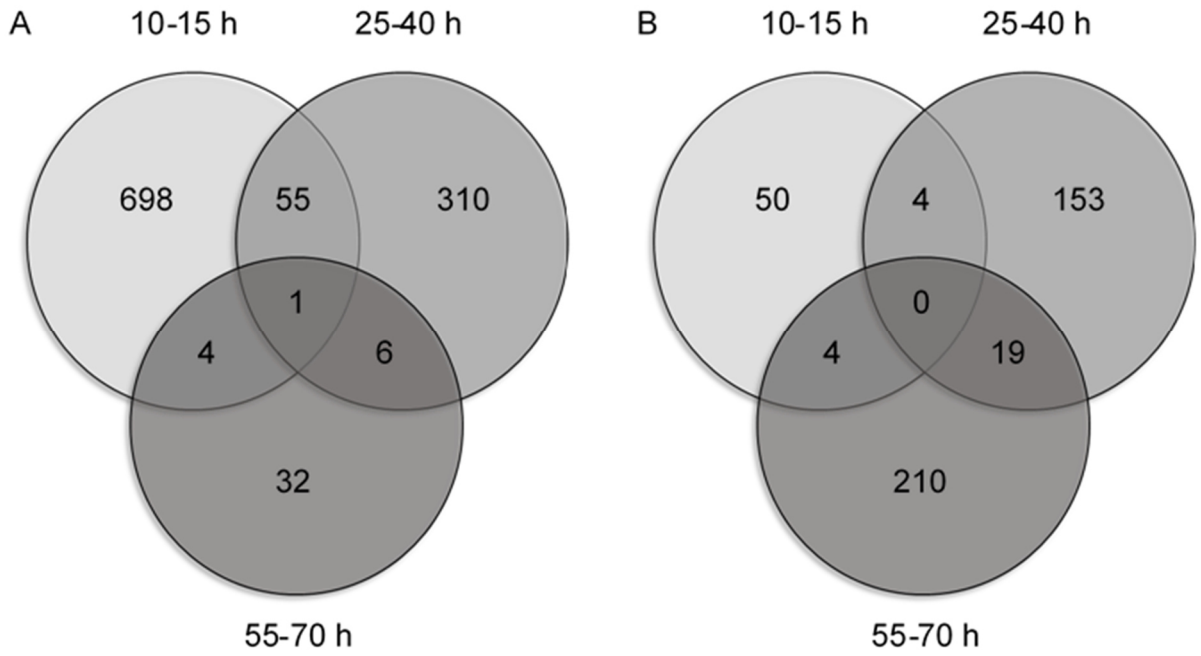

Figure 1: Comparison of differentially expressed (A) and alternatively spliced (B) genes in males and females at three embryonal stages.

Gene Ontology terms for the molecular function of nucleic acid binding are enriched in sexually regulated transcripts

We performed a GO term enrichment analysis on the differentially regulated genes in males and females that encode open reading frames. GO terms of molecular function were inferred from *D. melanogaster* orthologs and were tested for overrepresentation utilizing DAVID online resources (Supplementary tables 2-7; Huang da et al., 2009). We assigned GO terms for 43 out of 58 DEGs in embryos 10-15 h AEL and found that in this group the molecular function of phosphatase activity (GO:0004722) was overrepresented. For 108 of the 167 DSGs in male and female embryos 25-40 h AEL we observed that the molecular functions of protein binding and phospholipid binding (GO:0005515, GO:0005543) were enriched. GO terms for 161 out of 220 DEGs in 55-

70 h old embryos showed an enrichment for mRNA/nucleotide binding and actin/actin filament binding function (GO:0003729, GO:0003779, GO:0000166, GO:0051015).
For the differentially expressed genes in embryos 10-15 h AEL, 514 out of 739 protein encoding genes could be associated to a GO term. The molecular function of ATP binding and protein binding were overrepresented in this set of genes (GO:0005524, GO:0005515). In embryos 25-40 h AEL we observed an higher abundance of DEGs with the molecular function of DNA binding and transcription regulation (GO:0043565, GO:0003700, GO:0000977). Here, for 234 out of 346 genes a GO term could be assigned. We assigned GO terms for 21 out of 39 DEGs in embryos 55-70 h AEL and found that the function of cuticula formation was overrepresented in this group (GO:0042302, GO:0008010). In general, we see an overrepresentation of nucleotide binding functions in sex-biased spliced and differentially expressed genes in the two timespans, 25-40 h AEL and 55-70 h AEL.

Discussion

In this study we identified genes that are differentially regulated in the sexes either through differential expression and/or alternative splicing during the embryonic key processes of fertilization and sex determination in the honeybee.
In the time window 10-15 h AEL we observe the effect of fertilization on the embryonic transcription, which occurs prior to 6 h AEL during early cleavage (Fleig and Sander, 1985; Pires et al., 2016). For this stage, we mainly observed regulation differences at the level of differential expression of transcripts (DEGs: 758 versus DSGs: 58). Our findings imply that the fertilization process already induces substantial differences in gene expression between the sexes. The GO term overrepresentation of ATP binding and kinase activities in this gene set suggests that these differences may manifest in sex-biased changes of phosphorylation and dephosphorylation processes. Examples for the major role of phosphorylation on development is the Erk (extracellular signal-regulated kinase) gene, that controls the fate and posterior identity of embryonic cells of *D. melanogaster* (Johnson and Toettcher, 2019), or the role of Class I_A phosphoinositide 3-kinase in imaginal disc cell size, cell number and organ size regulation of *D.melanogaster* (Weinkove et al., 1999).
We found that the molecular functions of DNA binding and transcription regulation were overrepresented in the group of differentially expressed genes 25-40 h AEL. This

overrepresentation of DNA binding and transcription regulation function could represent genes that encode further developmental regulator activities for sexual differentiation. These genes may operate in parallel to the sex-determination pathway and may be differently expressed in response to the fertilization and non-fertilization process.

The increase of alternatively spliced genes in the sexes in embryos 10-15 h to 25-40 h AEL is consistent with the start of the sex determination cascade at 12 h AEL and the sex-specific splice activity provided by the *csd* and *fem* genes (Beye et al., 2003; Gempe et al., 2009). The number of genes that are alternatively spliced is further increased in the 55-70 h AEL stage, the next older embryonic stage we examined. At this stage we observed that 233 DSGs between the sexes as compared to 176 DSGs in 25-40 h old embryos. Further, we found that the differential expression of the genes declined in this later stage compared to 25-40 h old embryos, since only 43 DEGs were detected. The observed decline of DEGs but increase of DSGs throughout embryonal development suggests, that gene regulation is dominated by alternative splicing in the late honeybee embryo. Further, we found in the group of DSGs in embryos 55-70 h AEL an overrepresentation of mRNA binding proteins that may suggest that beside the identified components of the canonical sex-determination pathway *fem* and the *csd* gene, there are possibly other splice regulators involved in transducing and/or realizing the sexual signal (Hasselmann et al., 2008; Gempe et al., 2009; Gempe and Beye, 2011; Bopp et al., 2014). From the increase of alternative splice processes and overrepresentation of genes with RNA-binding functions with the progression of embryonic development, we conclude that the role of alternative splicing in sexual differentiation is more complex and important than previously thought. We identified 19 genes that were consistently sex–biased spliced in embryos 25-70 h AEL if we examined these stages combined. These genes could play a role in the maintenance of the sexual signal or be targets of the *fem* gene, that is expressed and sex-specific spliced from 33 h AEL on to pupal stage (Gempe et al., 2009; Gempe and Beye, 2011). We conclude that these 19 genes could contain new regulators that play a role in sexual differentiation.

The sex-specific transcriptome data of honeybee embryos presented here provides comprehensive information about early regulatory steps of embryonal sex differentiation. Using the recently developed CRISPR/Cas9 or the transgenic method in honeybees (Schulte et al., 2014; Roth et al., 2019) we can now systematically test

these candidate genes for their functions. This will allow us to determine which of the differentially spliced and expressed genes are required for which aspects of female and male differentiation. Indeed, studies that employed a similar approach and compared the transcriptome between males and females in *Bombyx mori* and *Anopheles gambiae* identified functionally important new regulators for sexual development (Kiuchi et al., 2014; Kawamoto et al., 2015; Krzywinska et al., 2016).

Material and Methods

Collection of embryos

Haploid and diploid embryos of *Apis mellifera carnica* were collected at the beekeeping facility of the Arizona State University (ASU, USA). We used sister-queens that were either inseminated by drones from a single colony or treated with CO_2 to induce the laying of unfertilized eggs. To gather embryos the queens were caged in a Jenter egg collection box (Jenter Queen Rearing Kit, Karl Jenter GmbH, Frickenhausen, Germany). After 5 h or 15 h the eggs were collected and reared in an incubator (Schulte et al., 2014). The embryos were immediately frozen in liquid nitrogen when they were 10-15 h, 25-40 h and 55-70 h old.

RNA isolation and sequencing

We pooled thirty 55-70 h, sixty 25-40 h and one hundred 10-15 h old embryos, which derived from three different mother queens for each sex. Total RNA was isolated according to a Trizol based protocol, which we combined with a further purification step using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) as previously described (Vleurinck et al., 2016). Quality checks for degradation were performed on 18S and 28S ribosomal RNAs using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Library preparation and 100 bp single-read sequencing was conducted by the Biological and Medical Research Center (BMFZ) at the Heinrich-Heine University Düsseldorf, Germany. Sequencing of two biological replicates for each condition was performed on an Illumina HiSeq 2500 system (Illumina, San Diego, USA). We achieved an average Phred score of 35 for all twelve samples, which was calculated using the CLCbio Genomics Workbench software (v8.0.2, Qiagen, Hilden,

Germany). The data are accessible via the Gene Expression Omnibus under the accession number GSE159387.

Bioinformatic analysis of RNA-Seq data

We used the honeybee genome Assembly v.4.5 from the NCBI data base (ftp://ftp.ncbi.nih.gov/genomes/Apis_mellifera/ARCHIVE/ANNOTATION_RELEASE.102/Assembled_chromosomes/seq/). The chromosomes were concatenated (Vleurinck et al., 2016). The reads were mapped to the genome using Tophat (2.1.1) and Bowtie2 (2.2.6; Langmead, 2010; Trapnell et al., 2012). We created *de novo* a transcript model from our data sets using the Cufflinks package (v2.2.1; Trapnell et al., 2012) that also integrated information from the NCBI annotations (http://biomirror.aarnet.edu.au/biomirror/ncbigenomes/Apis_mellifera/GFF//ref_Amel_4.5_top_level.gff3.gz).
DEGs we identified using the cuffdiff command implemented in the Cufflinks package (Trapnell et al., 2012). We normalized for sequencing depth using the upper quartile counts option instead of total read counts. Details on different parameter settings are found in Vleurinck et al. (2016). DEGs with $p < 0.001$ were further analysed. Sorting and visualization of data was performed with the R package CummeRbund (Trapnell et al., 2012). To detect differently spliced junctions we employed the Spanki software (v.0.5.0; Sturgill et al., 2013). We only studied splice junctions that met the anchor ≥ 8, hamming 5' ≥ 3 and entropy ≥ 2 criteria and that had an intron-exon boundary of GT-AG dinucleotides (Sturgill et al., 2013). Splice-junctions with a q-value <0.01 were analysed further. Gene IDs were manually assigned to the junctions via the Integrative Genomics Viewer (Thorvaldsdottir et al., 2013) and the NCBI database to obtain a list of DSGs.

Gene Ontology analysis

Annotated amino acid sequences of DEGs and DSGs were obtained from NCBI. Orthologs from *Drosophila melanogaster* were identified with the Blast2GO software (Conesa et al., 2005). Gene Ontology (GO) terms were assigned to identify overrepresented molecular functions of orthologs using the DAVID online tool (v.6.8, https://david.ncifcrf.gov/; Huang da et al., 2009). Genes with no assigned GO terms either had no ortholog in *D.melanogaster* or no molecular function has yet been annotated to the ortholog.

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Supplementary data

Supplementary table 1: Spearman correlation of expression values between the replicates and conditions

	M1a	M1b	M2a	M2b	M3a	M3b	W1a	W1b	W2a	W2b	W3a	W3b
M1a		0.95	0.79	0.77	0.67	0.65	0.92	0.91	0.71	0.70	0.62	0.64
M1b			0.79	0.77	0.66	0.64	0.93	0.91	0.71	0.70	0.62	0.64
M2a				0.93	0.77	0.75	0.77	0.75	0.90	0.87	0.71	0.74
M2b					0.80	0.76	0.74	0.72	0.91	0.88	0.75	0.76
M3a						0.92	0.64	0.63	0.83	0.83	0.89	0.92
M3b							0.63	0.62	0.81	0.81	0.90	0.94
W1a								0.93	0.69	0.69	0.60	0.62
W1b									0.67	0.67	0.59	0.61
W2a										0.93	0.78	0.80
W2b											0.79	0.81
W3a												0.93
W3b												

* F: female, M: male
1: 10-15 h old embryos; 2: 25-40 h old embryos; 3: 55-70 h old embryos
a and b replicates of the same condition

Supplementary table 2: Gene Ontology (GO) terms for molecular function of differential spliced genes in 10-15 h old male and female embryos.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0004722	protein serine/threonine phosphatase activity	6.12%	1.75E-02
GO:0030165	PDZ domain binding	4.08%	3.56E-02

*cut off value p < 0.05 (EASE score)

Supplementary table 3: Gene Ontology (GO) terms for molecular function of differential spliced genes between male and female embryos 25-40 h after egg deposition.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0005515	protein binding	12.41%	2.88E-03
GO:0005543	phospholipid binding	2.92%	4.37E-03
GO:0003779	actin binding	3.65%	4.02E-02
GO:0004115	3',5'-cyclic-AMP phosphodiesterase activity	1.46%	4.53E-02

*cut off value p < 0.05 (EASE score)

Supplementary table 4: Gene Ontology (GO) terms for molecular function of differential spliced genes between male and female embryos 55-70 h after egg deposition.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0003729	mRNA binding	6.74%	3.76E-05
GO:0003779	actin binding	4.15%	3.16E-03
GO:0005509	calcium ion binding	5.18%	1.04E-02
GO:0000166	nucleotide binding	5.18%	1.10E-02
GO:0051015	actin filament binding	2.07%	1.20E-02
GO:0005515	protein binding	9.84%	2.95E-02

*cut off value $p < 0.05$ (EASE score)

Supplementary table 5: Gene Ontology (GO) terms for molecular function of differential expressed genes between male and female embryos 10-15 h after egg deposition.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0005524	ATP binding	13.08%	2.05E-09
GO:0005515	protein binding	11.64%	4.95E-09
GO:0004674	protein serine/threonine kinase activity	3.83%	1.43E-05
GO:0004672	protein kinase activity	3.67%	3.25E-05
GO:0046872	metal ion binding	9.89%	3.29E-05
GO:0003677	DNA binding	8.61%	5.45E-05
GO:0003723	RNA binding	5.10%	8.81E-05
GO:0044822	poly(A) RNA binding	2.23%	1.35E-04
GO:0016887	ATPase activity	3.19%	5.84E-04
GO:0035091	phosphatidylinositol binding	1.28%	6.47E-04
GO:0070615	nucleosome-dependent ATPase activity	0.80%	1.48E-03
GO:0042803	protein homodimerization activity	2.87%	1.93E-03
GO:0008270	zinc ion binding	8.61%	1.98E-03
GO:0008017	microtubule binding	2.39%	2.01E-03
GO:0004004	ATP-dependent RNA helicase activity	1.44%	5.00E-03
GO:0043035	chromatin insulator sequence binding	0.80%	9.85E-03
GO:0043021	ribonucleoprotein complex binding	0.64%	1.10E-02
GO:0003682	chromatin binding	2.39%	1.17E-02
GO:0003777	microtubule motor activity	1.12%	1.31E-02
GO:0003676	nucleic acid binding	5.74%	1.45E-02
GO:0001078	transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	0.96%	1.73E-02
GO:0004402	histone acetyltransferase activity	1.12%	1.91E-02
GO:0005543	phospholipid binding	0.96%	1.99E-02
GO:0031625	ubiquitin protein ligase binding	1.44%	2.01E-02
GO:0004843	thiol-dependent ubiquitin-specific protease activity	1.12%	2.14E-02
GO:0001077	transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	1.28%	2.33E-02
GO:0070180	large ribosomal subunit rRNA binding	0.48%	2.73E-02
GO:0018024	histone-lysine N-methyltransferase activity	0.64%	3.17E-02
GO:0003713	transcription coactivator activity	1.28%	3.65E-02
GO:0010485	H4 histone acetyltransferase activity	0.48%	3.94E-02
GO:0005198	structural molecule activity	0.96%	4.63E-02
GO:0008157	protein phosphatase 1 binding	0.80%	4.63E-02

*cut off value $p < 0.05$ (EASE score)

Supplementary table 6: Gene Ontology (GO) terms for molecular function of differential expressed genes between male and female embryos 25-40 h after egg deposition.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0043565	sequence-specific DNA binding	11.15%	4.28E-13
GO:0003700	transcription factor activity, sequence-specific DNA binding	8.01%	1.45E-06
GO:0000977	RNA polymerase II regulatory region sequence-specific DNA binding	3.48%	1.23E-05
GO:0005509	calcium ion binding	5.57%	2.53E-04
GO:0042803	protein homodimerization activity	4.18%	8.22E-04
GO:0004872	receptor activity	2.79%	2.29E-03
GO:0042802	identical protein binding	1.74%	4.93E-03
GO:0046872	metal ion binding	9.41%	1.34E-02
GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	2.09%	2.47E-02
GO:0001078	transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	1.39%	3.22E-02
GO:0005515	protein binding	8.71%	3.45E-02
GO:0008233	peptidase activity	1.39%	3.53E-02
GO:0044212	transcription regulatory region DNA binding	1.39%	3.85E-02
GO:0008028	monocarboxylic acid transmembrane transporter activity	1.05%	4.08E-02
GO:0042043	neurexin family protein binding	1.39%	4.54E-02
GO:0004499	N,N-dimethylaniline monooxygenase activity	0.70%	4.96E-02
GO:0003858	3-hydroxybutyrate dehydrogenase activity	0.70%	4.96E-02
GO:0008332	low voltage-gated calcium channel activity	0.70%	4.96E-02
GO:0001227	transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence-specific binding	0.70%	4.96E-02

*cut off value $p < 0.05$ (EASE score)

Supplementary table 7: Gene Ontology (GO) terms for molecular function of differential expressed genes between male and female embryos 55-70 h after egg deposition.

