Die Regulation des geschlechtsspezifischen Spleißens des *Am-doublesex* Gens in der Honigbiene *Apis mellifera* und dessen Funktion bei der kastenspezifischen Morphogenese

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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.

Annika Roth Duisburg, im August 2019

Inhaltsverzeichnis

Kapitel I

Allgemeine Einleitung

1 Die Geschlechtsbestimmung der Honigbiene *Apis mellifera*

Die Geschlechtsbestimmung in der Honigbiene erfolgt durch Haplodiploidie. Dabei entwickeln sich Weibchen aus befruchteten Eiern mit doppeltem Chromosomensatz und Männchen, die man Drohnen nennt, aus unbefruchteten Eiern mit einfachem Chromosomensatz (s. Abb. 1). Das entscheidende Signal bei dieser Art der Geschlechtsbestimmung ist der allelische Zustand des Gens *complementary sex determiner* (*csd*) am geschlechtsbestimmenden Locus (sex determining locus, SDL). Liegen zwei unterschiedliche *csd*-Allele vor (heterozygot), entwickeln sich Weibchen. Da in unbefruchteten Eiern nur ein *csd*-Allel vorliegt, entwickeln sich diese zu Drohnen. In befruchteten Eiern, in denen zwei gleiche *csd*-Allele kombiniert vorliegen (homozygot), entwickeln sich ebenfalls Männchen. Diese werden jedoch vor dem Schlupf von den Arbeiterinnen im Stock erkannt und gefressen (Woyke, 1963), da diploide Drohnen triploide, sterile Nachkommen erzeugen würden. Um die Wahrscheinlichkeit für das Aufeinandertreffen zweier gleicher *csd*-Allele in einem Ei zu minimieren, paart sich eine Bienenkönigin bei ihrem Hochzeitsflug mit 5 - 20 Drohnen und sammelt deren Sperma in ihrer Spermathek (Estoup et al., 1994, Neumann et al., 1999, Franck et al., 2002, Schlüns et al., 2005, Koeniger and Koeniger, 2007). Da immer wieder nachreifende Eier mit Spermien aus der Spermathek befruchtet werden, kann eine Bienenkönigin über 3 - 5 Jahre bis zu 2000 befruchtete Eier am Tag legen (Franck et al., 2002).

Die Geschlechtsbestimmung per Haplodiploidie erfolgt in circa 20 % aller bekannten Tierarten (Bull, 1983, Crozier and Pamilo, 1996). Die haploiden Männchen besitzen dabei nur die Hälfte des maternalen Genoms, heterozygote Weibchen hingegen besitzen sowohl maternale als auch paternale Gene. Da der allelische Zustand des Gens *csd* über die Geschlechtsbestimmung entscheidet, spricht man von komplementärer Geschlechtsbestimmung (Cook, 1993). Beide Mechanismen zusammen ergeben die komplementäre Haplodiploidie.

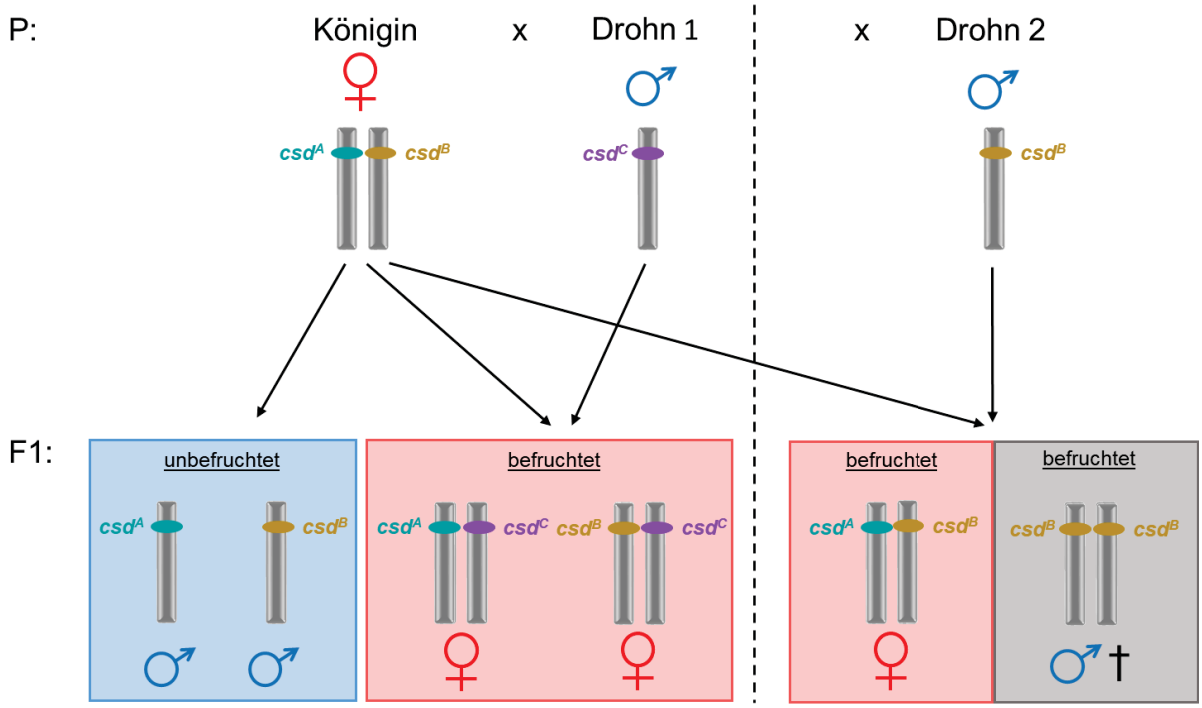

Abb. 1: Das Geschlecht wird in der Honigbiene durch komplementäre Haplodiploidie bestimmt. Dabei ist der allelische Zustand des Gens *complementary sex determiner* (*csd*) entscheidend. Aus unbefruchteten Eiern entstehen haploide Männchen mit nur einem *csd*-Allel (hier csd^A und csd^B). Aus befruchteten Eiern entstehen diploide Weibchen, sofern zwei unterschiedliche *csd*-Allele kombiniert vorliegen (hier csd^A mit csd^C und csd^B mit csd^C aus Kreuzung mit Drohn 1 und csd^A mit csd^B aus Kreuzung mit Drohn 2). Liegen in befruchteten Eiern zwei gleiche *csd*-Allele vor, entstehen diploide Männchen (hier csd^B aus Kreuzung mit Drohn B), die jedoch noch vor dem Schlupf von den Arbeiterinnen gefressen werden. (Beye, 2004)

csd leitet als initiales Signal eine Geschlechtsdeterminationskaskade ein (Beye et al., 2003). Csd-Heteromere sorgen dafür, dass die prä-mRNA des Gens *feminizer* (*fem*) weibchen-spezifisch gespleißt wird (s. Abb. 2A und Abschn. 2). Das Fem-Protein kann einerseits seine eigene Synthese autokatalytisch aufrechterhalten und leitet andererseits, zusammen mit dem Protein *Apis mellifera-Transformer2* (*Am-Tra2*), das weibchen-spezifische Spleißen der prä-mRNA von *Apis mellifera-doublesex* (*Am-dsx*) ein. Dieses Transkript kodiert für das weibchen-spezifische Protein *Am*-Dsx^F, das als Transkriptionsfaktor die weibliche Entwicklung induziert, wobei es die Aktivität von Genen weibchen-spezifisch reguliert.

In Männchen, in denen nur ein *csd*-Allel vorliegt, wird die *fem* prä-mRNA männchenspezifisch gespleißt. Dadurch entsteht kein funktionelles Fem-Protein und die *Am-dsx* prä-mRNA wird männchenspezifisch gespleißt. Das resultierende *Am*-

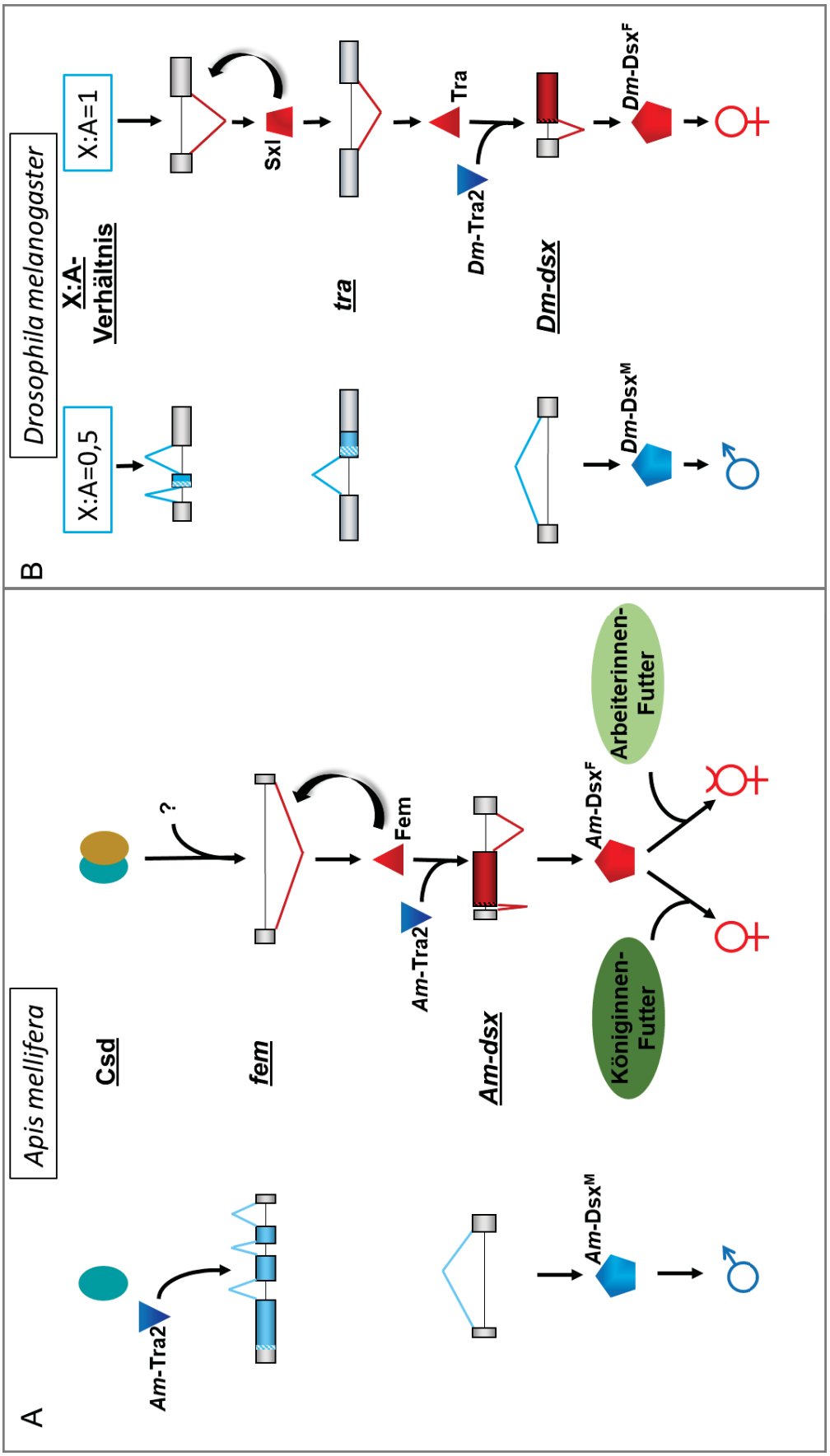

Abb. 2: Modelle der Geschlechtsbestimmungskaskaden von *Apis mellifera* (A) und *Drosophila melanogaster* (B). Exons sind durch Boxen dargestellt, Introns durch Linien,

Proteine durch geometrische Formen. Die Exon-Intron-Strukturen zeigen nur Ausschnitte der tatsächlichen Transkripte. Weibchenspezifische Exons, Spleißmuster und Proteine sind in rot, männchenspezifische in blau dargestellt, geschlechtsunspezifische Exons in grau. A: Der allelische Zustand des geschlechtsbestimmenden Gens *csd* ist das initiale Signal bei der Geschlechtsbestimmung der Honigbiene (*Apis mellifera*). Liegt *csd* heterozygot vor, wird die *fem*-prä-mRNA weibchenspezifisch gespleißt, was zur Produktion eines weiblichen Fem-Proteins führt. Fem und *Am*-Tra2 leiten zusammen das weibchenspezifische Spleißen von *Am-dsx* ein. Das entstehende Protein *Am*-Dsx^F führt zur weiblichen Entwicklung, wobei in Kombination mit Kasten-spezifischem Futter entweder Königinnen oder Arbeiterinnen entstehen. Liegt *csd* hemi- oder homozygot vor, wird die *fem*-prä-mRNA männchenspezifisch gespleißt und ein verkürztes, nicht funktionales Fem-Protein entsteht. Infolgedessen wird die *Am-dsx*-prä-mRNA männchenspezifisch gespleißt und es entsteht das männchenspezifische Protein *Am*-Dsx^M, das die männliche Entwicklung einleitet. B: In *Drosophila melanogaster* entscheidet das X:A-Verhältnis über die Bildung von funktionalem, weibchenspezifischem Sxl-Protein. Dadurch wird die *tra*-prä-mRNA geschlechtsspezifisch gespleißt. Tra und *Dm*-Tra2 leiten das geschlechtsspezifische Spleißen von *Dm-dsx* ein, wodurch die geschlechtsspezifischen Proteine *Dm*-Dsx^F und *Dm*-Dsx^M entstehen, die die weibliche bzw. männliche Entwicklung einleiten. Csd = complementary sex determiner, *fem* = *feminizer*, *Am*-Tra2 = *Apis mellifera*-Transformer2, *Am-dsx* = *Apis mellifera-doublesex*, Sxl = Sex lethal, *tra* = *transformer*, *Dm*-Tra2 = *Drosophila melanogaster*-Transformer2, *Dm-dsx* = *Drosophila melanogaster-doublesex*. (Gempe et al., 2009, Hasselmann et al., 2008, Saccone et al., 2002)

Dsx^M-Protein induziert die männliche Entwicklung (Beye et al., 2003, Hasselmann et al., 2008, Gempe et al., 2009, Nissen et al., 2012). Während alle für den männlichen Entwicklungsweg notwendigen Komponenten in beiden Geschlechtern exprimiert werden, sorgen nur in Weibchen exprimierte Proteine für die Einleitung des weiblichen Entwicklungswegs. Da die für die männliche Entwicklung notwendigen Transkripte und Proteine also basal vorhanden sind, wird dieser Entwicklungsweg als der voreingestellte bezeichnet.

In den folgenden Abschnitten werden die an der Geschlechtsbestimmungskaskade beteiligten Gene noch einmal genauer erläutert.

1.1 *complementary sex determiner (csd)*

Zur Zeit sind weltweit 116 - 145 verschiedene *csd*-Allele bekannt (Lechner et al., 2014), die sich in der sogenannten Hypervariablen Region (HVR) voneinander unterscheiden. Sie kodieren für SR-ähnliche Proteine, die eine Arginin-/Serin-reiche

Domäne (RS-Domäne) (Beye et al., 2003, Beye, 2004) sowie eine Prolin-reiche Region (PR) besitzen, welche die Protein-Protein-Interaktionen vermitteln. In Weibchen, in denen *csd* heterozygot vorliegt, vermitteln zwei Csd-Varianten das weibchenspezifische Spleißen von fem (Hasselmann et al., 2008, Gempe et al., 2009). *csd* ist vor ca. 120 Mio. Jahren aus einer Genduplikation von *fem* entstanden (Koch et al., 2014) und ist *fem* in der Geschlechtsbestimmungskaskade übergeordnet.

1.2 *feminizer (fem)*

Die männchenspezifische *fem*-mRNA kodiert für kein funktionales Protein, da die Translation des 12 Exons langen Transkripts durch ein Stopcodon (TGA: Thymin, Guanin, Adenin) in Exon 3 bereits frühzeitig terminiert wird. In Weibchen dagegen aktivieren Csd-Heteromere die Nutzung eines alternativen Spleißdonors in Exon 3. Dadurch werden der hintere Teil von Exon 3 – und somit das in Männchen genutzte Stopcodon – sowie Exon 4 und 5 der *fem*-prä-mRNA herausgespleißt. Stattdessen wird die Translation in Weibchen durch ein Stopcodon (TAA: Thymin, Adenin, Adenin) in Exon 12 beendet (Hasselmann et al., 2008).
Das so entstehende funktionale Fem-Protein mit zwei RS-Domänen und einer PR-Region ist ein Ortholog des *Drosophila melanogaster transformer* (*tra*)-Gens, das nachgewiesenermaßen an der Spleißregulation, unter anderem von *Drosophila melanogaster-doublesex* (Dm-*dsx*), beteiligt ist (Inoue et al., 1992).