Gene Ontology term ID	Description	Frequency in group	p-value
GO:0008010	structural constituent of chitin-based larval cuticle	13.33%	1.43E-03
GO:0042302	structural constituent of cuticle	13.33%	1.68E-03
GO:0008061	chitin binding	10.00%	2.83E-02

*cut off value $p < 0.05$ (EASE score)

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Author's Contribution: Manuscript II

Diversification of splice regulation during sexual development in honeybees (*Apis mellifera*), a haplodiploid system

Journal: Manuscript in preparation for submission to "Insect molecular biology"

Oksana Netschitailo, Stefan Raub, Osman Kaftanoglu, Robert E. Page, Martin Beye

1st author

Author's contribution:

- Concept of study
- Collection and preparation of samples for sequencing
- Experimental design
- Bioinformatic analysis of raw data
- Evaluation of processed data
- GO term analysis
- Authoring the manuscript

Manuscript III

A novel regulator for sexual eye development evolved by gain of female-specific sequences in the Glubschauge protein

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Manuscript in preparation for submission to “Nature Communications”

Abstract

Organisms show a large variety of secondary sex traits across all animal phyla. Despite decades of research we have little knowledge about the evolutionary and developmental mechanisms underlying the formation of these sexual traits. Previous studies in insects have shown that external sex-traits are largely controlled by the shared sex-specific regulator *doublesex* (*dsx*). Sex traits thereby evolve through the co-option of *dsx* into new functions which rely on genetic changes of *cis*-regulatory elements that effect the expression of target gene or the spatial expression of *dsx* itself. However, sex dimorphic head and eye morphology in the honeybee are not controlled by *dsx* suggesting that other mechanisms must be involved. Here, we report on a new developmental regulator for sexually dimorphic eye formation in the honeybee. We show that the gene *glubschauge* (*glu*) is sex-specific spliced and tissue specific expressed. The activity of Feminizer (Fem) protein is required for the female-specific splice process, which we show by analyzing *fem* loss-of function mutants. Females with CRISPR/Cas9 induced double deletion of the sequence from exon 2 to 8 in the *glu* gene showed an enlargement of the compound eyes, which was intermediate between the male and female phenotypes. The female-specific peptide encompasses a C2H2 zinc finger motif that is responsible for part of the feminization which we demonstrated by targeted mutations of the C2H2 motif. The evolutionary history analyses of sex-specific splicing and the zing finger motif revealed a gain of sex-specific splicing and the C2H2 zinc finger motif (the latter through coding changes) in the hymenopteran group of insects. Our findings reveal the emergence of a novel developmental regulator as an alternative genetic pathway to form sex traits.

Introduction

Morphological and structural differences in males and females are general features in animals. The sex traits can involve elaborate modifications such as horns in some male beetles and the color patterns of the peacock's tail. The emergence of these sex traits in different lineages establishes remarkable species-specific differences and contributes to phenotype diversity and to the reproductive success and fitness of its possessor. The debate whether these major phenotypic changes rely on evolutionary changes in coding or regulatory DNA sequences is long standing (Ohno, 1970; King and Wilson, 1975; Davidson, 2001; Carroll et al., 2005; Halfon, 2017). Intensive investigations during the last two decades, particularly in insects, have shed light on this question (Lints and Emmons, 2002; Zauner et al., 2003; Williams and Carroll, 2009; Rice et al., 2019).

Comparative analyses of key developmental components identified in *Drosophila melanogaster* found that pre-existing developmental pathways have been "co-opted" into the genetic programming of the new sex-dimorphic structure. Classical examples are the co-option of the conserved sex-specific regulator *doublesex* (*dsx*) into horn formation in some male beetles (Kijimoto et al., 2012; Ito et al., 2013; Ohde et al., 2018) or the evolution of the posterior lobe, a male genital outgrowth, in the *D. melanogaster* lineage (Glassford et al., 2015). The posterior lobe derived from the redeployment of a set of transcription factors and *cis*-regulatory elements (CREs) of their target genes that otherwise are employed in the development of the larval posterior spiracle. In *Drosophila* the evolutionary gain of CREs generated novel spatial expression of the *dsx* gene that is essential for male sex comb development, a structure that is involved in copulation behavior of *D. melanogaster* and related species (Tanaka et al., 2011a; Rice et al., 2019). Evolutionary changes in CREs that are targeted by Dsx protein generated modifications, gains and losses of sex-specific gene expression underlying the sex dimorphic pigmentation and diene hydrocarbon production in *D. melanogaster* (Williams et al., 2008; Shirangi et al., 2009a). The outcome of these studies demonstrated that the co-option of pathways into new developmental contexts relied on evolutionary changes of regulatory sequences. However, other genetic pathways underlying the emergence of sexual traits are largely unknown.

Here, we report evolutionary changes that resulted in the expression of sex-specific proteins and gave rise to a new developmental pathway for sexually dimorphic eye formation in the honeybee.

The compound eyes of honeybees (*Apis mellifera*) distinctly differ in size and function between males and females. Females, the queens and the workers, have oval shaped compound eyes that are dorsally separated and cover less than a quarter of the head. Honeybee males have large roundish shaped eyes covering one third and the dorsal part of the head (Supplementary figure 1; Winston, 1987; Menzel et al., 1991; Streinzer et al., 2013). The enlargement of the entire male eye with its pronounced dorsal expansion (3.8 times lager surface area than in workers; Winston, 1987; Streinzer et al., 2013) enhances the spatial resolution and spotting of objects in the air. This feature facilitates the detection of queens that are freely flying in the air and enhances the mating success (van Praagh et al., 1980; Gries and Koeniger, 1996; Ribi et al., 1989). The *dsx* gene, which is essential in *D. melanogaster* for the development of nearly all aspects of sex traits (reviewed in Christiansen et al., 2002), is not employed in the control of sexually dimorphic eye development of the honeybee. CRISPR/Cas9 mediated mutations in the *dsx* gene affected the reproductive organ development but not the sexually dimorphic eye morphology suggesting that another developmental regulator is required for eye differentiation (Roth et al., 2019).

To develop a screening strategy we assumed that *feminizer* (*fem*) regulates this currently unknown regulatory gene through alternative splicing as active Fem protein in essential for female development and a loss of *fem* function leads to a complete masculinization of the individual (Gempe et al., 2009; Roth et al., 2019). In females *fem* transduces the primary signal of sex determination initialized by *complementary sex determiner* (*csd*) and directs the female specific splicing of *dsx* (Figure 1; Beye et al., 2003; Gempe et al., 2009), suggesting that other targets of *fem* are also sex-specific spliced.

In this study we identified a key developmental regulator for dimorphic eye development and characterized the mechanisms underlying its regulation.

Results

The transcripts of the *glu* gene are sex-specific spliced and encode a C2H2 zinc finger motif only in females

To characterize the developmental regulation of secondary sex traits in honeybees we screened for sex-specific spliced transcripts that encode a possible transcriptional regulator under control of the sex-determining gene *fem* (Figure 2). We performed genome wide RNA-Seq studies using female and male embryos and identified transcripts that use alternative splice junctions in females and males. With this screening approach we found the gene *glubschauge* (*glu,* Gene ID 552468) that is sex-specific spliced in males and females and encodes a zinc finger motif (ZnF) only in females.

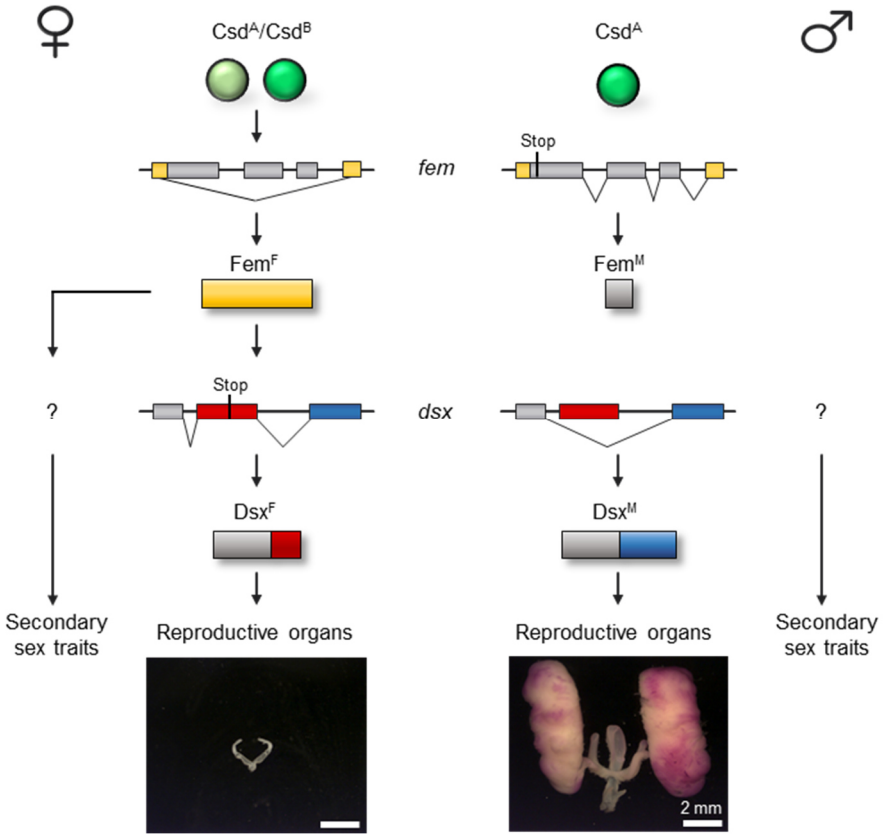


Figure 1: The search for new regulators of sex dimorphic development
Model of the sex determination pathway displaying the role of the known components in the honeybee. Part of the gene structures as well as the proteins are presented. For the primary signal *csd* only the protein variants are presented that derive form heterozygous (female) and hemizygous (male) genotypes.

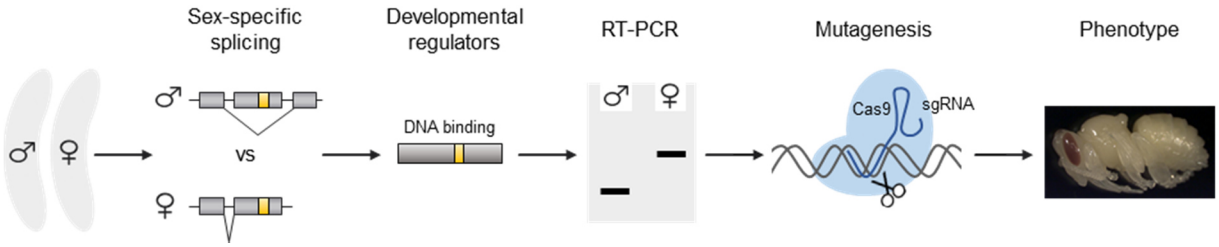


Figure 2: Experimental procedure to identify regulators for sex dimorphic development

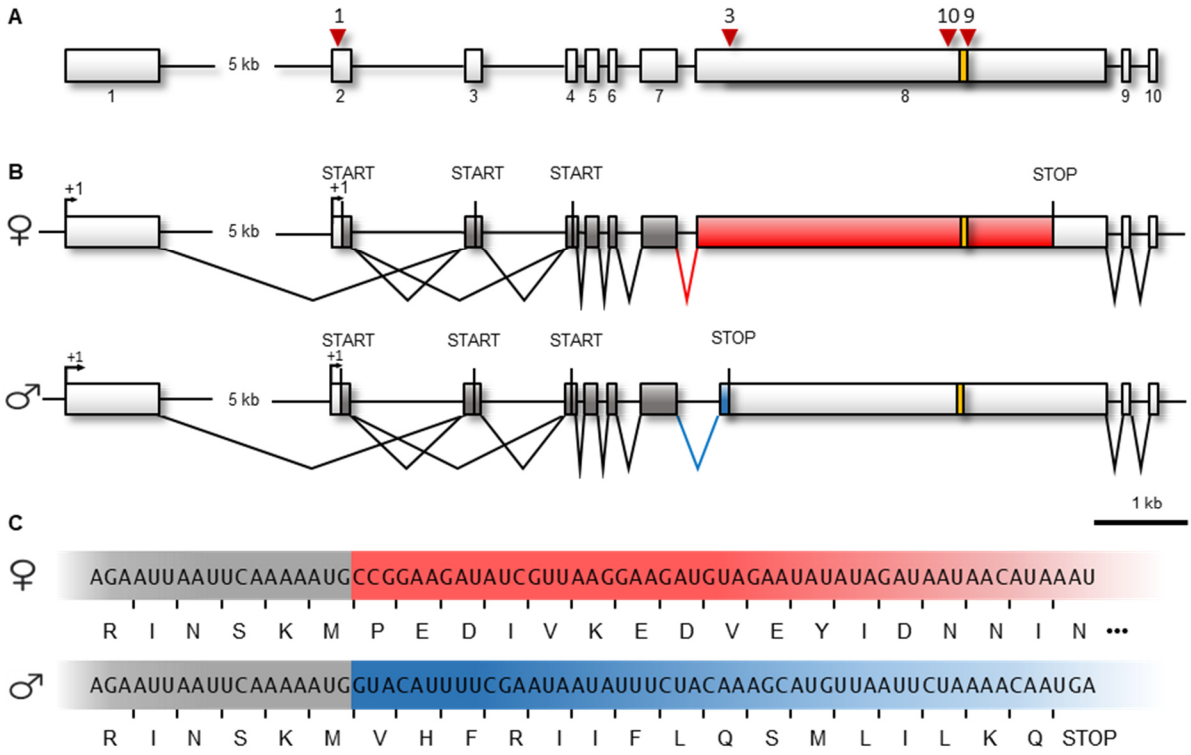
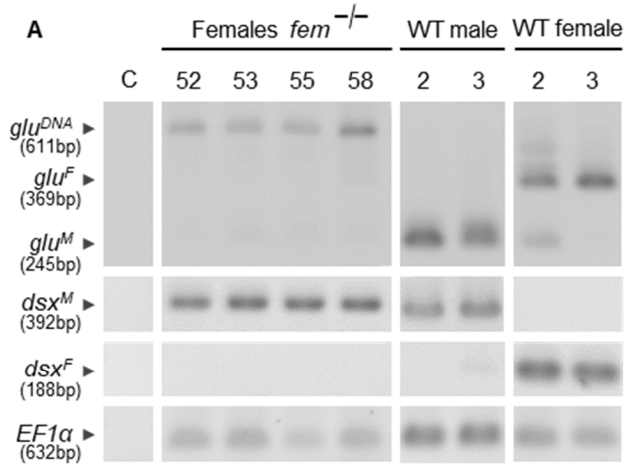


Figure 3: Scheme on the genomic organization and sex-specific splicing of *glubschauge* (*glu*)
(A) Genomic organization of the gene *glu*. The position of the target sites of the sgRNAs 1, 3, 9 and 10 used for CRISPR/Cas9 mutagenesis are shown above the genome structure (red arrows). Position of the sequence encoding the C2H2 zinc finger motif is displayed in yellow. (B) Female and male specific splicing of the *glu* transcripts. (C) The mRNA sequence at the exon 7 and 8 boundary that is sex-specific spliced. The deduced amino acid sequence with the shift of the ORF is shown. Boxes denote exons. Female specific parts of the ORF are shown in red, male ones are displayed in blue while common parts are shown in grey.

Mapping the RNA-Seq and RT-PCR amplicon sequence data on the honeybee genome showed that the *glu* gene consists of 10 exons (Figure 3A) and uses two transcriptional start sites (Figure 3B). Alternative splicing in both males and females results in inclusion or exclusion of exon 3. Together with the two transcriptional start sites this produces three alternatively spliced transcripts that encode three possible translation start sites, which are in exon 2, 3 and 4. Sex-specific splice acceptor sites

are used in exon 8 of females and males that lead to a shift of the open reading frame in exon 8 (ORFs, Figure 3C). In females the exon 8 ORF encodes a 1256 amino acids long peptide harbouring a C2H2 zinc finger (ZnF) motif. In males the exon 8 encodes only 16 amino acids since the shift of the ORF introduces an early stop codon (Figure 3C). These results suggest that in females the *glu* transcripts encode 1466 to 1572 amino acid long protein variants harbouring a C2H2 ZnF motif. In males shorter, 226 to 332 amino acids long, protein variants are expressed that lack the C2H2 ZnF motif. We conclude that the sex-specific regulation of the *glu* gene via splicing produces proteins containing a C2H2 ZnF domain only in females. The domains can possibly encode DNA binding abilities and be a component of a transcription factor activity regulating the transcription of target genes (Suske, 1999; Wolfe et al., 2000; Kim et al., 2003; Swamynathan, 2010).



B

	Larvae screened	Larvae with *glu*^{F} splicing	Larvae w/o *glu*^{F} splicing
fem $^{-/-}$	11	0	11 (100%)
WT male	15	0	15 (100%)
WT female	15	15 (100%)	0

Figure 4: *glu* mRNA splicing in *feminizer* (*fem*$^{-/-}$) female mutants
A) The female-specific (*glu*^{F}) and the male-specific (*glu*^{M}) parts of the *glu* transcripts were amplified by RT-PCR from individual first stage larvae. The *fem* double mutants (*fem*$^{-/-}$) possessed an early stop in the female ORF. The amplicon above the splice variants (*glu*^{DNA}) is derived from the genome or non-spliced *glu* transcripts. *glu*^{M} transcripts containing an early stop codon were not reliably amplified possibly due to nonsense mediated decay (Wagner and Lykke-Andersen, 2002; Houseley and Tollervey, 2009). The sex-specific transcripts of the *doublesex* (*dsx*^{M} and *dsx*^{F}) were separately amplified to demonstrate the loss of *fem* activity. EF1α: elongation factor 1α; C: null control for RT-PCR. The amplicons from single individuals were resolved by gel electrophoresis. (B) Total and relative numbers of individuals with nonsense mutations in the *fem* gene showing alterations in *glu* splicing compared to wildtype males and females.

To understand if the sex-specific splicing of the *glu* gene is controlled by the *fem* gene, we mutated *fem* and studied the effect on splicing of *glu* transcripts. The *fem* gene encodes a splice regulator that controls the entire development of the sexes by an activity that is only provided in females (Hasselmann et al., 2008; Gempe et al., 2009). Sex-specific splicing of *fem* transcripts introduces an early stop codon in male and a late one in female transcript (Figure 1), thus the active Fem protein is only encoded in females (Gempe et al., 2009). To inactivate the *fem* gene we induced frame shift mutations using CRISPR/Cas9 method and our efficient somatic mutation approach (Jinek et al., 2012, Roth et al., 2019). These mutations mimic the male regulatory state as they introduce early stop codons in exon 3 (Supplementary figure 2). We screened for $fem^{-/-}$ females, carrying stop codons in the absence of wildtype sequences (at the level of detection), which we identified by deep sequencing of amplicons for each individual (Supplementary table 1; Roth et al., 2019). We found that the $fem^{-/-}$ females had only the male and not the female *glu* splice variant while wildtype females had only the female splice variant (Figure 4). This shift from female to male splicing in response to early stop codons in exon 3 of *fem* suggests that the female splicing of *glu* transcripts is controlled by the *fem* gene. To validate that our frame shift mutations produced the non-active state of the *fem* gene, we studied the splicing of the *dsx* gene, a known target of the Fem protein. We observed that $fem^{-/-}$ females displayed only the male and not the female *dsx* splice variants (Supplementary table 2) supporting our conclusion of non-functionality of the *fem* gene in our mutants.

To further examine the regulation of the *glu* gene expression we studied sex-specific splicing and transcription at different stages and tissues. For 0-15 hours old embryos we found that only the male-specific variant was present in both genetic females and genetic males (Figure 5A). The female-specific transcript appears in females from 25 hours on while in males only the male-specific transcript is present. These results suggest that in females the splice pattern is switched into the female state with the onset of the sex determination cascade and Fem protein activity (Gempe et al., 2009). We conclude, that the generation of sex-specific activity is based on switching between a default (male) and the female splicing mode, which is regulated by Fem protein activity. Further, we studied the presence/absence of sex-specific spliced *glu* transcripts in tissues of pupae and adults. At red eye pupal stage (Figure 5B) we detected glu^{F} transcripts in the brain, the gonads and in hind legs but not in the tissue of the head capsule (without the brain), the thorax, the abdominal ganglia and the

abdomen (without the gonads and ganglia). In adult honeybee females we localised *glu*^{F} expression in the brain, the abdomen, the antenna and again in the hind legs (Figure 5B). We were not able to amplify *glu*^{F} transcripts from ganglia, the gonads and the head capsule. Also, we were not able to reliable amplify the male transcripts in different tissues possibly because the early stop codons in the male transcripts caused a nonsense mediated decay of this mRNAs (Wagner and Lykke-Andersen, 2002; Houseley and Tollervey, 2009). Taken together these results suggest that in addition to the sex-specific regulation via splicing, a tissue-specific regulation of expression is operating at the level of *glu* transcription.

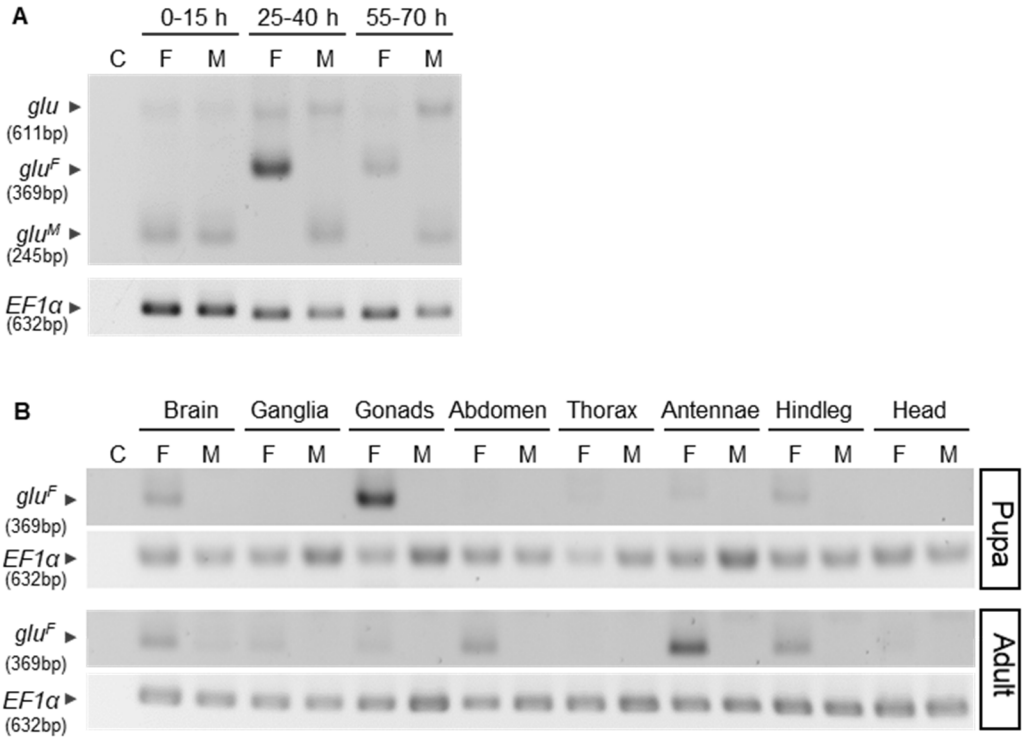


Figure 5: Sex-specific splicing and tissue-specific transcription of the *glu* gene
(A) Sex-specific amplicons of *glu* transcripts in 0-70 h old embryos were amplified using semi-quantitative RT-PCRs. Pools of 15-60 embryos were used. (B) Tissue-specific expression of *glu*^{F} in males and females at pupal (P4) stage and in 10 days old male and female imagos. One example of three performed replicates is shown. For semi-quantification RT-PCRs were adjusted across samples using amplicons of the transcript of the *EF1α* gene. *glu*^{M} with the early stop codons were not reliably amplified possibly due to nonsense mediated decay (Wagner and Lykke-Andersen, 2002; Houseley and Tollervey, 2009). The amplicons from single individuals were gel electrophoretically resolved by size. F: female; M: male; *EF1α: Elongation factor 1α*; C: null control for RT-PCR.

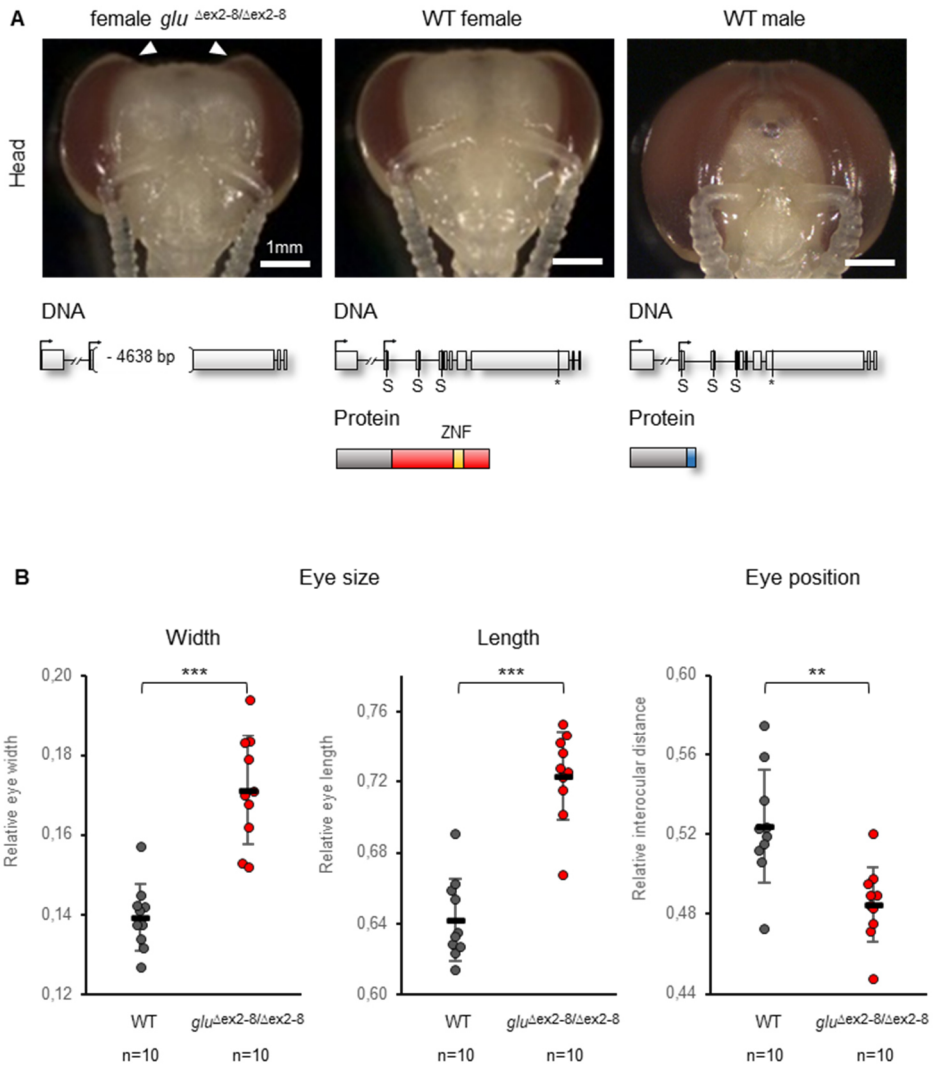


Figure 6: Eye morphology of *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ female mutants

(A) Head morphology of female *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ female mutant, wildtype (WT) females and males are shown. Pupae are form P4 stage. The introduced mutation of the DNA and the expected protein products are schematically presented. (B) Relative eye width and length together with relative interocular distances are presented (**p<0.01, ***p<0.001, one-tailed Mann-Whitney U test). Means and standard deviations are shown.

glu regulates sex-specific eye morphology

To understand the role of the *glu* gene in sexual differentiation we mutated *glu* in female embryos using the CRISPR/Cas9 method, reared larvae to pupal stage using worker nutrition and screened for individuals that were entirely mutated by deep sequencing of amplicons (Jinek et al., 2012; Roth et al., 2019). Genetic females, that carried the exon 2 to exon 8 deletion (*glu*$^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$) in the absence of the wildtype sequence (Supplementary table 3), developed larger eyes with a male-like dorsal bulb

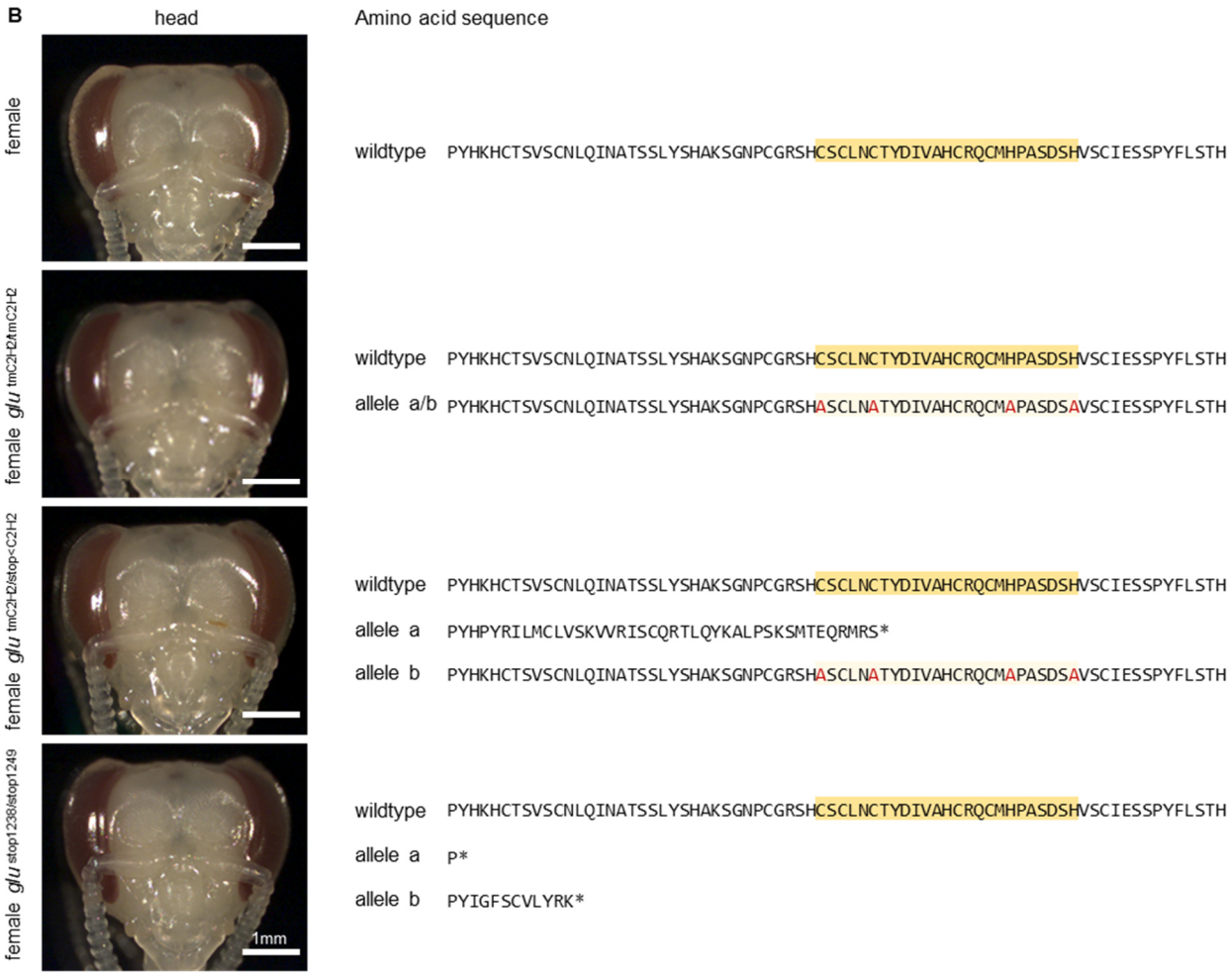
(Figure 6), while other characters of outer body morphology were wildtype (Supplementary figure 1). The relative compound eye length and width were significantly larger while the relative interocular distance of compound eyes was significantly smaller as compared to wildtype females, which are all traits that enhance similarity to the male eye (Figure 6). These results suggest a masculinization of eye development in the absence of *glu* activity. We conclude that *glu* is required for the female-specific differentiation of the compound eye. However, the eye morphology had an intermediate form and did not completely change into to the male phenotype, since, for example, the distance between the compound eyes in males is even smaller (Figure 6A, Supplementary figure 1). This intermediate form either suggests that the sex-specific Glu proteins are functional in both females and males, or, that beside *glu* another gene is required for sexual differentiation of the eye in both sexes. We can also not exclude, that the caste signal is involved in the regulation of eye development in the honeybee females (Roth et al., 2019). Unfortunately, despite major efforts such as collecting embryos from 15 virgin queens, we were not able to obtain enough male mutants to study *glu* function in males. Thus, we confined our examinations on *glu* in females and essential components of the female-specific peptide.

Transcription factors (TF) can activate downstream effector genes and are key regulators shaping developmental features. For the regulatory function of TFs an interaction with the DNA required. A major family of DNA binding domains are C2H2 zinc fingers (Suske, 1999; Wolfe et al., 2000; Kim et al., 2003; Swamynathan, 2010). To determine whether the female-specific expressed C2H2 ZnF motif can have such a developmental role, we mutated the C2H2 ZnF motif (Michael et al., 1992; Pabo et al., 2001; Krishna et al., 2003) using CRISPR/Cas9 mediated homologous repair and studied sexual development. We replaced the nucleotide codons encoding cysteine and histidine with those translating into alanine in order to disrupt Zn^{2+} complexation and domain structure (Figure 7; Supplementary table 4; Heyer et al., 2010; Beumer et al., 2013; Yang et al., 2013). We observed that those double mutant females (*glu*$^{tmC2H2/tmC2H2}$) had longer relative eye length than wildtype females suggesting that loss-of-function of the C2H2 ZnF motif induced masculinization. However, the other masculinization effects, such as relatively larger eye width and smaller interocular distance, were absent, which are found in the *glu*$^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ mutants that lack the N-terminal part of female specific peptide and all translation start codons. This result

indicates that at least one other functional domain is encoded in the female-specific peptide that is required for female differentiation.

TFs of the ZnF family usually have more than one ZnF motif (Wolfe et al., 2000; Krishna et al., 2003). We identified no other ZnF motifs of the canonical type but a possible motif of the non-canonical type (Wolfe et al., 2000; Krishna et al., 2003) further C-terminal, close to the end of the female-specific peptide (Supplementary figure 3). To find evidence for this other functional element near the C-terminus we studied individuals in which one allele carried a stop codon upstream of the C2H2 encoding sequence while in the other allele only the C2H2 was compromised. We observed that these *glu*$^{tmC2H2/stop<C2H2}$ mutants had also a relative shorter interocular distance, beside the larger relative eye length that we observed in our C2H2 double mutants (*glu*$^{tmC2H2/tmC2H2}$; Figure 7C). However, the relative eye width was not influenced. This result provides evidence that the presence of one copy of the in C-terminal sequence harboring a non-functional C2H2 motif produces a phenotype intermediate between *glu*$^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ and *glu*$^{tmC2H2/tmC2H2}$ mutants. From this mutational approach we also obtained one double mutant, *glu* $^{stop1238/stop124}$, that lacked the C2H2 zinc finger motif and the female peptide C-terminal of it (Figure 7B, C). This mutant displayed male-like positional and size parameters and resembled the phenotype that we observed in the *glu*$^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ mutants. This result suggests that the C-terminal part of the female peptide harbors a further functional element that is necessary for female eye differentiation.