1.3 *Apis mellifera-transformer2 (Am-tra2)*

Apis mellifera-transformer2 besteht aus 5 Exons, aus denen durch alternatives Spleißen von zwei Exons sechs verschiedene prä-mRNAs gebildet werden. Keines der daraus synthetisierten *Am*-Tra2-Proteine ist geschlechtsspezifisch, alle Varianten finden sich sowohl in Männchen wie auch in Weibchen (Nissen et al., 2012). Alle Isoformen besitzen die für SR-Proteine charakteristische RNA-Bindedomäne und die RS-Domäne für die Interaktion mit anderen Proteinen. Durch Nutzung unterschiedlicher Spleißstellen in Exon 2 entstehen drei Hauptvarianten von *Am*-Tra2. Drei weitere Varianten entstehen durch die Nutzung unterschiedlicher

Spleißakzeptoren zu Beginn von Exon 5, was zu einem Unterschied von einer einzelnen Aminosäure in der hinteren RS-Region führt (Nissen et al., 2012). Das *Am-tra*2-Gen der Honigbiene ist ein Ortholog des Gens *Dm-tra2* aus *Drosophila melanogaster* (Amrein et al., 1990, Nissen et al., 2012).

1.4 *Apis mellifera-doublesex (Am-dsx)*

Die *Apis mellifera-doublesex* (*Am-dsx*)-prä-mRNA besteht aus 7 Exons. Der offene Leserahmen (open reading frame; ORF) beginnt mit einem Startcodon (ATG: Adenin, Thymin, Guanin) in Exon 2 und endet in Männchen mit einem Stopcodon (TGA) in Exon 6, da das weibchen-spezifische Exon 5 in Männchen herausgespleißt wird. Die Exon 5-Inklusion in Weibchen führt dort zur Nutzung eines früheren Stopcodons (TGA). Die entstehenden unterschiedlichen ORFs mit 1080 bp in Männchen und 831 bp in Weibchen kodieren für die männchen- und weibchen-spezifischen Proteine *Am*-Dsx^M und *Am*-Dsx^F. Die ersten 750 bp der ORFs kodieren für die gleichen 250 Aminosäuren, sodass beide Proteine im N-terminalen Bereich die gleiche Oligomerisierungsdomäne 1 (OD1) aus zwei Zinkfingern besitzen. Im C-terminalen Bereich dagegen bilden die 27 weibchen-spezifischen und die 110 männchen-spezifischen Aminosäuren eine unterschiedliche OD2 aus (Cho et al., 2007).
Da die homologen *Dm*-Dsx-Proteine in *D. melanogaster* als Transkriptionsfaktor die Aktivität von strangabwärts gelegenen Genen männchen- und weibchen-spezifisch regulieren (Burtis et al., 1991, Erdman and Burtis, 1993), geht man davon aus, dass auch *Am*-Dsx^M und *Am*-Dsx^F in der Honigbiene als Transkriptionsfaktoren agieren. Vermutet wird, dass sie als Multimere an die DNA binden und so die Expression von Genen geschlechtsspezifisch beeinflussen (Bayrer et al., 2005).

2 Spleißen

Bei der Honigbiene und bei Insekten im Allgemeinen spielt das alternative Spleißen eine entscheidende Rolle bei der Geschlechtsbestimmung (Burtis and Baker, 1989, Shearman and Frommer, 1998, Suzuki et al., 2001, Ohbayashi et al., 2001, Kuhn et al., 2001, Scali et al., 2005, Ruiz et al., 2005). Dabei können aus zwei identischen prä-mRNAs verschiedene reife mRNAs werden. Hierzu werden Spleißdonoren

und/oder –akzeptoren aktiviert oder reprimiert und zusätzliche oder alternative Spleißstellen verwendet, was zur Kürzung, Verlängerung, Integration und dem Überspringen von Exons führen kann. Entscheidend dabei ist die Stärke der Spleißstellen, die durch meist angrenzende Sequenzen bestimmt wird, sowie die Beeinflussung dieser Spleißstellenstärke durch Aktivatoren und Repressoren.
Der Mechanismus des Spleißens sowie die spleißregulatorischen Elemente und Proteine werden daher im Folgenden näher erläutert.

2.1 Der Mechansimus des Spleißens

Als Spleißen bezeichnet man das Herausschneiden von nicht-kodierenden Sequenzen der RNA (Introns) unter der Verknüpfung von kodierenden Sequenzen (Exons) (Berget et al., 1977, Chow et al., 1977, Breathnach et al., 1977, Jeffreys and Flavell, 1977), (s. Abb. 3). In einer Umesterung werden Phosphodiesterbindungen unter Verwendung von Hydroxyl (OH)-Gruppen zwischen Exons und Introns aufgebrochen und zwischen Exons und Exons neu gebildet. Ein Spleißakzeptor (SA; 3‘-Spleißstelle) markiert dabei den Beginn eines Exon, ein Spleißdonor (SD; 5‘-Spleißstelle) das Ende eines Exons (Mount, 1982). Diese Stellen sind essentiell für das Spleißen und finden sich in verschiedenen Organismen mit zum Teil konservierten Konsensussequenzen (Padgett et al., 1986). Da das Honigbienengenom aus sehr langen Introns aus teilweise mehreren tausend Basenpaaren besteht, werden hier für das Spleißen die Exons definiert, während in anderen Organsimen auch häufig Intron-Definition stattfindet (De Conti et al., 2012, Robberson et al., 1990, Sakharkar et al., 2005, Fox-Walsh et al., 2005).

Der Spleißdonor setzt sich aus elf Nukleotiden zusammen, wobei ein GU (Guanin, Uracil) an Position 4 und 5 das Ende des Exons markieren. Am Spleißakzeptor wird der Beginn des Exons durch ein AG (Adenin, Guanin) an Position 19 und 20 einer 22 Nukleotide langen Sequenz definiert (Mount, 1982). Durch einen Polypyrimidintrakt direkt strangaufwärts, also per Definition im Intron, wird die Stärke des SA bestimmt. In dieser 10-12 Nukleotide langen Sequenz finden sich fast ausschließlich Pyrimidine. Je mehr Pyrimidine dieser Trakt enthält, desto besser erkennen die Proteine der Spleißmaschinerie den SA (Burtis and Baker, 1989). Noch weiter strangaufwärts des Polypyrimidintraktes befindet sich der 7 Nukleotide lange