A
C2H2 zinc finger: C - X2–4 - C - X12 - H - X3-5 - H
sgRNA 10
sgRNA 9
WT
CCATATCATAAACATTGCACTTCG-81nc-TGCTCTTGTTTAAATTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCGGCATCGGATTCTCATGTGTCT
P Y H K H C T S -27AS- C S C L N C T Y D I V A H C R Q C M H P A S D S H V S
sgRNA 10
sgRNA 9
glu tmC2H2
CCATACCACAACCACTGCACTTCG-81nc-GCGTCTTGTTTAAATGCGACTTATGATATCGTTGCGCATTGCAGACAATGTATGGCGCCGGCCAGCGATTCTGCGGTGTCT
P Y H K H C T S -27AS- A S C L N A T Y D I V A H C R Q C M A P A S D S A V S


B
head
Amino acid sequence
female
wildtype PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHCSCLNCTYDIVAHCRQCMHPASDSHVSCIESSPYFLSTH
female glu tmC2H2/tmC2H2
wildtype PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHCSCLNCTYDIVAHCRQCMHPASDSHVSCIESSPYFLSTH
allele a/b PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHASCLNATYDIVAHCRQCMAPASDSAVSCIESSPYFLSTH
female glu tmC2H2/stop<C2H2
wildtype PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHCSCLNCTYDIVAHCRQCMHPASDSHVSCIESSPYFLSTH
allele a PYHPYRILMCLVSKVVRISCQRTLQYKALPSKSMTEQRMRS*
allele b PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHASCLNATYDIVAHCRQCMAPASDSAVSCIESSPYFLSTH
female glu stop1238/stop1249
wildtype PYHKHCTSVSCNLQINATSSLYSHAKSGNPCGRSHCSCLNCTYDIVAHCRQCMHPASDSHVSCIESSPYFLSTH
allele a P*
allele b PYIGFSCVLYRK*
1mm

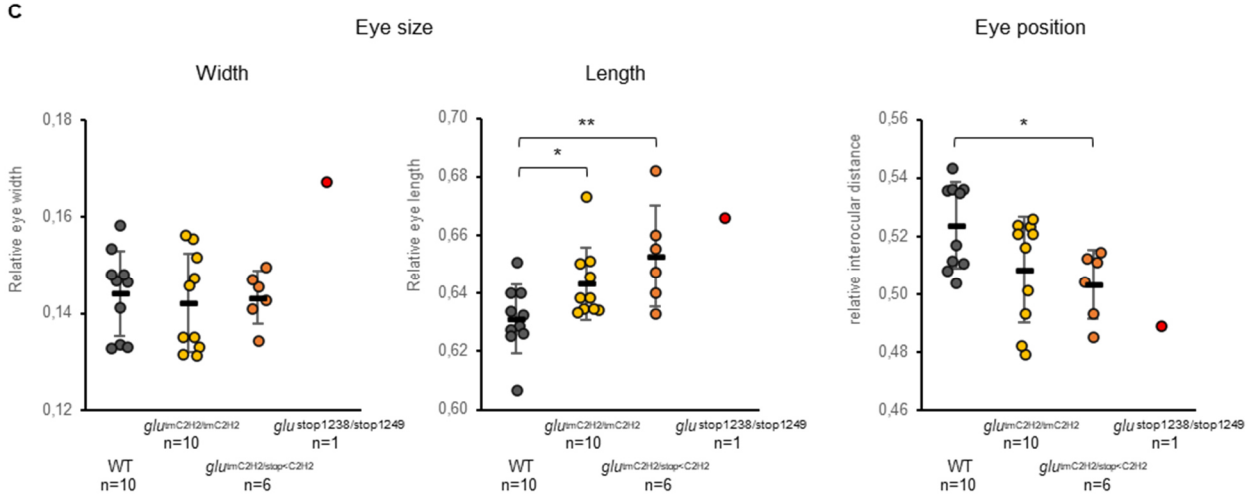

C
Eye size
Eye position
Width
Length
Relative eye width
Relative eye length
relative interocular distance
0,18
0,16
0,14
0,12
0,70
0,68
0,66
0,64
0,62
0,60
0,56
0,54
0,52
0,50
0,48
0,46
**
*
glu tmC2H2/tmC2H2 n=10
glu stop1238/stop1249 n=1
WT n=10
glu tmC2H2/stop<C2H2 n=6

Figure 7: Eye morphology of *glu* $^{tmC2H2/tmC2H2}$, *glu* $^{tmC2H2/stop<C2H2}$ and *glu* $^{stop1238/stop1249}$ female mutants

(A) Scheme of the mutations that were introduced by the CRISPR/Cas9 method using homologous repair. C2H2 consensus motif C-X_{2-4}-C-X_{12}-H-X_{3-5}-H (Michael et al., 1992; Pabo et al., 2001; Krishna et al., 2003) is shown together with the corresponding nucleotide and amino acid sequences of the wildtype (WT) and the designed *glu*^{tmC2H2} mutations. The *tmC2H2* mutation changed the codons for cysteine (C) and histidine (H) into alanine (A). Red letters mark the codons and amino acids of the C2H2 motif. We introduced silent mutations (orange letters) to prevent sgRNA target site recognition (underlined sequence, protospacer adjacent motif in bold) and to exclude further double-strand breaks. (B) Eye morphology of *glu* $^{tmC2H2/tmC2H2}$, *glu* $^{tmC2H2/stop<C2H2}$, the *glu* $^{stop1238/stop1249}$ female and a WT female at pupal stage P4. Examples of the deduced amino acid sequences for the mutants are shown to the right as compared to the wildtype sequence. The sequence harboring the zinc finger motif are highlighted in yellow while the induced amino acid changes are shown in red. (C) Relative eye sizes and positions of *glu* $^{tmC2H2/tmC2H2}$, *glu* $^{tmC2H2/stop<C2H2}$, *glu* $^{stop1238/stop1249}$ mutants (*p<0.05, **p<0.01, one tailed Mann-Whitney U test)**.** Means and standard deviations are shown.

The evolution of *glu* splice control and the C2H2 ZnF motif

We next examined how *glu*'s role in the regulation of sexual developmental has evolved. Our mutational studies revealed that the sex-specific spliced exon 8 of *glu* is essential for the sexually dimorphic development of the compound eye. To get knowledge about the evolutionary history of this splice function in insects, we examined the splicing of *glu* homologs in different insect lineages. We observed that in *Drosophila melanogaster*, which belongs to the insect order Diptera, and in the bed bug *Cimex lectularius*, which is member of the order Hemiptera, the transcripts of the *glu* homologs were not sex-specific spliced (Figure 8A). However, in *Nasonia vitripennis*, which is, as the honeybee, a member of the insect order Hymenoptera, the *glu* homolog showed a female-specific spliced transcript (*LOC100678462*^{F}; Figure 8A). Next, we examined if the splicing of the *glu* homolog in *N. vitripennis* is under control of the *tra* gene, which is the homolog of the *fem* gene. Down regulation of the *tra* gene in females using RNAi resulted in strong increase of the amount of transcript that is present in both sexes (*LOC100678462*^{C}) while the amount of the female-specific transcript (*LOC100678462*^{F}) was reduced compared to the *gfp* dsRNA treated controls (Figure 8B) suggesting that the *tra* gene directs female splicing of the *glu* homolog in *N. vitripennis*. Reconstructing the evolution of this trait using maximum parsimony inference provides evidence that the ancestral state was an absence of the sex-specific splice control, which was at least once gained in hymenopteran insects (Peters et al., 2014; Misof et al., 2014). The functional testing showed that this splice regulation

evolved under the control of the homologous *fem*/*tra* genes, which supports the assumption of common ancestry.

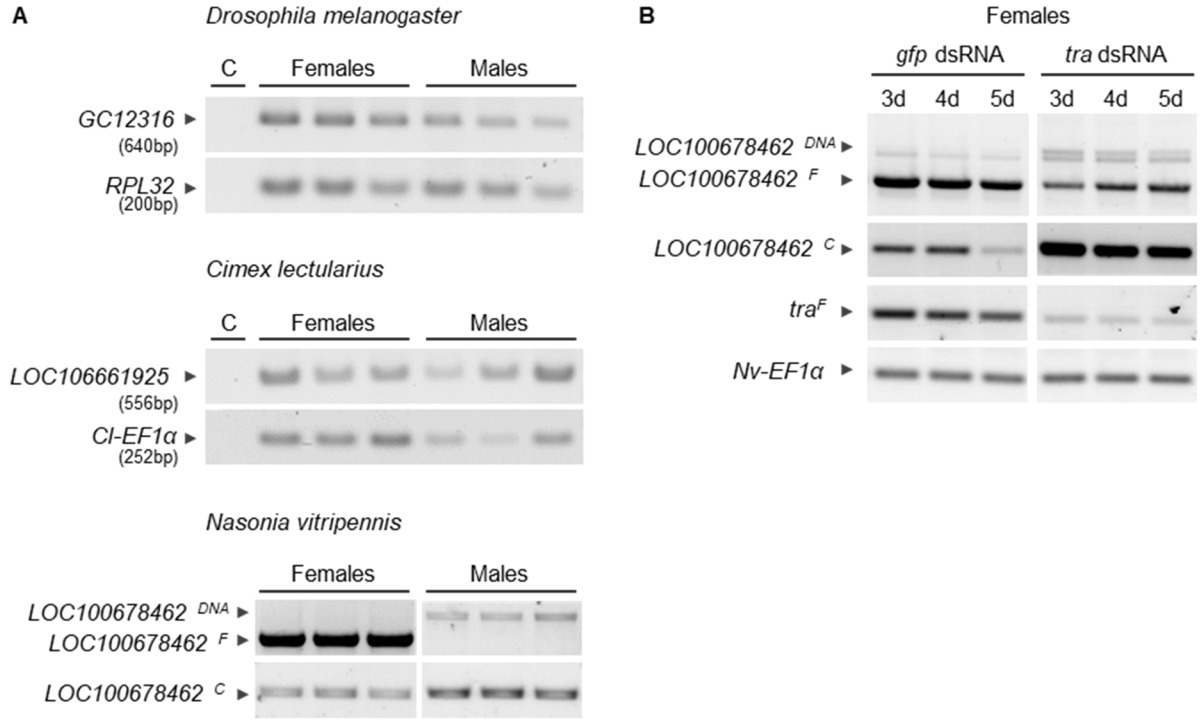


Figure 8: The sex-specific splicing and function of *glu* homologous genes in other insects
(A) Testing of sex-specific splicing of *glu* orthologs in *D. melanogaster* (*CG12316*), *Cimex lectularius* (*LOC106661925*) and *N. vitripennis* (*LOC100678462*). The splice-junctions homologous to the exon 7/8 boundaries of *glu* were tested for sex-specific splicing. The genes *Elongation factor 1-alpha* for *C. lectularius* (*Cl-Ef1α*) and the *ribosomal protein L38* for *D. melanogaster* (*RPL38*) were employed to adjust PCRs across samples and to perform semi-quantitative PCRs. *N.vitripennis* has one splice variants that is confined to females (*LOC100678462^{F}*). The other variant is found in males and females with strong abundance in males (*LOC100678462^{C}*). (B) The sex-specific splicing of the *glu* homolog *LOC100678462* in response to *tra* knockdown in *N. vitripennis*. Females were treated with *tra* dsRNA or *gfp* dsRNA. The females were collected 3-5 days after dsRNA treatment and pooled (n=5) into separate samples. Semi-quantitative RT-PCRs were performed using *Elongation factor 1-alpha* (*Nv-Ef1α*) gene as a control to adjust PCRs across samples. Samples 3d, 4d and 5d show a partial knockdown of the *tra^{F}* transcript. These samples show a high abundance of male *LOC100678462^{C}* splice variants and a reduction of the *LOC100678462^{F}*compared to the controls. Amplicons were gel electrophoretically resolved by size. C: null control of RT-PCR.

Our mutational studies revealed that the female-specific expressed C2H2 ZnF domain is another essential component for the sexual differentiation of the compound eye. To understand how this functional domain originated, we examined the sequence evolution of the core C2H2 ZnF motif of the *glu* gene. We inferred the likelihood of the ancestral states for the amino acid positions using maximum parsimony method from a set of 49 homologous sequences from the same number of hymenopteran species. The parsimony analysis provided evidence that in the ancestral state the C2H2 core

motif was absent (Supplementary figure 4). We provide representative examples of the full analysis in figure 8. The core motif was gained in the stinging wasps (Aculeata) that is after the divergence the stinging and parasitoid wasp groups, the latter group including *N. vitripennis* (Figure 9; Supplementary figure 4). The maximum parsimony analysis further suggest a series of changes underlying the emergence of this motif within the hymenopteran insects (Figure 9; Supplementary figure 4) that is, (i) the gain of the required spacing between the 2nd cysteine (C2) and 2nd histidine (H2), (ii) the origin of a hydrophobic isoleucine (I), and (iii) the evolution of the 2nd histidine. These findings suggest a de novo origination for the sex-specific function of the C2H2 ZnF domain by a series of mutations in the coding sequence.

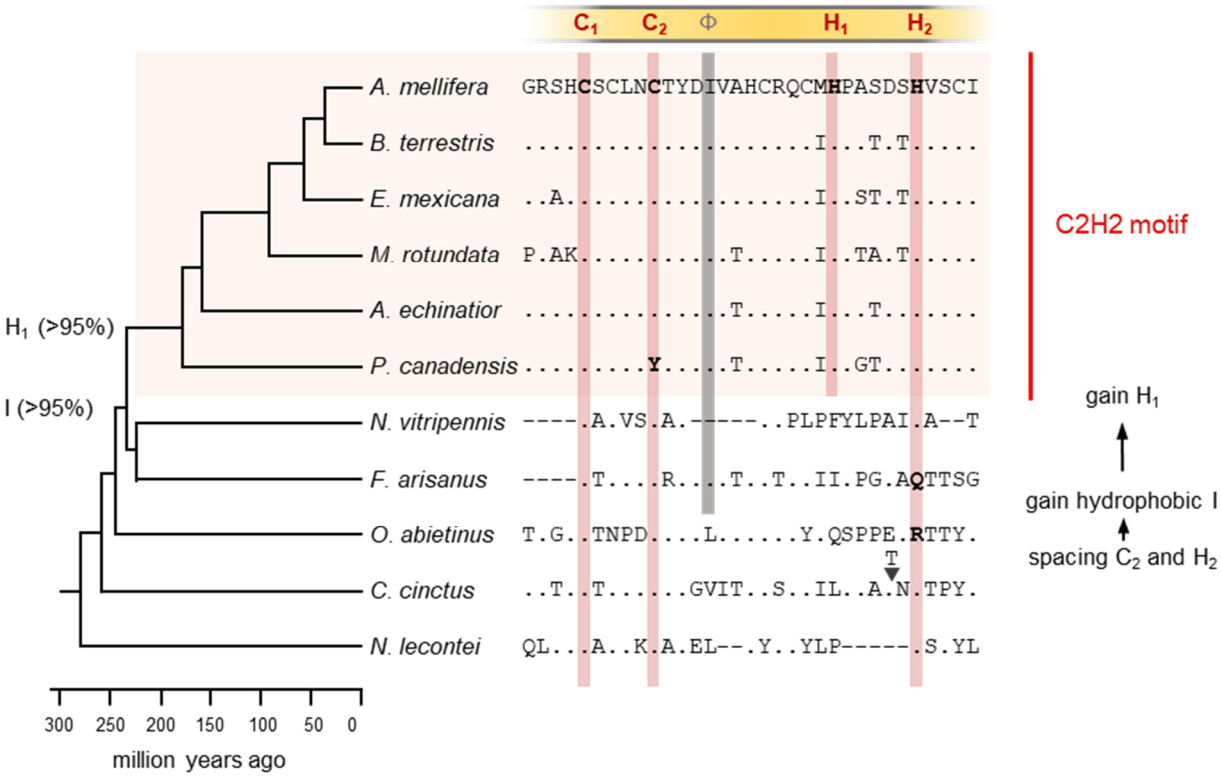


Figure 9: The evolutionary gain of a female-specific C2H2 zinc finger motif in stinging wasps
Amino acid sequence alignment and phylogenetic relationship of the *glu* homologs are shown. The amino acid sequences are representative examples of the different subgroups and the 49 hymenopteran sequences that we studied (Supplementary figure 4). The phylogenetic relationship and evolutionary divergence time are presented according to Peters et al. (2017). The canonical C2H2 motif (Michael et al., 1992; Pabo et al., 2001; Krishna et al., 2003) is shown above the sequences while amino acids matching this motif are marked by red (C2H2 motif) and grey boxes (hydrophobic core). For the two key nodes, the amino acid changes are shown. The percentage next to them indicate the inferred likelihood using parsimony method. The following sequences are presented: *Apis mellifera* XP_026299695.1; *Bombus terrestris* XP_012165493.2; *Eufriesea mexicana* OAD55885.1; *Megachile rotunda* XP_012146010.1; *Acromyrmex echinatior* XP_011060010.1; *Polistes canadensis* XP_014601325.1; *Nasonia vitripennis* XP_003425013.1; *Fopius arisanus* XP_011314024.1; *Orussus abientinus* XP_012279333.1; *Cephus cinctus* XP_015598325.1; *Neodiprion lecontei* XP_015517082.1.

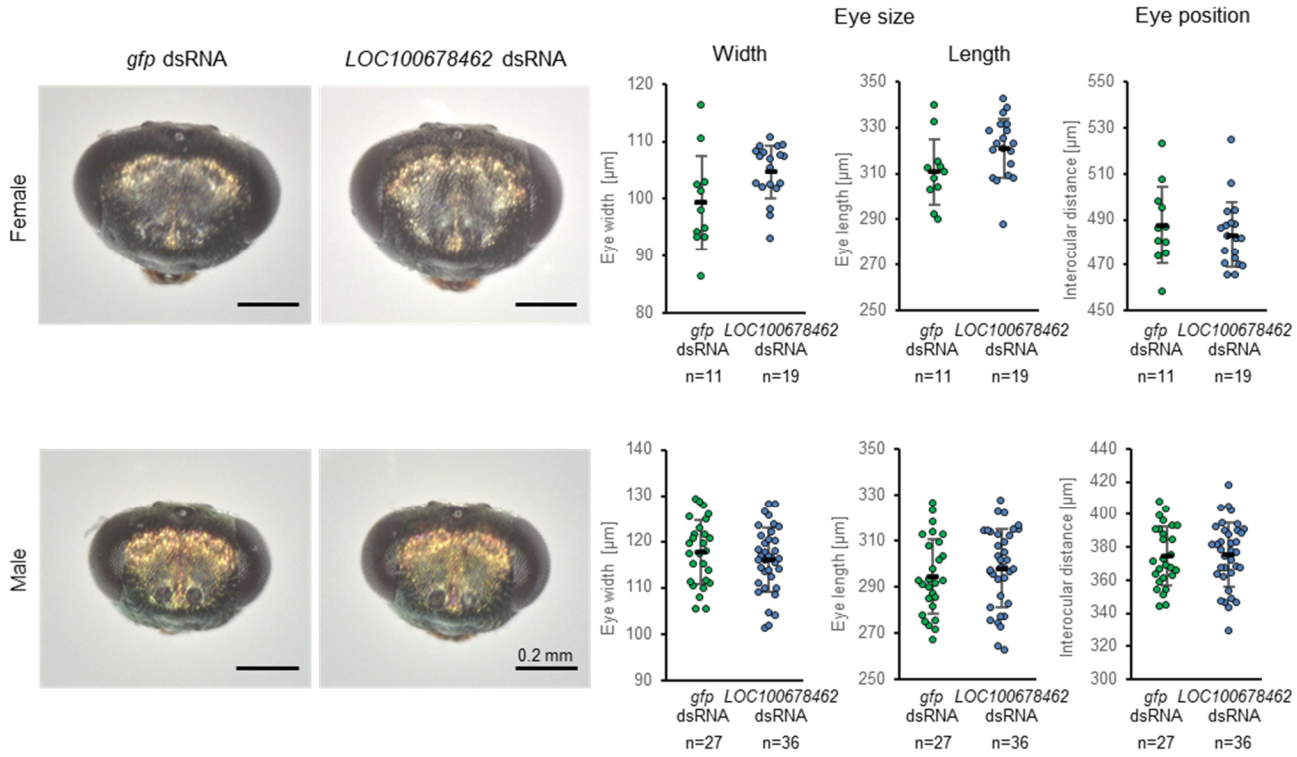


Figure 10: Eye morphology of *N. vitripennis* males and females in response to *LOC100678462* knockdown.
Knockdown of *LOC100678462* transcript was demonstrated in a sample of individuals by qRT-PCR (Supplement figure 7). Absolute instead of relative eye size and position parameters were shown, since the *LOC100678462* and *gfp* dsRNA treatments affected head width and length as well as the inter-individual variances (Supplementary figure 5, treated individuals compared to wildtype $p<0.001$, two-tailed Mann-Whitney U test). For comparison the absolute values and significant differences of these parameters for the honeybee *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ mutants are shown in Supplementary figure 6.

The *glu* homolog of *D. melanogaster* (*CG12316*) has no role on sex-dimorphic eye development (Mummery-Widmer et al., 2009; Neely et al., 2010; Schnorrer et al., 2010) and is not sex-specific spliced. In *N. vitripennis* compound eyes of males are slightly larger than those of males (Figure 10) while the *glu* homolog *LOC100678462* is sex-specific spliced. To understand if the *glu* gene has a conserved role for sexual eye development that associates with the gain of sex-specific splicing in the hymenopteran insects, we examined eye size development in *N. vitripennis* using RNAi mediated knockdowns (Supplementary figure 7). We observed that the width, length and intraocular distances of the compound eyes were similar between individuals that were either treated with *LOC100678462* dsRNA or control dsRNA irrespective of the sexes (Figure 10). This result suggests that the *glu* homolog *LOC100678462* has not a

conserved role in sexual development that is shared between the *N. vitripennis* and the honeybee.

Together our evolutionary and functional studies demonstrate that the *glu* gene is a new sexual developmental regulator that de novo evolved its sex-specific function. The gene gained its sex-specific expression by changes in splice regulation and evolved at least a new C2H2 ZnF domain by changes in the coding sequence. The evolution of these sex-specific peptides lead to a novel protein function and the co-option of the gene into the process of sexual differentiation. Hence, the sexual dimorphic eyes evolved in honeybees by the origin of a novel developmental regulator.

Discussion

Our study on dimorphic eye development in honeybees identified a new regulator of sexual development. The sex-specific expression of the *glu* gene is controlled by the *fem* gene and alternative splicing generating a long peptide only in females. This peptide encodes elements, including a C2H2 ZnF domain, that are essential for the female-specific eye differentiation. Previous studies, particularly in invertebrates, showed that external sexual dimorphic development is largely controlled by a shared developmental regulator, the *dsx* gene and its homologs. The Dsx proteins are TFs of the ZnF type that share a DM DNA binding domain. This domain consists of intertwined CCHC and HCCC zinc-binding sites and is expressed in both sexes (Erdman and Burtis, 1993; An et al., 1996). They regulate for example the tail tip morphology of *Caenorhabditis elegans* (the paralogs dmd-3 and mab-3; Mason et al., 2008), the elongation of the 1st antenna in *Daphnia magna* (Kato et al., 2011), the horn development in some beetles (Kijimoto et al., 2012; Ito et al., 2013; Ohde et al., 2018) and the formation of sex combs in *D. melanogaster* (Tanaka et al., 2011b; Rice et al., 2019). To gain sex-specific activity the *dsx* homologs are expressed in only one sex (*D. magna*, *C. elegans*; Mason et al., 2008; Kato et al., 2011). Or, sex-specific spliced transcripts (usually under control of *tra/fem* genes) encode DM containing dsx proteins that differ in females and females only by short peptides at its C-terminal end (Shukla and Nagaraju, 2010; Verhulst and van de Zande, 2015). Our study now identified another dimorphic control mechanism that employs sex-specific splicing to produce proteins that contain the ZnF domain only in females.

Our study further demonstrated the de novo evolution of a regulator for sexual development. We found evidences that a pre-existing gene was co-opted into the sex-specific control and development. The first step of this genetic pathway was that sex-specific splicing of the *glu* common ancestor transcript was gained that relies on regulatory genetic changes. This resulted in the sex-specific expression of a short, possibly common and a long female-specific peptide. In a second step, sex-specific developmental activity was evolutionary gained in the female peptide by genetic changes of the coding sequence. These changes resulted in a gradual gain of a C2H2 ZnF domain. These results show that the evolutionary mechanism by which the gene was co-opted for the control of the sexual dimorphic eye development of the honeybee relied on both regulatory and coding sequence changes that affected sex-specific proteins.

Previous work, in particular in insects, in which the evolutionary mechanisms that underlie sexual traits have been uncovered, showed that pre-existing sex-specific activity of the *dsx* gene was redeployed for the new dimorphic functions. This co-option was realized by temporal and spatial changes of *dsx* transcription and by modification, gains and losses of transcription activities of *dsx* target genes (Williams and Carroll, 2009; Shirangi et al., 2009b; Tanaka et al., 2011b; Kijimoto et al., 2012; Ito et al., 2013; Ohde et al., 2018; Rice et al., 2019). Hence, our study on a novel sexual developmental regulator demonstrates other evolutionary routes and developmental functions for the control of sexual differentiation. This suggests that other developmental and evolutionary routes are accessible that expand the possibility to control the diversity of sex-dimorphic traits.

Material and Methods

Animal sources

The bees used in this study were feral colonies of the *Apis mellifera carnica* strain. Diploid female embryos were collected from naturally inseminated queen mothers. Haploid male eggs were collected from non-mated queens treated with CO_2 to induce laying of unfertilized eggs. Embryos were collected using the Jenter egg collection box (Jenter Queen Rearing Kit, Karl Jenter GmbH) and either directly injected or kept in the incubator at 34 °C until required age (Schulte et al., 2014). Older wildtype pupae

and adults were collected directly from the combs at the stated age and dissected. Male controls for phenotyping were also collected at the required age from the hive.
Wildtype *Cimex lectularius* adult males and females were purchased from the Insect Services GmbH. Adult *Drosophila melanogaster* from the isogenic ^{w1118} strain were provided by Hermann Aberle.
The lab strain AsymCx of *Nasonia vitripennis* was cured from *Wolbachia* infection and constantly reared on *Calliphora sp.* hosts under the condition of 25 °C and 16 h light/8 h dark. Based on the sex-specific trait (forewing size), male and female wasps were separated before eclosion. Given the haplodiploid reproduction character of *N.vitripennis*, male offspring were directly generated by offering hosts to virgin females. To generate female offspring, a virgin female was given a single male to mate for one day. Afterwards, two hosts per day were provided to individual females to initiate oviposition. Since mated *N.vitripennis* tend to lay extremely female-biased eggs, offspring from mated females was considered female (Hamilton, 1967).

DNA isolation, RNA isolation and cDNA synthesis

For genotyping genomic DNA was isolated from *A.mellifera* L1 larvae or pupal hindleg tissue with the innu prep DNA kit (Analytik Jena). For RNA-Isolation from dissected honeybee tissue, pools of three sex typed *D.melanogaster* or *C.lectularius* adults we applied the TRIZOL method (Thermo Fisher Scientific). RNA of whole L1 larvae or pools of embryos (30 embryos 55-70 h old, 60 embryos 25-40 h old, 90 embryos 0-15 h old) was isolated with the innu prepDNA/RNA kit (Analytik Jena).
cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit and oligo dT or random hexamer primers (Thermo Fisher Scientific). Subsequently, second strand synthesis was performed by addition of 10 µl of 10× DNA Polymerase Buffer, 40 U DNA Polymerase I, 0.8 U Ribonuclease H (Thermo Fisher Scientific), and 65 µl of dH2O to 20 µl cDNA first-strand synthesis product. Purification of cDNA was performed using the EZNA Cycle Pure kit (Omega Bio-Tek. Inc.).
RNA from *N.vitripennis* was extracted using ZR Tissue & Insect RNA MicroPrep™ (Zymo) following manufacturer's instructions. On column DNase treatment step was added. Subsequently, cDNA was synthesized by using 1 µg RNA template with a standard reaction mix (SensiFAST™ cDNA Synthesis Kit, Bioline). Reactions were carried out in a thermal cycler with 5 minutes priming at 25 °C, 30 minutes reverse transcription at 46 °C and 5 minutes reverse transcriptase inactivation at 85 °C.

RT-PCR

RT-PCR reactions were carried out using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) for follow up sequencing or using GoTaq® G2 Flexi DNA Polymerase (Promega) following manufacturer's instruction. A standard PCR profile for GoTaq® G2 Flexi DNA Polymerase (Promega) includes: 3 minutes at 95 °C, 35 amplification cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, 50 seconds at 72 °C and a final extension of 5 minutes at 72 °C in a thermal cycler. PCR condition were adjusted to avoid saturation of product. The housekeeping genes *elongation factor 1-alpha* (*Nv-EF1α, N.vitripennis; Cl-EF1α, C.lectularius; EF1α, A.mellifera*) and *ribosomal protein L32* (*RPL32; D.melanogaster*) were used as reference. Used oligonucleotides are listed in supplementary table 5.

Characterisation of *glu* in *A.mellifera*

Potential exon structure of *glu* (*LOC552468*) was visualised using RNA-Seq data available in the NCBI databases (https://www.ncbi.nlm.nih.gov/gene/) and junctions were verified by RT-PCR on transcripts of male and female embryos and pupal brain tissue. Amplicons from at least two reactions for each fragment were sequenced (Sanger sequencing; Eurofins). The deducted amino acid sequences were searched for potential domains with PROSITE (de Castro et al., 2006).

Analysis of *glu* homologs

Homologous proteins were identified by BLAST analysis. Based on the sequence available in the NCBI database of *glu* homologs in *N.vitripennis* (*LOC100678462*), *C.lectularius* (*LOC106661925*) and *D.melanogaster* (*GC12316*) we designed primers for the exons homologues to the sex-specific spliced junction in *A.mellifera* (Supplementary table 5). Ancestral states were inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

CRISPR/Cas9 mutagenesis in *A.mellifera*

Target sites for sgRNAs were identified with the Benchling CRISPR tool (https://benchling.com/). Sequences were chosen to be 20 nt's long, starting with a 5' guanidine and with at least three mismatches to alternate, off-target sequences. A PCR

was used to generate a DNA fragments containing a T7 RNA polymerase transcription start site, the chosen target sequence and a Cas9 protein binding sequence. sgRNA (Supplementary table 6) was synthesized using the DNA fragment as a template and the Ribo Max Kit (Promega). The sgRNA was purified with the MEGAclear Kit (Thermo Fisher Scientific) and mixed in a molar ratio 2:1 with 500 ng/µl Cas9 protein (New England Biolabs) prior to injection. 0-1.5 h old honeybee embryos were injected with 400 pl sgRNA/Cas9 mixture each and kept in the incubator until hatching as previously described (Beye et al., 2002; Schulte et al., 2014). Hatched treated L1 Larvae as well as not injected controls were directly frozen for DNA/RNA analysis or transferred onto 170 mg of worker diet (w/v in sterile water: 50% royal jelly, 15% glucose, 15% lactose, 1% yeast extract; Kaftanoglu et al., 2011) and incubated at 34 °C and 94% humidity until 5th larval stage. During defecation larvae were transferred to Petri dishes with filter paper and kept at 34 °C and 75% humidity until red eye pupal stage (Schmehl et al., 2016).

Identification of CRISPR/Cas9 induced mutations

Individuals were screened for mutations in the *glu* gene by amplification of the area of the DNA surrounding the sgRNA target site. Mutational screens on the *fem* gene were performed on cDNA by fragment length analysis (Roth et al., 2019). Used oligonucleotides (Eurofins Genomics) are listed in supplementary table 5. Deletions in *glu*$^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ individuals were detected by electrophoresis of the amplicons, smaller insertions/deletions in *fem* and *glu* were detected by fragmentlenght analysis of hexachloofluorescein labeled PCR products (Roth et al., 2019). We identified *glu*^{tmC2H2} mutations by amplification of the genomic area and a subsequent treatment of the amplicons with the restriction enzyme *PdiI* (Thermo Fisher Scientific) targeting the introduced restriction site.

DNA sequence of mutated individuals was determined using next generation sequencing. Amplicons of relevant regions were used for index PCR with the Nextera XT Index Kit (Illumina) followed by purification with the Agencourt AMPure XP beads (BeckmanCoulter). Library preparation and sequencing of 2x250bp reads using the MiSeq System with the MiSeq Reagent Kit v2 (500 cycles; Illumina) was performed following the Illumina protocols by the Center for Biological and Medical Research (BMFZ, Heinrich-Heine University). A minimum of 78000 reads per sample was generated. Raw sequences were processed and analysed using the Galaxy online

toolset (http://usegalaxy.com; Afgan et al., 2018) Sequences with an abbundance of lower than 5% per sample were removed during sequence data evaluation.

RNAi of *tra^F* and *LOC100678462* gene

To test whether *LOC100678462^F* is spliced under the control of *tra* and to verify the function of *LOC100678462*, MEGAscript RNAi Kit (Thermo Fisher Scientific) was used to produce dsRNAs to target *tra^F* and *LOC100678462* mRNA. *Gfp* dsRNA was used as a control which was generated from the vector pOPINEneo-3C-GFP, a gift from Ray Owens (Addgene plasmid # 53534; http://n2t.net/addgene: 53534; RRID: Addgene_53534). Primers of *LOC100678462* and *tra^F* RNAi are provided in supplementary table 5. Before microinjection, dsRNAs were diluted to the final concentration 4000 ng/µl (NanoDrop™ 2000 Spectrophotometer, Thermo Fisher Scientific).

For dsRNA targeting *tra^F*, injection was performed on 4th instar females. RNAi on *LOC100678462* mRNA was performed in 2nd instar males and females. Both 2nd and 4th instar larvae were collected from parasitoid hosts and placed on 1X PBS agar plates before injection. Microinjection in *N.vitripennis* larvae was carried out with FemtoJet® 4i (eppendorf) and followed the protocols described by Werren et al. (2009) with minor changes. Red colour food dye was added to the dsRNA solution in 1:9 ratio to guide the injection. After injection, 2nd instar larvae were transferred back to the foster hosts (6-8 larvae per host) to resume feeding. All foster hosts with injected samples were placed back on 1X PBS plates to prevent dehydration and incubated at rearing conditions. Female samples were collected 3d, 4d and 5d after 4th instar larva *tra^F* knockdown. All samples were fast frozen in liquid Nitrogen.

To test silencing efficiency of *LOC100678462* dsRNA injection, samples were collected at the white pupa stage. Four to five pupae were pooled to produce one biological replicate. SensiFASTTM SYBR® No-ROX Kit manual (Bioline) was used to conduct the qPCR in order to verify the silencing efficiency of *LOC100678462* knockdown. *LOC100678462* qPCR primers were designed outside the RNAi region (Table S1). *Nv-EF1a* transcripts were used as reference. qPCR was carried out using the CFX96TM Real-Time System (Bio-Rad) with CFX Manager 3.1 Software (Bio-Rad). The standard qPCR profile consists of 95 °C for 3 minutes, 45 amplification cycles of 15 seconds at 95 °C ,15 seconds of 55 °C, 30 seconds of 72 °C and a final standard dissociation curve step to check for non-specific amplification.