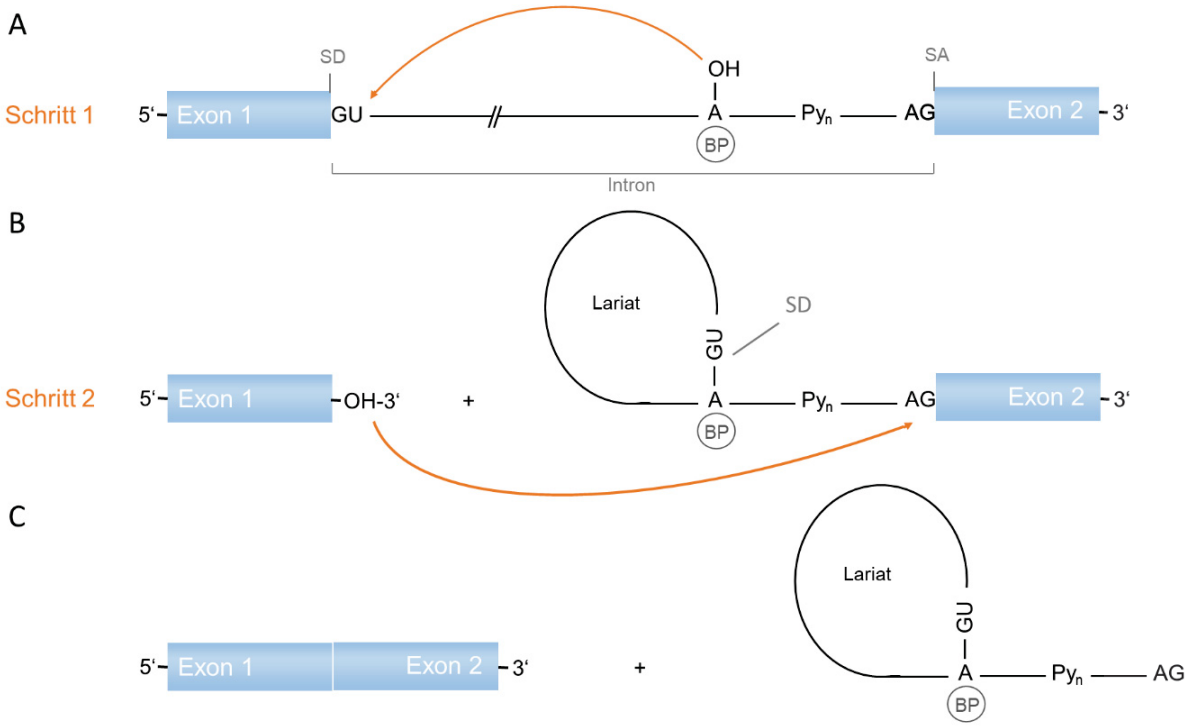

Abb. 3: Der Spleißvorgang folgt einem Zweischrittmechanismus. Bei einer Umesterung werden Phosphodiesterbindungen aufgebrochen und neue gebildet. Im ersten Schritt erfolgt ein nukleophiler Angriff des Branchpoint-Adenins auf die 5‘-3‘-Phosphodiesterbindung zwischen Exon 1 und dem Intron (A). Diese Bindung bricht auf und eine neue Bindung zwischen Branchpoint und dem Intron-Start entsteht. Diese Struktur wird als Lariat-Intermediat bezeichnet. Im zweiten Schritt greift die nun freie 3‘-OH-Gruppe von Exon 1 die Phosphodiesterbindung zwischen Intron und Exon 2 an (B). Die beiden Exons werden verknüpft und die Lariat-Struktur wird entlassen (C). G = Guanin, U = Uracil, A = Adenin, BP = Branchpoint (Verzweigungspunkt), Py = Pyrimidin.

Branchpoint (BP; Verzweigungspunkt), der ein an Position 6 konserviertes A besitzt, welches essentiell für den ersten Schritt der Umesterung ist. Während des Spleißprozesses erfolgt zuerst ein nukleophiler Angriff des Sauerstoffatoms der 2‘-OH-Gruppe des BP-Adenins auf die 5‘-3‘-Phosphodiesterbindung zwischen Exon und Intron am SD, die dadurch aufgebrochen wird (Abb. 3A). Das nun unverbundene Ende des Intron-Starts verbindet sich über eine 5‘-2‘-Phosphodiesterbindung mit dem BP-Adenin, wodurch eine Schlaufen-Struktur entsteht, die als Lariat-Intermediat bezeichnet wird (Ruskin et al., 1984), (Abb. 3B).
Im nächsten Schritt bricht die freie 3‘-OH-Gruppe des Exons die 5‘-3‘-Phosphodiesterbindung zwischen dem Exon und dem Intron auf und verbindet sich anstelle des Introns mit dem strangabwärts gelegenen Exon. Das Intron wird als Lariat frei (Staley and Guthrie, 1998), (Abb. 3C).

Der Mechanismus des Spleißens wird durch einen Ribonukleoproteinkomplex katalysiert (Lerner et al., 1980), der sich Spleißosom nennt. Dieses setzt sich aus fünf nukleären Ribonukleoproteinpartikeln und vielen mit ihnen assoziierten Proteinen zusammen (Zhou et al., 2002, Mount et al., 2007) und erkennt spezifische Sequenzen an den Spleißstellen.

2.2 Spleißregulatorische Elemente

Für die in der Honigbiene bestehende Exon-Definition muss das Spleißosom erkennen, welche Spleißstellen miteinander verknüpft werden sollen. Da deren Markierung durch jeweils nur zwei Nukleotide – GU am SA und AG am SD – bei der oft großen Zahl an möglichen Spleißstellen meist nicht für eine eindeutige Definition der zu verwendenden Spleißstellen ausreicht, müssen weitere regulatorische Sequenzen dafür sorgen, dass die Umesterung an den korrekten Stellen in der Nukleinsäuresequenz stattfindet. Diese *cis*-regulatorischen Elemente (CREs) können Spleißstellen aktivieren oder reprimieren. CREs, die im Exon liegen, werden als ESEs bzw. ESSs (exonic splicing enhancers/silencers) bezeichnet, CREs, die im Intron liegen, als ISEs bzw. ISSs (intronic splicing enhancers/silencers). Die aktivierende oder reprimierende Wirkung dieser CREs wird durch an diese Sequenzen bindende Proteine oder Proteinkomplexe vermittelt, die wiederum das Spleißosom zu diesem Sequenzabschnitt hin dirigieren oder die Erkennung oder Bindung erschweren (Zuo and Maniatis, 1996). Beispiele für solche Spleißaktivatoren sind die SR-Proteine (Serin-/Arginin-reiche Proteine), wie auch *Am*-Tra2 eines ist.

2.3 Spleißregulatorische Proteine

Spleißregulatorische Proteine binden an spleißregulatorische Elemente, die CREs, und sorgen so dafür, dass eine Spleißstelle mehr oder weniger wahrscheinlich genutzt beziehungsweise aktiviert oder reprimiert wird. Die häufigsten Proteingruppen dabei sind SR-Proteine (wie *Am*-Tra2) oder SR-ähnliche Proteine (wie Fem und Csd), die als Aktivatoren im Exon binden und hnRNPs (heterogenous nuclear ribonucleoproteins) als Repressoren oder Silencer. Solche Proteine binden an ihre meist spezifischen Bindesequenzen auf der prä-mRNA und blockieren so die vom Spleißosom genutzten Bindestellen oder rekrutieren seine Untereinheiten zu der

zu aktivierenden Spleißstelle (Eperon et al., 1993, Graveley et al., 2001, Roscigno and Garcia-Blanco, 1995).

SR-Proteine besitzen im N-terminalen Bereich mindestens eine RNA-Bindedomäne (RBD) und im C-terminalen Bereich eine RS (Arginin-/Serin-reiche)-Domäne, die Protein-Protein-Interaktionen vermittelt (Zahler et al., 1992, Wu and Maniatis, 1993, Kohtz et al., 1994, Tacke and Manley, 1995, Shi et al., 1997, Graveley, 2000). Interaktionspartner sind dabei häufig andere SR-Proteine oder SR-ähnliche Proteine, die ohne eine eigene RBD nicht selbst an die RNA binden können, sondern nur indirekt über einen Interaktionspartner. Da Fem und Csd SR-ähnliche Proteine sind, benötigen sie einen solchen Interaktionspartner mit RNA-Bindedomäne (Beye et al., 2003, Hasselmann et al., 2008).

3 Geschlechtsbestimmung bei *Drosophila melanogaster*

Weitaus detaillierter als in der Honigbiene ist die Geschlechtsbestimmung in der Taufliege *Drosophila melanogaster* erforscht. Viele Komponenten der geschlechtsbestimmenden Kaskade sind konserviert beziehungsweise es existieren orthologe Gene (s. Abb. 2B).

Der entscheidende Faktor bei der Geschlechtsbestimmung in *Drosophila* ist das Verhältnis von X-Chromosomen zu Autosomensätzen. Weibliche Individuen tragen 2 X-Chromosomen und einen doppelten Satz Autosomen (2X:2A=1). Männliche Individuen tragen nur ein X-Chromosom (1X:2A=0,5) und ein Y-Chromosom, welches keine Rolle bei der Geschlechtsbestimmung spielt (Bridges, 1916). Da die X-chromosomalen Gene in Weibchen in doppelter Menge vorliegen, erfolgt zur Dosiskompensation eine Hypertranskription dieser Gene in Männchen (Muller, 1932, Mukherjee and Beermann, 1965).

Das initiale Signal in *D. melanogaster* ist das Gen sex let*hal* (*sxl*). In der Hauptsache existieren drei Sxl-Isoformen: eine späte weibchen-spezifische Form, eine späte männchen-spezifische Form und eine frühe weibchen-spezifische Form. Die beiden späten Isoformen werden vom *Sex lethal „maintenance“-Promotor* (*SxlPm*) exprimiert und unterscheiden sich durch eine männchen-spezifische Exon L3-Inklusion. Ein frühes Stopcodon in diesem Exon führt dazu, dass in Männchen kein funktionales Protein entsteht. In Weibchen wird Exon L3 dagegen durch alternatives Spleißen

übersprungen und das Stopcodon so herausgespleißt (Bell et al., 1988, Bopp et al., 1991). Das weibchen-spezifische Spleißen des *SxlPm*-Transkripts wird durch die frühe weibchen-spezifische Isoform induziert. Diese wird durch die Expression am *Sex lethal „establishment"-Promotor* (*SxlPe*) bereitgestellt und seine Synthese durch positive Autoregulation aufrechterhalten (Bell et al., 1991). Aktiviert wird der *SxlPe* in Weibchen durch die Expressionslevel einiger X-chromosomaler Proteine (Cline, 1988, Duffy and Gergen, 1991, Jinks et al., 2000).

Neben seiner eigenen prä-mRNA reguliert Sxl auch das Spleißen von *transformer* (*tra*) in *D. melanogaster*. Dabei entsteht in Männchen ohne funktionales Sxl-Protein ein durch ein frühes Stopcodon stark verkürztes Tra-Peptid ohne Funktion. In Weibchen aktiviert Sxl die Nutzung eines alternativen Spleißakzeptors in Exon 1 von *tra*, wodurch das frühe Stopcodon herausgespleißt wird und ein funktionales, SR-ähnliches Protein gebildet wird, das als Spleißenhancer wirkt (Boggs et al., 1987).

Da Tra in *D. melanogaster* als SR-ähnliches Protein über keine eigene RNA-Bindedomäne verfügt, bildet es über die RS-Region einen Protein-Protein-Komplex mit dem SR-Protein *Drosophila melanogaster*-Transformer2 (*Dm*-Tra2). Dieser Komplex wirkt als Spleißenhancer und bindet an spezifische Bindesequenzen auf der *Drosophila melanogaster-doublesex* (*Dm-dsx*)-prä-mRNA und sorgt so dafür, dass *Dm-dsx*-Transkripte weibchen-spezifisch gespleißt werden (McKeown et al., 1988, Goralski et al., 1989).

Die *Dm-dsx*-prä-mRNA besteht aus 6 Exons, von denen Exon 1 – 3 in der reifen mRNA beider Geschlechter vorkommen. Ohne die Spleißenhancer Tra und *Dm*-Tra2 wird der SA an Exon 4 übergangen und von Exon 3 auf Exon 5 gespleißt. Das männchen-spezifische *Dm-dsx*-Transkript endet mit Exon 6.
Tra und *Dm*-Tra2 aktivieren in Weibchen einen konstitutiv inaktiven SA zu Beginn von Exon 4, sodass von Exon 3 auf Exon 4 gespleißt wird, an dessen Ende sich eine weibchen-spezifische Polyadenylierungsstelle befindet, die zur Transkriptionstermination der weiblichen *Dm-dsx*-mRNA führt (Burtis and Baker, 1989). Dazu bindet Tra/*Dm*-Tra2 an sechs Sequenzwiederholungen eines 13 Nukleotide langen Bindemotivs in Exon 4 und aktiviert so den 300 Nukleotide strangaufwärts gelegenen, inaktiven SA (Inoue et al., 1992, Tian and Maniatis, 1993, Sciabica and Hertel, 2006). Dieses sechsfach wiederkehrende ESE-Motiv hat die

Konsensussequenz: TC(T/A)(T/A)C(A/G)ATCAACA (Burtis and Baker, 1989, Inoue et al., 1992).

Durch das geschechtsspezifische Spleißen entstehen in Männchen und Weibchen verschiedene *Dm*-Dsx-Proteine, die sich in ihrem C-terminalen Bereich unterscheiden. *Dm*-Dsx^{M} und *Dm*-Dsx^{F} regulieren als Transkriptionsfaktoren die Expression somatischer Gene geschlechtsspezifisch (Burtis et al., 1991, Erdman and Burtis, 1993). Beide Proteine besitzen eine identische N-terminale OD1 mit zwei Zinkfingern, die die Bindung an die DNA realisieren, und eine geschlechtsspezifische C-terminale OD2. Dieser Unterschied sorgt für die männchen- bzw. weibchen-spezifische Aktivierung von untergeordneten Zielgenen, deren Expression entsprechend zur männlichen oder weiblichen Entwicklung führt (Burtis et al., 1991, An et al., 1996, Williams et al., 2008).

4 CRISPR/Cas9

Das CRISPR/Cas9-System ist ursprünglich ein Mechanismus in Prokaryoten zum Schutz vor in die Zelle eindringender Fremd-DNA durch Viren oder Bakteriophagen. Diesen Mechanismus hat man sich in der Molekulargenetik zu Nutze gemacht, um zielgerichtet Mutationen ins Genom von eukaryotischen Zellen einzubringen. Im Folgenden wird zunächst der natürliche CRISPR/Cas-Mechanismus in Bakterien erläutert. Anschließend wird das System dargelegt, mit dem das CRISPR/Cas-System als molekulargenetisches Werkzeug zum Editieren von Genen verwendet wird.

4.1 Das CRISPR/Cas-System in Bakterien

Mit Hilfe des CRISPR/Cas-Systems können Prokaryoten sich vor eindringender Fremd-DNA schützen. Es stellt eine Art Immunsystem in Bakterien dar, da Fremd-DNA, deren Sequenz der Zelle bekannt ist, abgebaut wird und so ihre Funktion verliert (Barrangou et al., 2007) (s. Abb. 4).

Im Genom von vielen Bakterien- und Archaen-Arten finden sich sogenannte CRISPR-Arrays (CRISPR: clustered regularly interspaced short palyndromic repeats) von 23 - 47 bp, die sich mit sogenannten Spacern von 21 - 72 bp Länge abwechseln,