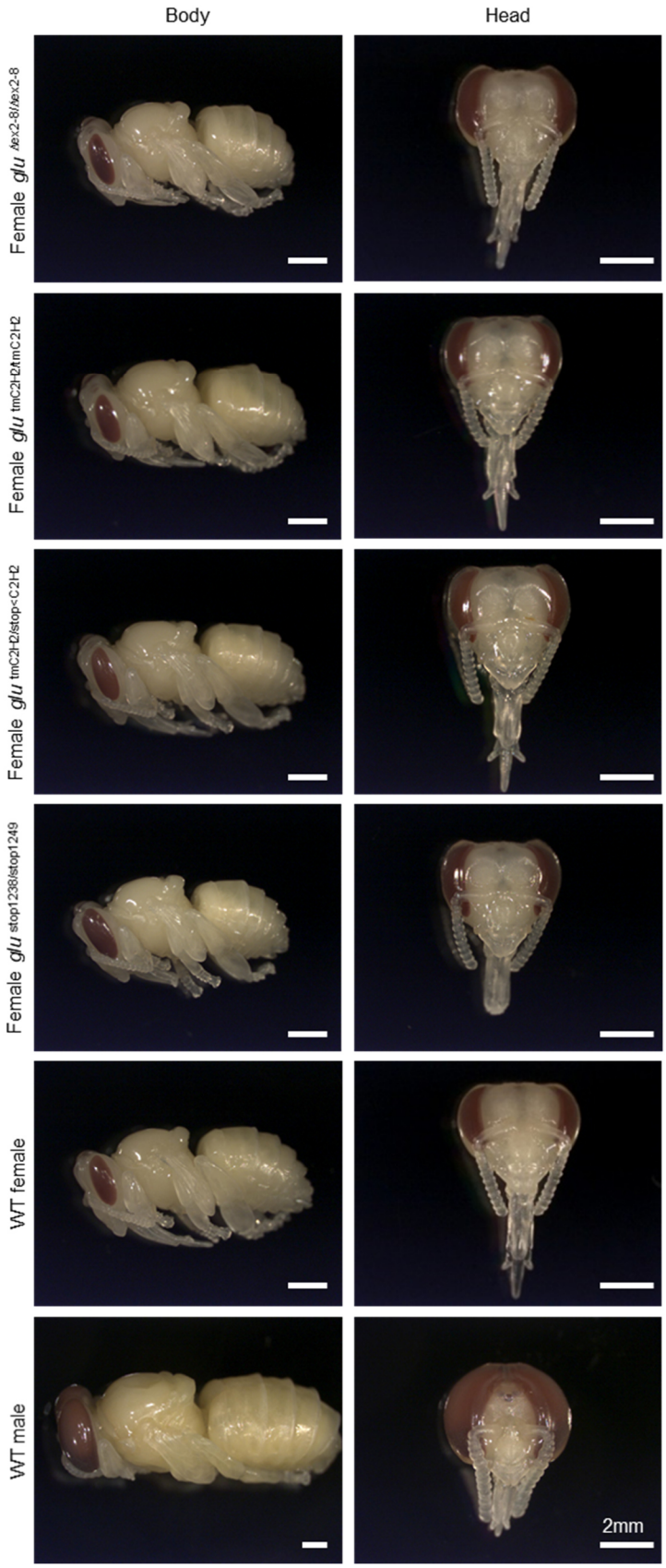
qPCR data was first imported to LinRegPCR software (LinRegPCR, 2017.1.0.0, HFRC, Amsterdam, The Netherlands; Ramakers et al., 2003). After baseline correction, the initial number of templates (N0) were calculated based on the average PCR efficiency of each amplicon. Relative expression levels of *LOC100678462* in each sample was obtained by dividing the N0 value of *LOC100678462* by N0 value of *Nv-EF1a*. For *N.vitripennis* males *LOC100678462* knockdown was performed in two separate experiments to increase sample size. The individuals from both experiments were used for head measurements, as the effect of silencing (Supplementary figure 7) was the same.

Head measurements

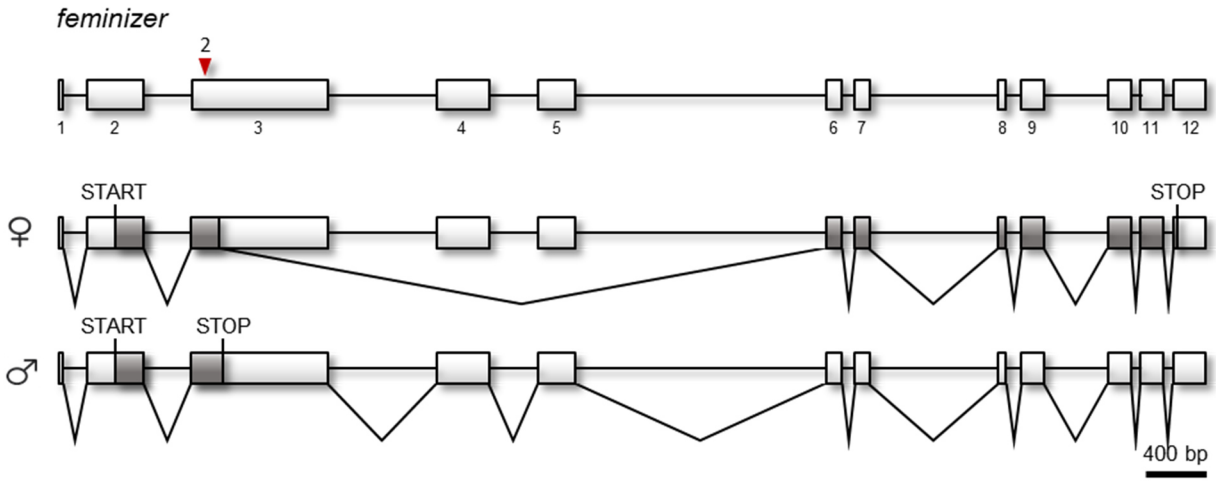
For the measurements of the head, heads were dissected on clean glass slides and positioned using tweezers. Heads of honeybee pupae were photographed with a S8 APO binocular (Leica), a UI-1240LE-C-HQ camera and the software uEye Cockpit (IDS). Head length was measured from top to bottom and maximum head width at a 90° angle on the level of the antennal basis (Supplementary figure 8). Eye length was measured as the longest possible linear measurement across each eye and was seen relative to the total head length. Relative interocular distance was measured as the distance between the eyes at the position of the upper two ocelli in relation to the maximum head width. Eye width and head width position for the eye/head width ratio was obtained at a 90° angle at half the length between top of the head and antennal basis.

Photos of *N.vitripennis* adult heads were taken with a Dino-Lite Edge 5MP digital microscope and the DinoCapture 2.0 software. Interocular distance and eye width at maximum head width, maximum width of the head and length from top of head to bottom of head (Supplementary figure 8) was measured based on the standard methods described by Werren et al. (2016). Measurements were performed using ImageJ (National Institute of Mental Health, USA). Comparison of parameters in *N.vitripennis* males and females was performed on absolute values, since the dsRNA treatment affected head width and length as well as the inter-individual variances. Individuals with a head width and head length value outside 1.5 times the standard deviation from the mean of *LOC100678462* dsRNA treated individuals were not analyzed further to exclude outliers.

Supplementary data



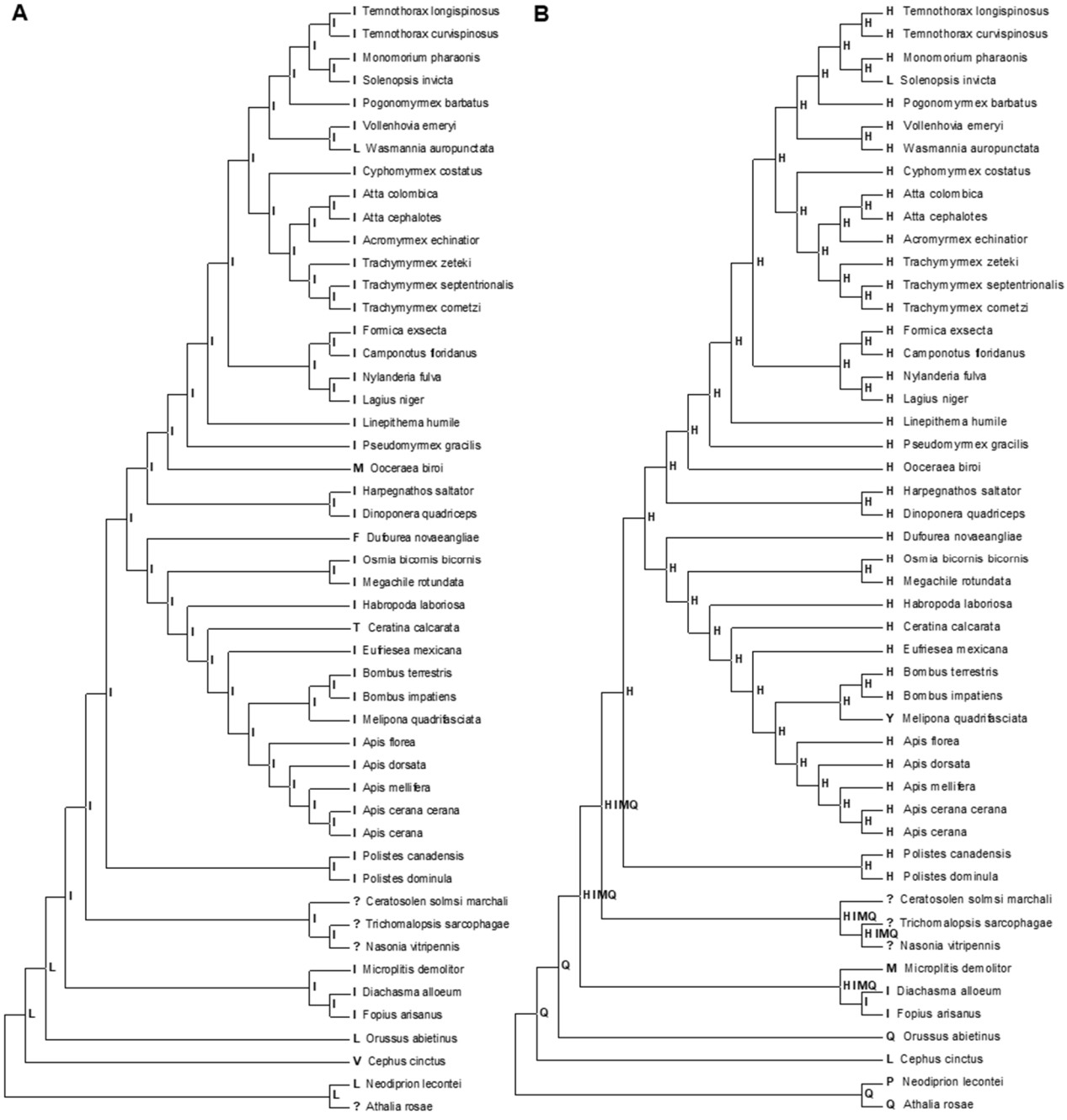
Supplementary Figure 1: The outer morphology of the mutants (full view)



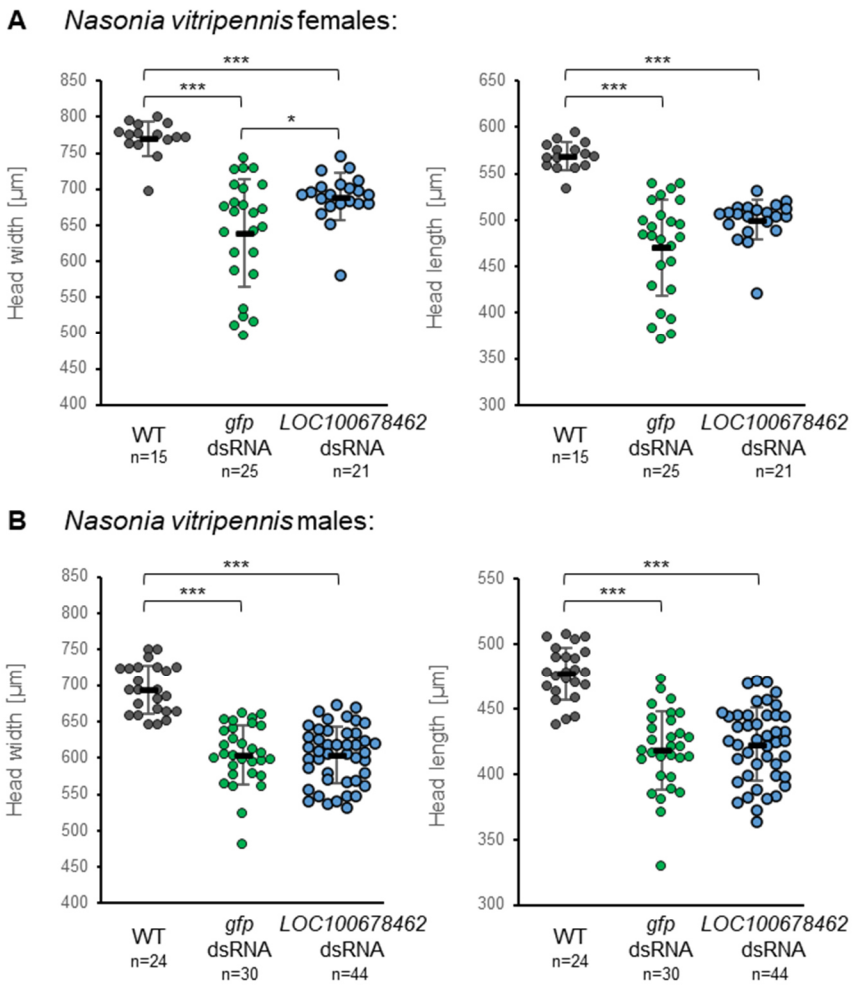
Supplementary Figure 2: Genomic organization of the *fem* gene with its female- and male - specific splice forms. Boxes indicate the exons and grey fillings the open reading frame. The red arrow indicates the position of the target site for *fem* sgRNA2 and the CRISPR/Cas9 mediated mutations.

...HMASNCAIPGFSGIHSHPREVDSQHPLHIPATHPTTLPAIYGNCADNTELCVPYHKHCTSVSCNLQINATSSLYS
HAKSGNPCGRSHCSCLNCTYDIVAHCRQCMHPASDSHVSCIESSPYFLSTHSSVQSPAVQEHDRAKNEVIEKLYDD
QLLCKIEKNLLQNNSLEKLEVQCDSERMFNKAAENKLPLKKRLKAHAMAYGEVQIKAKNVDNYPAMPMMSIAALEA
LDNTRKGSDQIVKSEYEVSVGKKEESHNDYHCSSNLIRRNYYKDMHVANSHQNLAKENTRKIECQFRSTNNQTDNT
ICQESCLQRTVKTSAQRKEINLSDVASLKQFDIEPMEQEGTYKKIKKTQSPLRQTRSSKRNVPKVNYSYTDVDPEW
NPSGESKRKRKKTSR*

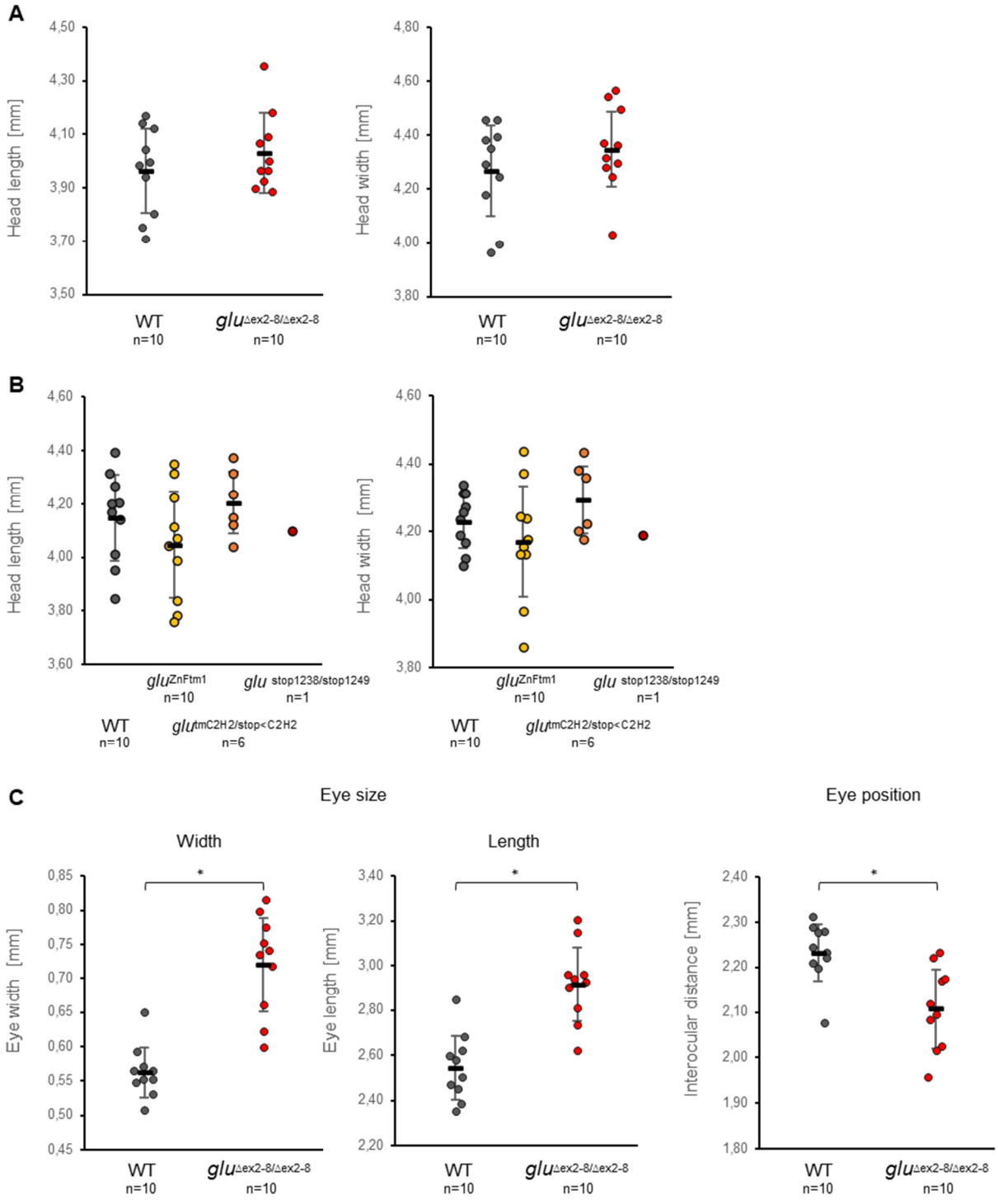
Supplementary Figure 3: Non-canonical C2H2 motif in the C-terminal part of the female Glu peptide. A possible non-canonical zinc finger motive is underlined. The examined canonical C2H2 zinc finger motif is highlighted in yellow. The amino acids relevant for zinc ion complexion, H and C, are shown in red letters.