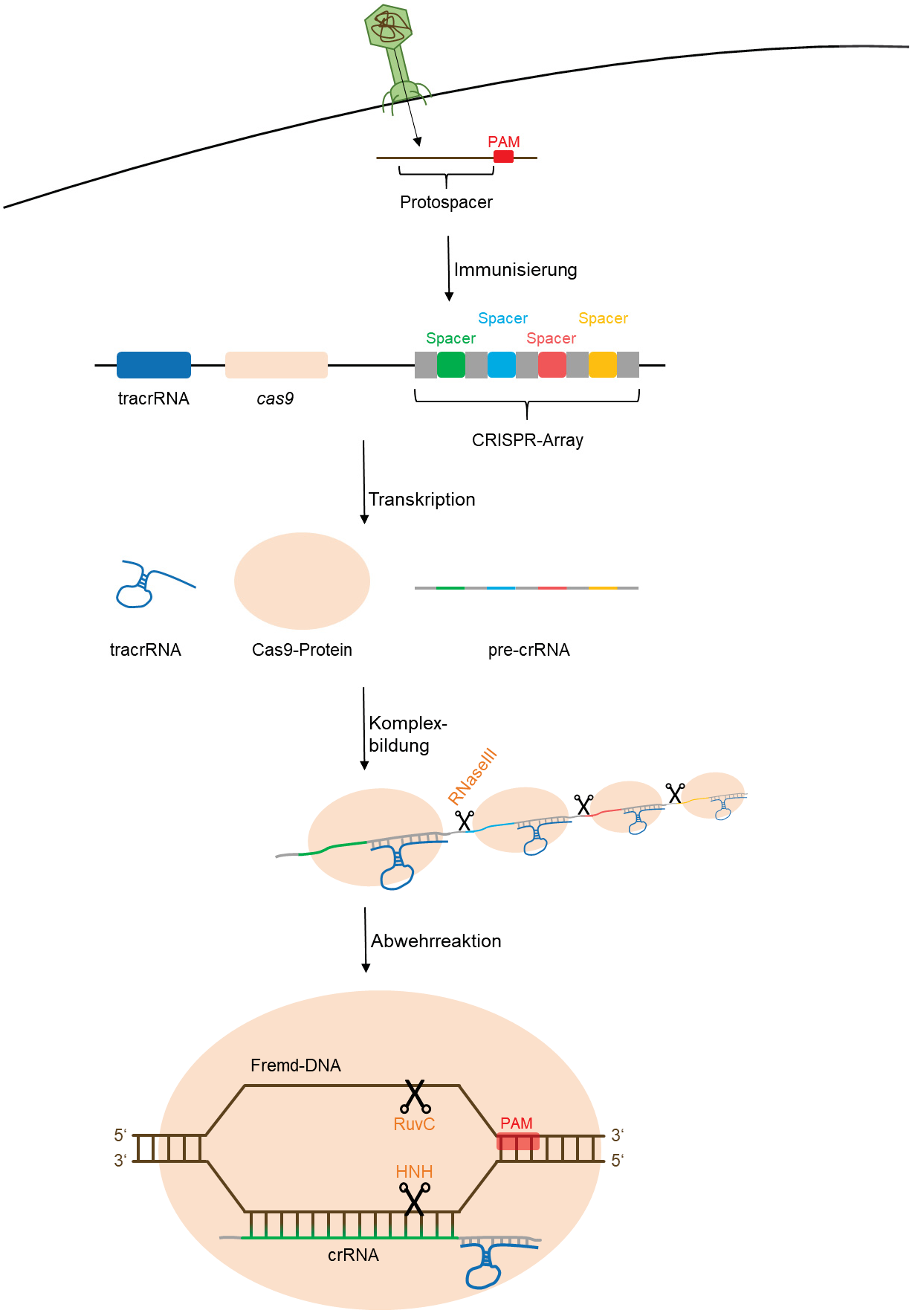

Abb. 4: Ein Teil von in Prokaryoten eindringender Fremd-DNA wird in einem Immunisierungsschritt als Spacer in den CRISPR-Array ins Genom des Bakteriums

eingebaut. Als Protospacer fungiert dabei eine Sequenz direkt angrenzend an ein PAM. Die Zelle transkribiert aus dem CRISPR-Array die pre-crRNA. Diese bindet an die tracrRNA, sodass es zur Komplexbildung mit dem Cas9-Protein kommt. RNaseIII-Domänen zerschneiden die zusammenhängende pre-crRNA in kurze crRNAs. In einer Abwehrreaktion kann nun bereits bekannte, da als Spacer vorhandene, Fremd-DNA abgebaut werden. Dazu bindet die crRNA an ihre komplementäre Sequenz auf der Fremd-DNA, wodurch die Endonuklease-Domänen des Cas9-Proteins einen Doppelstrangbruch in der Fremd-DNA erzeugen und diese somit abbauen. (Horvath and Barrangou, 2010, Makarova et al., 2011, Sorek et al., 2013, Chylinski et al., 2014, Peng et al., 2016)

und in der Nähe dieser Sequenzen verschiedene *cas* (*CRISPR-associated*)-Gene, die auf einen oder mehrere Loci verteilt sind (Ishino et al., 1987, Mojica et al., 2000, Jansen et al., 2002, Horvath and Barrangou, 2010). Die Spacer entsprechen Sequenzabschnitten von Fremd-DNA, die in einem Immunisierungsschritt aus eindringenden Viren oder Plasmiden mit Hilfe von Cas-Proteinen herausgeschnitten und in den CRISPR-Locus integriert werden (Bolotin et al., 2005, Barrangou et al., 2007). Dabei wird der Sequenzabschnitt auf der Fremd-DNA so gewählt, dass der Spacer-Vorläufer (Protospacer) direkt benachbart zu einem sogenannten PAM (protospacer adjacent motif) liegt. Diese meist drei Nukleotide lange Sequenz hat die Basenfolge NGG (beliebige Base, Guanin, Guanin) und ist essentiell für die Abwehrreaktion auf Fremd-DNA (Mojica et al., 2009, Sorek et al., 2013). Bei der Transkription des CRISPR-Arrays entsteht zunächst eine pre-crRNA (precursor-CRISPR-RNA) aus zusammenhängenden Spacer- und CRISPR-Abschnitten. Die sich wiederholenden, die Spacer flankierenden Sequenzen dienen dabei als Erkennungssequenzen für RNaseIII-Domänen, die die Spacer ausschneiden und so die crRNA (CRISPR-RNA) bereitstellen, welche die eigentliche Abwehrreaktion erzeugt (Brouns et al., 2008).

Dringt Fremd-DNA, deren Sequenz bereits als Spacer vorhanden ist, in die Zelle ein, vermittelt eine weitere kurze RNA, die tracrRNA (trans-acting-CRISPR-RNA), eine Komplexbildung zwischen crRNA und der Cas9-Endonuklease. Die crRNA leitet den Komplex zu ihrem komplementären Sequenzabschnitt auf der Fremd-DNA, in der das Cas9-Protein einen Doppelstrangbruch mit blunt ends (glatten Enden) erzeugt, der die Fremd-DNA zerstört (Chylinski et al., 2014). Für die Ausführung des Doppelstrangbruchs in der DNA ist das PAM unerlässlich (Jinek et al., 2012). Den Strangbruch am zur crRNA komplementären Strang führt dabei die HNH-

Nukleasedomäne des Cas9-Proteins aus. Der nicht-komplementäre Strang wird von einer RuvC-ähnlichen Domäne aufgebrochen (Jinek et al., 2012, Sorek et al., 2013).

Neben dem CRSIPR/Cas9-System, das die Abwehrreaktion mit Hilfe des Cas9-Proteins realisiert, existieren noch zwei andere Mechanismen, an denen andere Cas-Proteine und weitere Moleküle beteiligt sind (Makarova et al., 2011, Sorek et al., 2013). Auf diese soll hier allerdings nicht näher eingegangen werden, da das hier beschriebene Typ II-CRISPR-System jenes ist, welches als Werkzeug für die Molekulargenetik adaptiert wurde.

4.2 Das CRISPR/Cas9-System als molekulargenetisches Werkzeug

Mit Hilfe des CRISPR/Cas9-Systems als molekulargenetischem Werkzeug können Doppelstrangbrüche und in der Folge Mutationen an einem bestimmten Ort im Genom eukaryotischer Zellen induziert werden (Abb. 5). Dazu wurde das CRISPR/Cas-System Typ II als molekulargenetisches Werkzeug adaptiert (Jinek et al., 2012, Cong et al., 2013). Die funktionellen Komponenten dieses Werkzeugs sind die sgRNA (synthetic guide RNA), ein synthetischer Hybrid aus crRNA und tracrRNA, die das Cas9-Protein zur Zielsequenz leitet, und die Cas9-Endonuklease, die den DNA-Doppelstrangbruch ausführt. Das 3‘-Ende der sgRNA ist dabei komplementär zu der genomischen Zielsequenz, die an ein PAM (NGG) angrenzt. Das 5‘-Ende besteht aus einer spezifischen Sequenz, die eine mit der Cas9-Endonuklease interagierende Sekundärstruktur ausbildet (Bassett et al., 2013). Dabei kann das sgRNA-Molekül entweder zusammen mit rekombinantem Cas9-Protein (Gratz et al., 2013) oder mit synthetisch erzeugter *cas9*-mRNA in die Zelle eingebracht werden (Bassett et al., 2013).