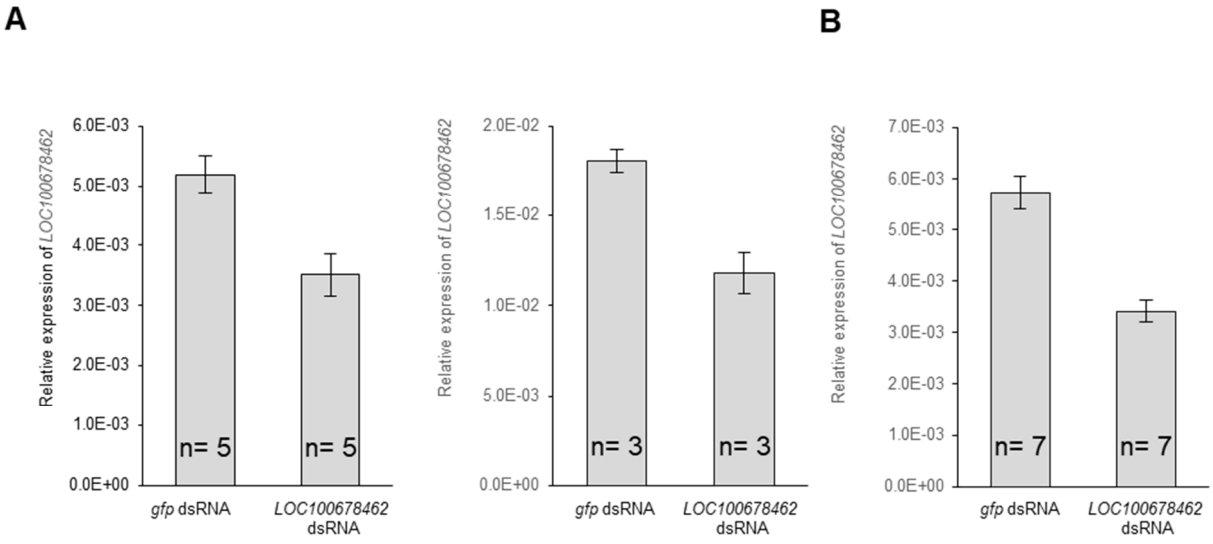
Supplementary Figure 4: Inferred evolutionary changes of amino acids at core sites of the C2H2 ZnF motif. Ancestral states were inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). The tree shows a set of possible amino acids (states) at each ancestral node based on their inferred likelihood at (A) the hydrophobic isoleucine at the core position and (B) the 2nd histidine of the canonical C2H2 motif. The set of states at each node is ordered from most likely to least likely, excluding states with probabilities below 5%. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The phylogenetic relationship presented in the tree derived from Peters et al. (2017). The analysis involved 49 amino acid sequences. There were a total of 129 positions in the final dataset.



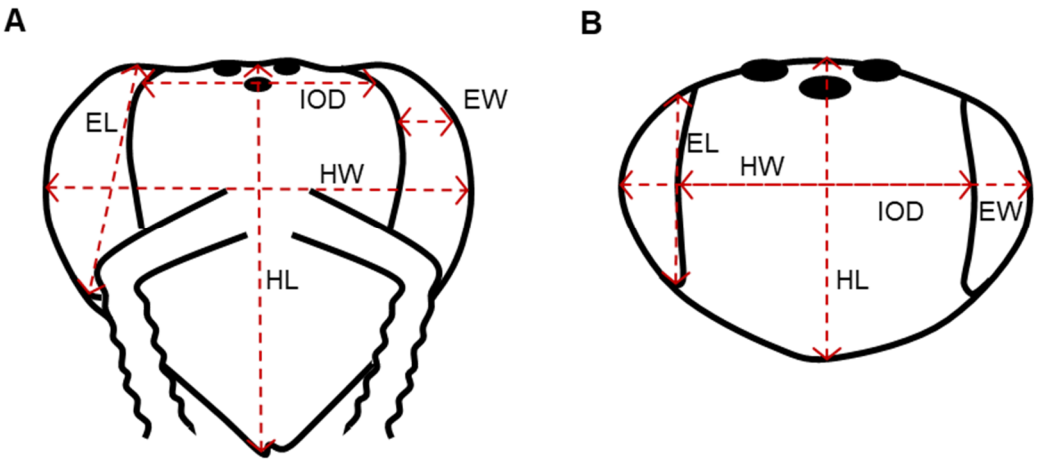
Supplementary Figure 5: *N.vitripennis* head width and length. Measured values for head width and head length of (A) *N.vitripennis* females and (B) of *N.vitripennis* males treated with *gfp* dsRNA or *LOC100678462* dsRNA (*p<0.05, ***p<0.001, two tailed Mann-Whitney U test). Means and standard deviations are shown.



Supplementary Figure 6: Absolute values of head parameters for mutant honeybee pupa. Measured values for head width and head length of (A) *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ and (B) of *glu* $^{tmC2H2/tmC2H2}$, *glu* $^{tmC2H2/stop<C2H2}$, *glu* $^{stop1238/stop1249}$ mutants. (C) Absolute values for eye width, eye length and eye position of *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ (*p<0.05, one tailed Mann-Whitney U test). Means and standard deviations are shown.



Supplementary Figure 7: relative Expression levels of *LOC100678462* after RNAi in *N.vitripennis* males (A) and females (B). Individuals injected with *gfp* dsRNA were used as a reference. Expression levels were standardized to *Nv-Ef1a* expression levels between samples. The dsDNA injection was performed in two separate experiments to increase total gained sample size. Error bars show standard error.



Supplementary Figure 8: Measurements of head parameters in (A) *A.mellifera* and (B) *N.vitripennis.* EL: eye length; IOD: interocular distance; HW: head width; EW: eye width; HL: head length.

Supplementary Table 1: Genotype sequences of *fem*$^{-/-}$ individuals. The deletions were sequenced for each individual using amplicons. Wildtype sequences are provided as reference. Target sites of *fem* sgRNA2 for CRISPR/Cas9 mediated double strand brakes are underlined.

Individual	sequence at target site aligned to wildtype sequence
# 27	wildtype AGAGGACCAGAAG----------GTACTCAAGTTAGT sequence 1 ··········----------·········· sequence 2 ··········----------·········· sequence 3 ··········.GTACTCAGAGAAG.········· sequence 4 ··········.---------TTAG.··········
# 36	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1/2 ··········-··········
# 37	wildtype AGAGGACCAGAAG-GTACTCAAGTTAGT allele 1/2 ··········.TC··········
# 44	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1 ··········-·········· allele 2 ··········--··········
# 56	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1/2 ··········.----·····
# 51	wildtype AGAGGACCAGAA----------GGTACTCAAGTTAGT allele 1 ··········.CTCAAGTAGGACC········· allele 2 ··········.GTTATTTAGA-----··········
# 52	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1/2 ··········-··········
# 53	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1/2 ··········----·····
# 55	wildtype AGAGGACCAGAA----------GGTACTCAAGTTAGT allele 1 ··········----------·········· allele 2 ··········.GGTACTCAGAGAAG.··········
# 58	wildtype AGAGGACCAGAAGGTACTCAAGTTAGT allele 1/2 ··········----·····
# 89	wildtype AGAGGACCAGAA----GGTACTCAAGTTAGT

	allele 1/2 ....AGTT..............

Supplementary Table 2: Splicing of *dsx* transcripts in *fem*$^{-/-}$ individuals. The sex-specific transcripts of the *doublesex* (*dsx*^{M} and *dsx*^{F}) were amplified in *fem*$^{-/-}$ first stage larvae and in wildtype (WT) male and female controls.

	Individuals screened	Individuals with *dsx*^{F} splicing	Individuals with *dsx*^{M} splicing
Females *fem* $^{-/-}$	6	0	6 (100%)
WT male	6	0	6 (100%)
WT female	6	6 (100%)	0

Supplementary Table 3: Genotype sequences of *glu* $^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ individuals. The deletions were sequenced for each individual using amplicons. Wildtype sequences are provided as reference. Treatment with sgRNA1 and sgRNA3 resulted in deletion of 4638 bp.

Individual	*glu* sequence at target site aligned to wildtype sequence	frequency
M62	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA .....---[-4638 bp] ----..... .....------[-4638 bp]	allele 1: 49% allele 2: 50%
M88	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ..... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA .....------[-4638 bp] ....------[-4638 bp]	allele 1: 48% allele 2: 51%

M89	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 .... allele 2 .... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] -------CGCAGGTGTA ....------[-4638 bp] -------......... ....[-4638 bp] GGAAAGA.........	allele 1: 64% allele 2: 36%
M90	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 .... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp] ....------[-4638 bp]	allele 1: 52% allele 2: 48%
M91	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 .... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp]	allele 1/2: 100%
M92	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC ....------ CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp] ..... ----------[-4638 bp] -.....	allele 1: 43% allele 2: 57%
M93	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp]	allele 1/2: 100%
M94	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 .... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp] ....------[-4638 bp]	allele 1: 50% allele 2: 50%

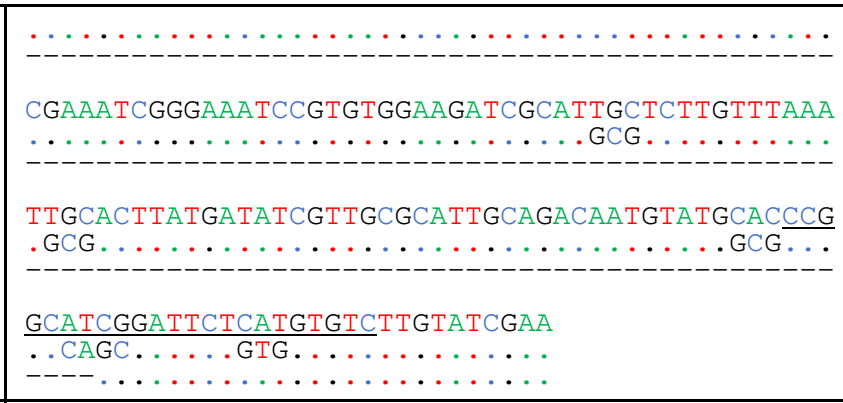
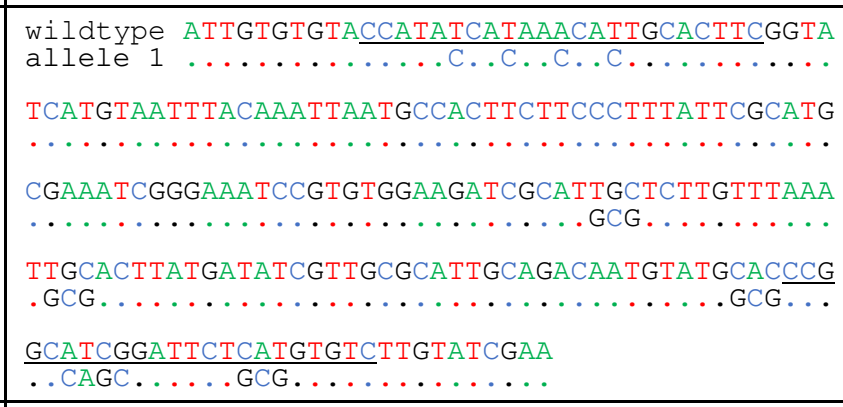
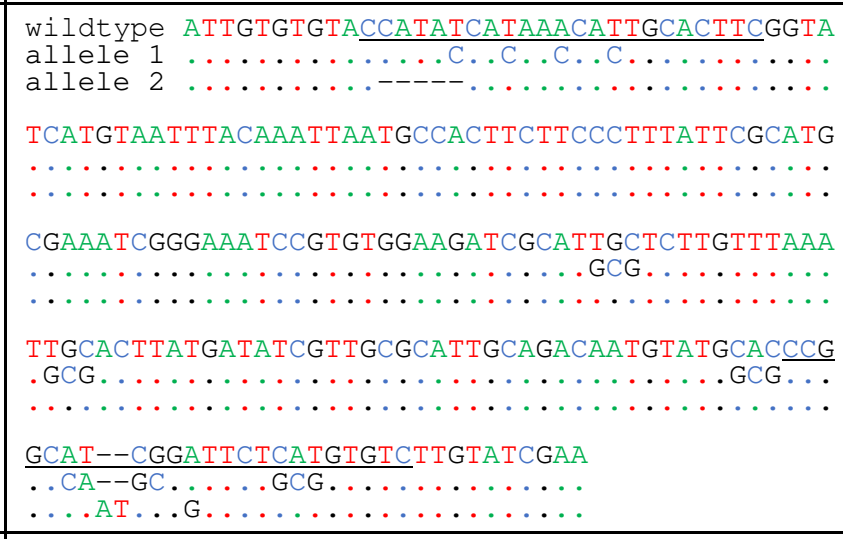
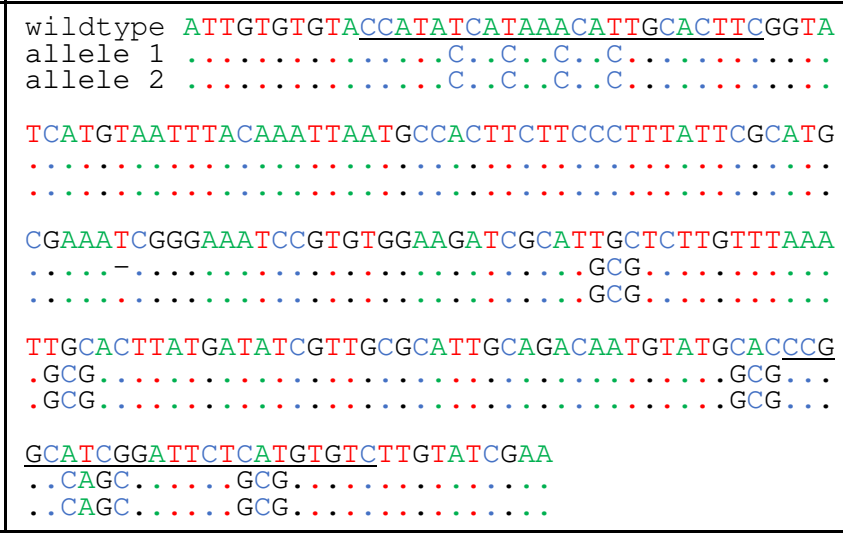
M227	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... allele 2 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] ---------CGCAGGTG ....------[-4638 bp] ---------....... ....----AT[-4638 bp] TTGAAGAAG..T..A..	allele 1: 60% allele 2: 30%
M242	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC ..... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp]	allele 1/2: 100%
M244	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... allele 2 ..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC .... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp] ....------[-4638 bp]	allele 1: 46% allele 2: 54%
Mut3	wildtype AGAATAAATGAGAACTTGGTAACAGTGGGATGTAGAG allele 1 ......G..... GACGCGCTCTCGTGGTGGGTATACCAAATGCCAGTGTCAATATGGC ..... CGTTGCCGAGAGCGCAGT [4638 bp] CGCAGGTGTAAATAATA ....------[-4638 bp]	allele 1/2: 100%

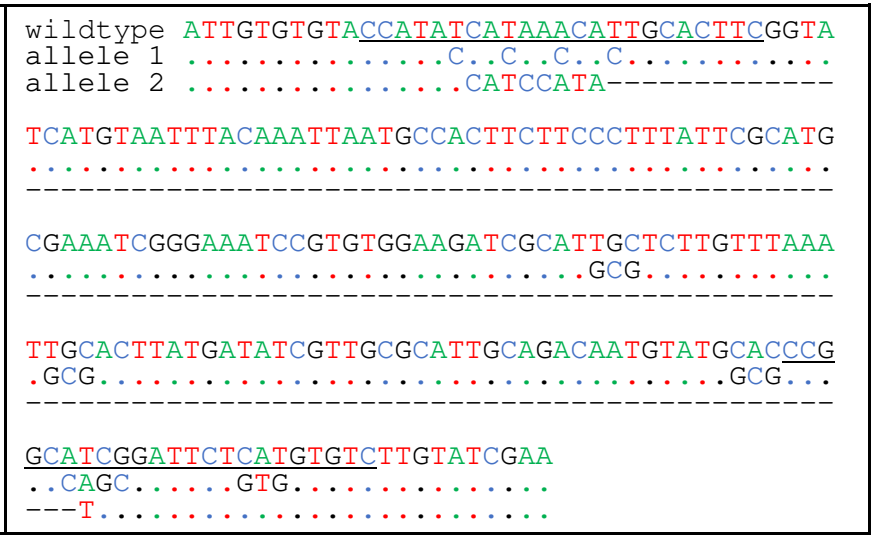
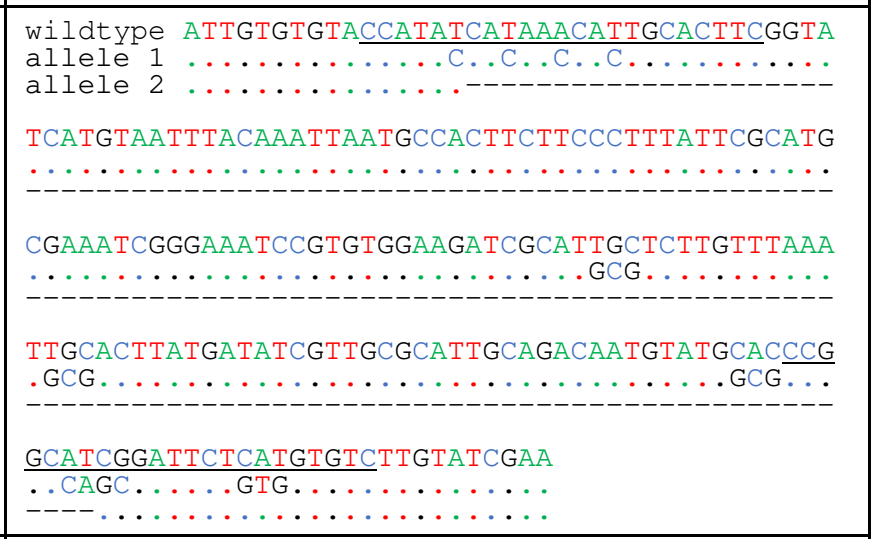
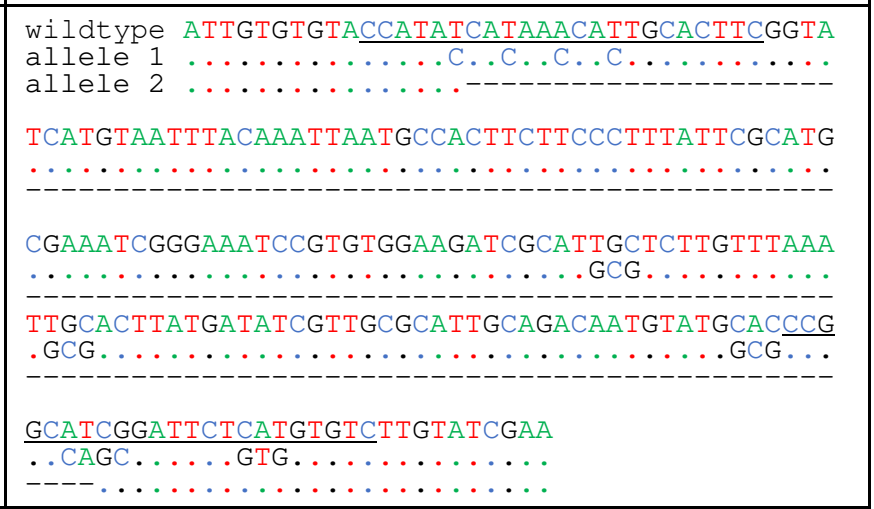
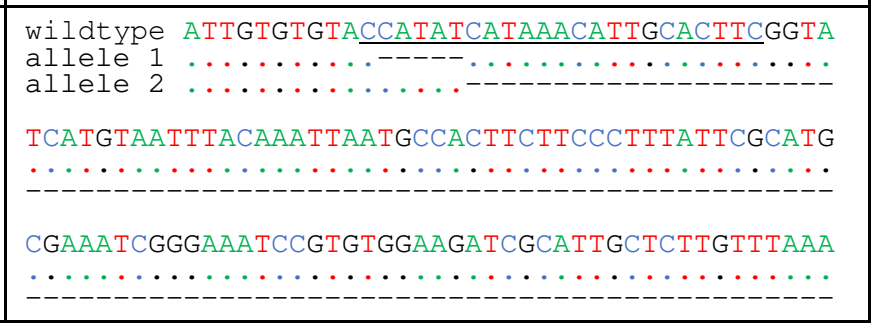
Supplementary Table 4: Genotype sequences of *glu* $^{tmC2H2/stop<C2H2}$, *glu* $^{tmC2H2/tmC2H2}$ and *glu* $^{stop1238/stop1249}$ individuals. The deletions were sequenced for each individual using amplicons. Wildtype sequences are provided as reference. Target sites of sgRNA9 and sgRNA10 for CRISPR/Cas9 mediated double strand brakes are underlined.