Die eigentlichen Mutationen werden nach dem induzierten Doppelstrangbruch durch den zellautonomen Reparaturmechanismus erzeugt. In eukaryotischen Zellen werden Doppelstrangbrüche üblicherweise durch einen von zwei Mechanismen repariert: homologe Rekombination (HR) oder nicht-homologe Endverknüpfung (non-homologous end joining; NHEJ). Während der Mechanismus der homologen Rekombination meist fehlerfrei verläuft, kommt es beim NHEJ häufig zu Deletionen und/oder Insertionen, die eine Verschiebung des Leserahmens (frame shift), die

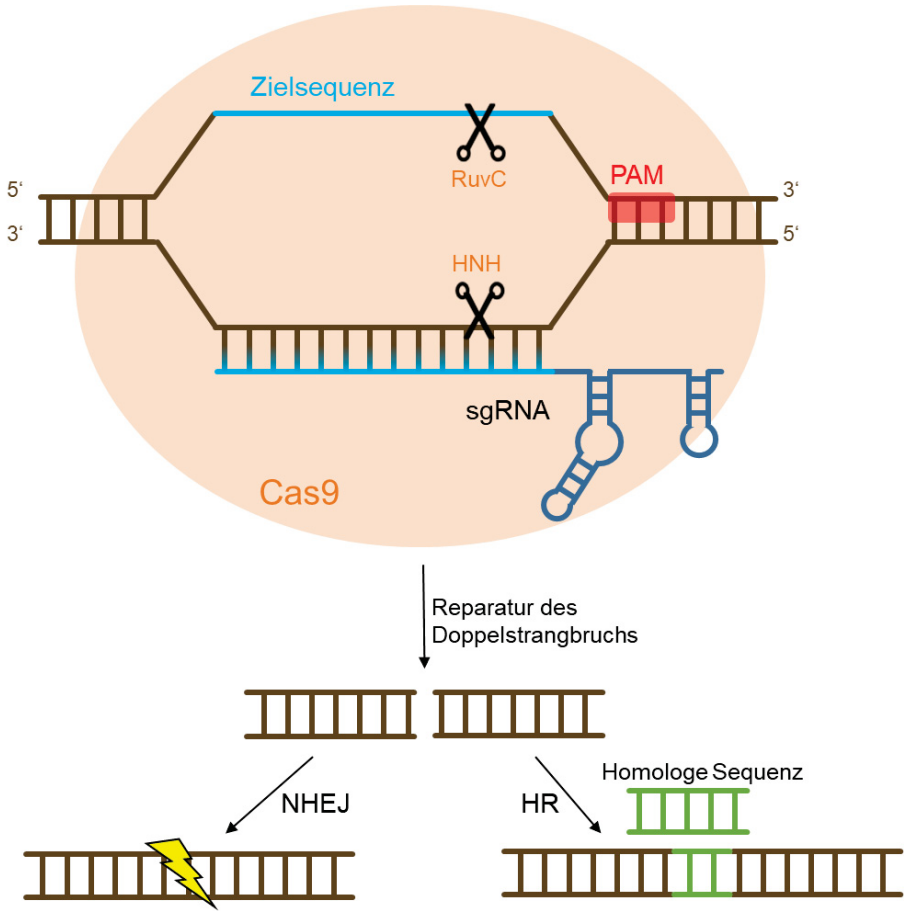

Abb. 5: Das CRISPR/Cas9-System wurde als Werkzeug für die Molekulargenetik adaptiert. Als funktionelle Komponenten wirken die sgRNA, ein synthetischer Hybrid aus tracrRNA und crRNA, und das Cas9-Protein. Ein Teil der sgRNA ist identisch mit einer ~20 nt langen Zielsequenz, die an ein PAM angrenzt. Die HNH- und RuvC-Domänen der Cas9-Endonuklease erzeugen einen Doppelstrangbruch im Genom. Dieser Doppelstrangbruch wird von zelleigenen Mechanismen mittels homologer Rekombination (HR) oder nicht homologer Endverknüpfung (NHEJ, non-homologous end joining) repariert. Bei der NHEJ kommt es sehr häufig zu Deletionen und Insertionen, die eine Verschiebung des Offenen Leserahmens zur Folge haben. (Khanna and Jackson, 2001, Jinek et al., 2012, Bassett et al., 2013, Hwang et al., 2013b, Peng et al., 2016)

Umwandlung essentieller Aminosäuren oder die Deletion von einzelnen Aminosäuren oder ganzen Sequenzabschnitten zur Folge haben können (Khanna and Jackson, 2001). Auf diese Weise kann mit dem CRISPR/Cas9-System der Funktionsverlust von Proteinen oder deren gesamter Expression induziert werden. Geschieht diese Genomveränderung im Einzellstadium von Organismen, ist die Mutation dauerhaft im gesamten Erbgut des Organismus verankert.

Bisher wurde das System bereits in einigen eukaryotischen Organismen wie dem Zebrabärbling *Danio rerio* (Hwang et al., 2013b), der Taufliege *Drosophila*

melanogaster (Bassett et al., 2013), der Honigbiene *Apis mellifera* (Kohno et al., 2016) und in menschlicher Zellkultur (Cong et al., 2013) erfolgreich angewendet.

5 Die Entwicklung der Honigbiene

5.1 Kastendifferenzierung

Ein Honigbienenvolk besteht aus tausenden von Individuen, wovon die meisten weibliche Arbeiterinnen sind. Daneben besteht es aus ein paar hundert männlichen Drohnen und einer einzelnen weiblichen Königin. Drohnen und Königin sind für die Reproduktion zuständig, während die sterilen Arbeiterinnen sich ausschließlich um die Aufrechterhaltung der Kolonie kümmern. Da mit den Arbeiterinnen und der Königin zwei weibliche Formen existieren, die sich in Phänotyp und Verhalten stark unterscheiden, muss in weiblichen Honigbienen neben der Geschlechtsdifferenzierung zusätzlich noch die Kaste differenziert werden. Dieses Signal wird nach dem Geschlechtssignal in die Entwicklungssignalkaskade integriert und durch das Futter während der larvalen Entwicklung bestimmt (Haydak, 1943, Haydak, 1970, Vleurinck et al., 2016). In frühen Larvenstadien ist der Zuckergehalt im Arbeiterinnenfutter geringer (Leimar et al., 2012, Buttstedt et al., 2016). Königinnen erhalten als Larve durchgehend ein Überangebot an Futter mit hohem Zuckergehalt (Wheeler et al., 2006, Patel et al., 2007), wodurch sie sich, verglichen mit Arbeiterinnen, in kürzerer Zeit größer und schwerer entwickeln. Außerdem nimmt ihre Fruchtbarkeit zu, da ihre Ovarien sich voll ausbilden und sie bis zu 200 Ovariolschläuche pro Ovar entwickeln können, während die Ovarienentwicklung in Arbeiterinnen unterdrückt wird.

Drei Tage nach der Eiablage durch die Königin schlüpfen die ca. 1 – 1,5 mm großen Larven, die fortan von den Arbeiterinnen gefüttert werden (Crailsheim, 1991, Crailsheim, 1992, Cash et al., 2005). In insgesamt 16 – 24 Tagen ab dem Schlupf, abhängig von Geschlecht und Kaste, entwickeln sie sich über ein Puppenstadium hin zum Imago, dem adulten Tier.

5.2 Drohnenentwicklung

Drohnen haben mit 24 Tagen die längste Entwicklungszeit unter den drei Honigbienenmorphen. Nach dem Schlupf aus dem Ei durchlaufen sie in 11 - 13

Tagen fünf Larvenstadien, bevor sie sich verpuppen. Als Puppe durchlaufen sie in 8 – 9 Tagen eine Metamorphose, in der die Zellen der Larve sich neu anordnen und die Strukturen des adulten Tieres annehmen (von Rhein, 1933, Dade, 1994).

Drohnen sind größer und schwerer als die weiblichen Bienenmorphen. Der auffälligste Unterschied im Vergleich mit Arbeiterinnen und Königin ist der große, runde Kopf mit den doppelt so großen Augen wie bei Weibchen (Streinzer et al., 2013), (vgl. Abb. 6A, B und C). Die Antennen als sensorische Organe für olfaktorische Reize sind dicker und bestehen mit 13 Segmenten aus einem mehr als bei Arbeiterinnen. Der Thorax ist ebenfalls größer und trägt eine ausgeprägtere Flugmuskulatur und im Vergleich zur Körperlänge größere Flügel. Das dorsale Abdomen besteht mit sieben Tergiten aus einem mehr als bei Weibchen (Dade, 1994).

Die männlichen Gonaden sind paarig angeordnet und bestehen aus einer großen Anzahl an Testiolen, die zu Testes angeordnet sind. Die Testes vereinigen sich in einem Tubulus zum *vas deferens*, der in den *vesicula seminalis* mündet, in dem die Spermatozoen gespeichert werden. Bevor sie im *ductus ejaculatorius* in den Endophallus gelangen, werden sie mit Sekret aus den ebenfalls paarigen akzessorischen Geschlechtsdrüsen zum Ejakulat vermischt. Der Endophallus liegt kolbenförmig im inneren des Abdomens und wird bei der Begattung ausgestülpt (Snodgrass, 1910, Gempe et al., 2009), (Abb. 6F). Unmittelbar nach der Begattung sterben Drohnen, da der eventerierte Endophallus aus dem Hinterleib gerissen wird.