Individual	treatment	*glu* sequence at target site aligned to wildtype sequence
Z2	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....

Z5	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....
Z6	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....
Z7	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....
Z8	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... allele 2 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG .....A.... ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG..... ..CAGC.....GCG.....
Z9	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG .....

		CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....
Z13	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... allele 2 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG .... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG..... ..CAGC.....
Z15	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... allele 2 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG .... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG..... ..CAGC.....
Z18	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG ..... CGAAATCGGGAAATCCGTGTGGAAGATCGCATTGCTCTTGTTTAAA .....GCG..... TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .GCG.....GCG... GCATCGGATTCTCATGTGTCTTGTATCGAA ..CAGC.....GCG.....
Z21	sgRNA 9/10, dsDNA	wildtype ATTGTGTGTACCATATCATAAACATTGCACTTCGGTA allele 1 .....C..C..C..C..... allele 2 .....---------- TCATGTAATTTACAAATTAATGCCACTTCTTCCCTTTATTCGCATG

		
Z25	sgRNA 9/10, dsDNA	
Z4	sgRNA 9/10, dsDNA	
Z11	sgRNA 9/10, dsDNA	

Z19	sgRNA 9/10, dsDNA	
Z20	sgRNA 9/10, dsDNA	
Z26	sgRNA 9/10, dsDNA	
Z39	sgRNA 9/10, dsDNA	

		TTGCACTTATGATATCGTTGCGCATTGCAGACAATGTATGCACCCG .... -- GCATCGGATTCTCATGTGTCTTGTATCGAA ...-----...................... --A...........................

Supplementary Table 5: List of oligonucleotides

Application	Oligo Name	sequence (5‘ → 3‘)
reference gene for RT-PCR in *A.mellifera*	#EM033 [a]	CGTTCGTACCGATCTCCGGATG
	#EM034 [a]	GCTGCTGGAGCGAATGTTAC
glu RT-PCR of sex specific splicing	1314: *glu* fw1	CCAGGACGGACCTGCAACTC
	1316: *glu* rev1	TTTGCCTCTTCTAACCATGC
RT-PCR of dsx^F/ dsx^M splicing	#417 [a]	CTATTGGAGCACAGTAGCAAACTTG
RT-PCR of dsx^F splicing	#419 [a]	GAAACAATTTTGTTCAAAATAGAATTCC
RT-PCR of dsx^M splicing	#418 [a]	GGCTACGTATGTTTAGGAGGACC
screen for deletions in $glu^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ females	1370: *glu* fw4	TACCAAATGCCAGTGTCAAT
	1371: *glu* rev5	CGATAATGTTATCTTATCGG
	1405: *glu* fw6	GCATGGTTAGAAGAGGCAAA
screen for mutations in $glu^{tmC2H2/stop<C2H2}$ females; PCR for amplicon sequencing	*glu* NGS fw 1p	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCTATAAATTCACAAATATATCAGG
	glu NGS rev 2p	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCCATTGCATGCGCTTTCAACC
	glu NGS rev 1p	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTGCAATGCGCAACGATATC
	glu NGS fw 2p	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTCCCTTTATTCGCATGCGAAATCG
	glu NGS del fw	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCCACGAGAAGTTGATAGTCAACAT
amplicon sequencing of mutations in fem^- females	*fem* NGS fw 1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAAGCCGAGGTAGAAGTAATG
	fem NGS rv 2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCCGTTTAAGTTCATCAGA
amplicon sequencing of mutations in $glu^{\Delta ex2\text{-}8/\Delta ex2\text{-}8}$ females	*glu* NGS fw4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAAATAATAAAAAGTATTC
	glu NGS rv1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATATATAAATACCATTTTTG
glu RT-PCR	1317: *glu* rev2	GCCTGGATTTCTTATTTCGC
	1485: *glu* fw16	TGTGCAAACTGTGAAAGAAAATACA
	1448: *glu* fw14	TGCAAAAATCTCCTAATAGC
	1449: *glu* rev8	CCCAGAGATGCAATTGCTGC
	1548: *glu* ex4 rev	CATTAAAGAGTCACTAATAAAATCC
	1546: *glu* ex1 fw2	TTCTTTCGAGTTACGTCGTAATGCG
	1315: *glu* fw2	TCCCACACATCCAGAATCAG
	1547: *glu* ex2 fw2	ATGTGGAAAGACGTGGTCGCGATAG
	1447: *glu* fw13	GCGCATGCAATGGCATATGG
	1483: *glu* rev12	CCACAATCTTCAGTTTGAGATATCC
	1369: *glu* rev4	TCCTTAACGATATCTTCCGG
detection of insertion/deletions in	*fem* 1_fw3-NotI	GATCGCGGCCGCCTTCTTCGGTATTTATCATAAAAATGAAACGG

fem caused by sgRNA2	*fem* 2_rev3 (HEX)	CTTCTTTTGAGCATCACATCTTCAGG
reference gene for RT-PCR in *D.melanogaster*	*Dmel_RLP32*_fw1	CCCAAGATCGTGAAGAAGCG
	*Dmel_RLP32*_rv1	TTCTTGAATCCGGTGGGCAG
reference gene for RT-PCR in *C.lectularius*	*Cilec_Ef1a*_fw1	ACATCGTCGTGATCGGACAC
	*Cilec_Ef1a*_rv1	GGGCATCAATGATGGTCACA
LOC106661925 RT-PCR	*Cilec_ LOC106661925*_fw1	GGCAAAATGTAGCTGGGAGC
	*Cilec_ LOC106661925*_rv2	CACAGGTGTTTTCAAGACAT
CG12316 RT-PCR	*Dmel_ CG12316*_fw1	TCCTCGCTGCACCCAAGCTC
	*Dmel_ CG12316*_rv2	CCATCTACGTTGGCAGGCAC
tra dsRNA	Nv_Tra_RNAi_F1[b]	[TAATACGACTCACTATAGGG]CGAGACATC AGTTAGAAGAT
	Nv_Tra_RNAi_R1[b]	[TAATACGACTCACTATAGGG]GTCTTGTGG TCCTATGAAAC
LOC100678462 dsRNA	*LOC100678462* RNAi_F3	[TAATACGACTCACTATAGGG]AAACACGAA CAATTAGGCGC
	LOC100678462 RNAi_R3	[TAATACGACTCACTATAGGG]ATCTGTACA AACTTGGCGCA
LOC100678462 RT-PCR for male1 splicing	*LOC100678462*_M&F_ Forward	TACGACTGATCCCGGCTAAT
	*LOC100678462*_M_ Reverse	CTGTTGGAGCTTGTTGGAGT
LOC100678462 RT-PCR for male2 splicing	*LOC100678462*_M2_ Forward	AAGTTTTACCGTCTGCTGCA
	*LOC100678462*_M2_ Reverse	CGCGTACCGAATTGTTTTGT
LOC100678462 RT-PCR for female splicing	*LOC100678462*_M&F_ Forward	TACGACTGATCCCGGCTAAT
	*LOC100678462*_F_ Reverse	ACGACTTTGATTCGAGCTCC
LOC100678462 qPCR	*LOC100678462*_qpcr_ Forward	AGTCGAGAGTTTTCCGCCT
	*LOC100678462*_qpcr_ Reverse	GGGAAACTGTATGTCAACGTTC
reference gene for qPCR and RT-PCR in *N.vitripennis*	Nvit_EF1a_qPCR_F1[b]	CACTTGATCTACAAATGCGG
	Nvit_EF1a_qPCR_R1[b]	GAAGTCTCGAATTTCCACAG
RT-PCR for tra^F & tra^M splicing	Nv_Tra_F2[b]	GACCAAAAGAGGCACCAAAA
	Nv_Tra_R3[b]	GGCGCTCTTCCACTTCAAT

Sequences in '[]' indicate the T7 adaptor sequence that provided by the MEGAscript RNAi Kit (Thermo Fisher). Underlined are adaptor sequences for follow up index PCR prior to amplicon sequencing (Nextera XT Index Kit, Illumina).

a. Nissen et al., 2012

b. Verhulst et al., 2010

Supplementary Table 6: Nucleotide sequences of the sgRNAs and dsDNAs used for homologues recombination. Sequences of sgRNAs matching target site in the genome are shown in bold letters.

molecule	nucleotide sequence
fem sgRNA2	**GCACUAACUUGAGUACCUUC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
glu sgRNA1	**GUUGCCGAGAGCGCAGUAUG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
glu sgRNA3	**GAUCUAGAAACAGAAUGCGC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
glu sgRNA9	**GACACAUGAGAAUCCGAUGC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
glu sgRNA10	**GAAGUGCAAUGUUUAUGAUA**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
dsDNA for mutation	TATAAATGTTATGAACAAATCGATAGACAAGAGGAAATCAAATTCTACTTTTCGACAA AATATCAAGCAAGAGTTCAATATCTCCAACCACATGGCATCGAATTGTGCAATTCCAG GATTCAGTGGAATTCACTCGCATCCACGAGAAGTTGATAGTCAACATCCCTTGCATAT TCCCGCTACTCATCCAACGACCTTACCAGCGATTTATGGAAATTGTGCTGACAACACG GAATTGTGTGTACCATACCACAACCACTGCACTTCGGTATCATGTAATTTACAAATTA ATGCCACTTCTTCCCTTTATTCGCATGCGAAATCGGGAAATCCGTGTGGAAGATCGCA TGCGTCTTGTTTAAATGCGACTTATGATATCGTTGCGCATTGCAGACAATGTATGGCG CCGGCCAGCGATTCTGCGGTGTCTTGTATCGAAAGTAGTCCGTATTTCTTGTCAACGC ACTCTTCAGTACAAAGCCCTGCCGTCCAAGAGCATGACAGAGCAAAGAATGAGGTCAT AGAAAAATTATACGACGATCAATTATTGTGTAAGATAGAGAAGAATCTTTTGCAAAAT AATTCATTGGAAAAATTGGAAGTACAATGTGATTCCGAAAGGATGTTTAATAAAGCTG CAGAAAATAAATTACCTTTGAAGA

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Author's Contribution: Manuscript III

A novel regulator for sexual eye development evolved by gain of female-specific sequences in the Glubschauge protein

Journal: Manuscript in preparation for submission to "Nature Communications"

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Author's contribution:

- Concept of study
- Experimental design
- Design and synthesis of sgRNAs for CRISPR/Cas9 mutagenesis
- Injection of honeybee embryos
- *In vitro* rearing of honeybee pupae
- Phenotyping of honeybees
- Genotyping of mutant honeybees
- Transcriptional studies of *glu*
- Dissections of honeybees
- Measurements of head parameters
- Bioinformatic analysis of amplicon sequencing data
- Identification of homologs and phylogenetic analysis
- Data analysis
- Statistical analysis
- Authoring the manuscript

Kapitel III

Zusammenfassung

In der Honigbiene lassen sich ein ausgeprägter Sexualdimorphismus zwischen den Geschlechtern und ein Kastendimorphismus innerhalb der Weibchen, zwischen Arbeiterinnen und Königinnen beobachten. Merkmale wie Körpergröße und Kopfmorphologie unterscheiden sich zwischen den drei Morphen. Während der Kastendimorphismus in Weibchen durch das Futter im Larvenstadium ausgelöst wird, wird der Sexualdimorphismus zellautonom durch die Geschlechtsdeterminations-kaskade kontrolliert. In der Fruchtfliege wird die Entwicklung von primären und sekundären Geschlechtsmerkmalen durch die Transkriptionsfaktoren Dsx^M und Dsx^F gesteuert, die am Ende der Geschlechtsdetermination stehen. Im Rahmen dieser Arbeit wurde gezeigt, dass die Differenzierung von primären Geschlechtsmerkmalen in Arbeiterinnen der Honigbiene durch das *dsx* Gen reguliert wird. Mit Hilfe der CRISPR/Cas9 Methode erzeugte Mutationen im *dsx* Gen hatten keinen Einfluss auf sekundäre Geschlechtsmerkmale, wie die Kopfmorphologie. Dies impliziert, dass in der Honigbiene an der Realisierung der sekundären Geschlechts-merkmale weitere Gene beteiligt sind. Der Vergleich des männlichen und weiblichen Transkriptoms von Embryonen hat ergeben, dass Spleißregulation bei der Diversifizierung der Geschlechter eine komplexere Rolle spielt als bisher vermutet. Es wurde eine Zunahme geschlechtsspezifisch gespleißter Transkripte mit fortschreitender Embryonalentwicklung beobachtet. Zusätzlich waren differenziell regulierte Gene, die mit RNA-Bindefunktionen assoziiert sind und potenziell spleißregulatorisch wirken können, kurz vor dem Schlupf des Embryos überrepräsentiert. Innerhalb der geschlechtsspezifisch gespleißten Gene während der embryonalen Entwicklung wurde das *glu* Gen identifiziert, das die geschlechtlich dimorphe Augenentwicklung in Arbeiterinnen steuert. Es wurde gezeigt, dass das weibliche Spleißen von *glu* mRNA durch die Geschlechtsdeterminationskaskade über das *fem* Gen reguliert wird. Funktionale Untersuchungen haben gezeigt, dass bei Mutationen in beiden Genkopien von *glu* die Augen in Arbeiterinnen vergrößert und der dorso-frontale Augenabstand verkleinert ist, was einer tendenziellen Vermännlichung entspricht. Die Funktion in Weibchen ließ sich partiell auf die durch das weibchenspezifische Transkript von *glu* kodierte C2H2 Zinkfinger-Domäne zurückführen. Weiterhin konnte gezeigt werden, dass sich die C2H2-Domäne innerhalb der Gruppe der Aculeata, der Stechimmen, über nicht-synonyme Mutationen der kodierenden Sequenz entwickelt hat, während die geschlechtsspezifische Regulation des Spleißens bereits früher entstanden ist.

Summary

In the honeybee, a pronounced sexual dimorphism between the sexes and a caste dimorphism within the females, between workers and queens, can be observed. Characteristics like body size and head morphology differ greatly between the three morphs. While the caste dimorphism in females is caused by nutrition in the larval stage, the sexual dimorphism is controlled cell autonomous by the sex determination cascade. In the fruit fly, the development of primary and secondary sex traits is controlled by the sex-specific transcription factors Dsx^M and Dsx^F, which are the result of the primary sex determination. In this study I showed that the differentiation of primary sex characteristics in female honeybee workers is regulated by the *dsx* Gene. However, CRISPR/Cas9 induced mutations in *dsx* had no influence on secondary sex characteristics such as head morphology. This implies that other genes are involved in the realization of secondary sex traits in the honeybee. The comparison of the male and female transcriptome of embryos revealed that splice regulation plays a more complex role in sex diversification than previously thought. As the embryonic development progressed, an increase in sex-specific spliced transcripts was observed. In addition, differentially regulated genes associated with RNA-binding functions and potentially splice-regulatory effects were over-represented in embryos close to hatching. I identified the *glu* gene, which controls sexually dimorphic eye development in workers, within the group of sex-specific spliced genes during embryonic development. I was able to show that female splicing of *glu* mRNA is regulated by the sex determination cascade via the *fem* gene. Functional studies have shown that mutations in both gene copies of *glu* increase the size of eyes in workers and decrease the dorso-frontal interocular distance, which corresponds to a tendency towards the male phenotype. Feminizing function of *glu* in females could be partially attributed to the C2H2 zinc finger domain encoded by the female-specific transcript. Furthermore, I demonstrated that the C2H2 zinc finger domain developed within the Aculeata, the stinging bees, via non-synonymous mutations of the *glu* coding sequence, whereas the sex-specific regulation of splicing was established earlier.

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