5.3 Arbeiterinnenentwicklung

Die Entwicklung von Arbeiterinnen dauert im Schnitt insgesamt 21 Tage. Während der ersten 8 – 10 Tage steht den Larven eine begrenzte Menge an Futter zur Verfügung, die von den Arbeiterinnen im Stock reguliert wird. Nach 8 – 9 Tagen als Puppe schlüpft der Imago. Frisch geschlüpfte adulte Arbeiterinnen wiegen im Schnitt etwa 120 mg (von Rhein, 1933, Dade, 1994).

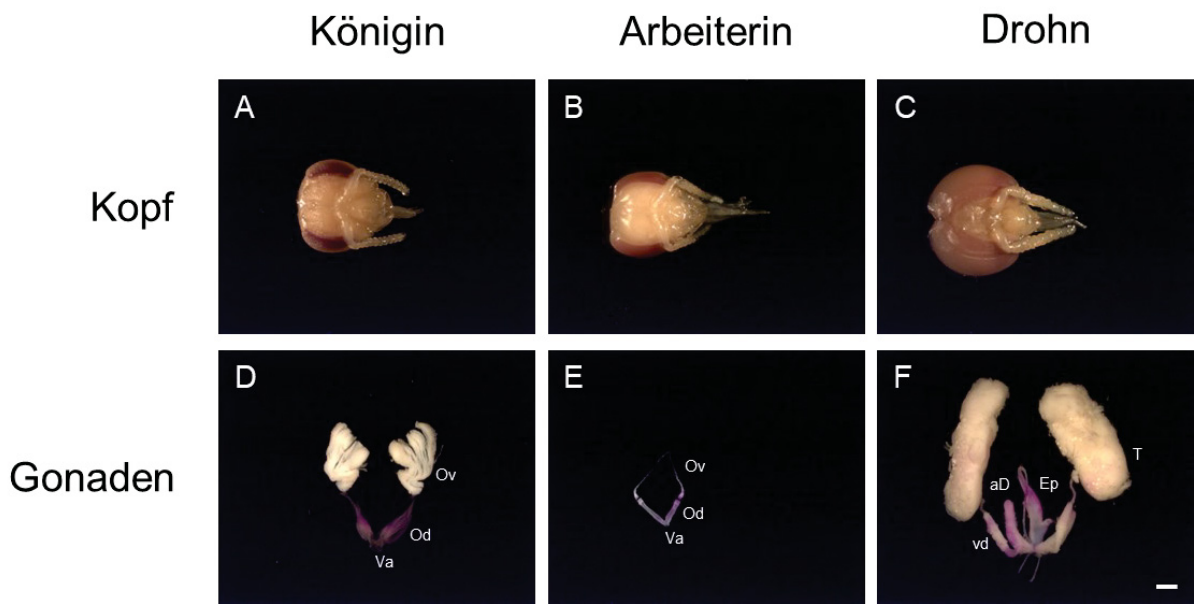

Abb. 6: Vergleich der Kopfstruktur (A – C) und der Gonaden (D – F) der drei Honigbienenmorphen Königin, Arbeiterin und Drohn im Rotaugenstadium. Ov = Ovariolen, Od = Ovidukt (Eileiter), Va = Vagina, T = Testis, vd = vas deferens (Samenleiter), aD = akzessorische Drüse, Ep = Endophallus. Maßstabsbalken = 1 mm.

Der Kopf von Arbeiterinnen ist im Vergleich zu dem von Drohnen triangulär und besitzt nur halb so große Augen (Streinzer et al., 2013), (vgl. Abb. 6B und C) . Die Antennen sind mit 12 Segmenten ein Segment kürzer als die von Drohnen und insgesamt dünner. Die Proboscis, der Rüssel, ist deutlich länger, um bis in tief liegende Blütenkelche und Wabenzellen zu reichen.

Die Hinterbeine von Arbeiterinnen sind mit anatomischen Werkzeugen zum Pollensammeln ausgestattet. An der äußeren Oberfläche des distalen Endes der Tibia sitzt der Pollenkamm. Das proximale Ende des ersten tarsalen Segments bildet ein Pollenkörbchen aus. Am gesamten Segment befinden sich symmetrisch angeordnete Haarreihen, die die Pollenbürste bilden. Mit Hilfe des Pollenkamms kämmt die Arbeiterin die Bürste des gegenüberliegenden Beines aus und schiebt so Pollen in das Pollenkörbchen, der sich als sogenannte Pollenhöschen sammelt (s. Abb. 7).

Mit sechs Tergiten besitzen Arbeiterinnen ein dorsales Abdominalsegment weniger als Drohnen (Snodgrass, 1910, Gempe et al., 2009). An der ventralen Seite der Segmente drei bis sechs befinden sich acht paarig angeordnete Wachsdrüsen, die

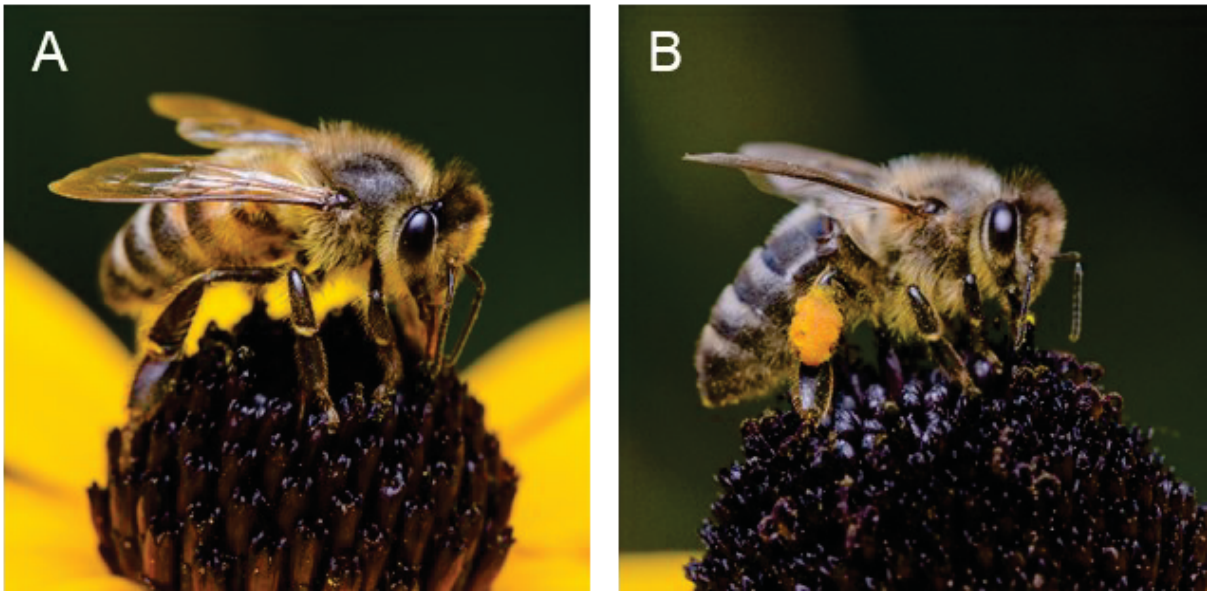

Abb. 7: Honigbienenarbeiterinnen beim Furagieren. A: Arbeiterin ohne Pollenhöschen. B: Arbeiterin mit Pollenhöschen an den Hinterbeinen. *Fotos: Christian Verhoeven*

nur bei jungen Arbeiterinnen aktiv sind. Sie sind mit dem Fettkörper verbunden und dienen dem Wabenbau.

Da die Ovarienentwicklung in Arbeiterinnen unterdrückt wird, sind diese verglichen mit denen der Königin verkümmert. Sie sind paarig angeordnet und bestehen aus etwa 2 – 12 leeren Ovariolen (Makert et al., 2006, Munday et al., 2012), die jeweils in einen Oviduct münden, die sich wiederum in der Vagina vereinigen (Snodgrass, 1910), (Abb. 6D und E).
Direkt benachbart zu den Ovarien befindet sich der Stachelapparat, bestehend aus Stachel und Giftdrüse. Der Stachel liegt in der Stachelhöhle im Körperinneren und kann von dort aus schnell ausgefahren werden. Da der Stachel Widerhaken besitzt, bleibt er beim Stich in der Haut von Säugern stecken. Dabei reißt der mit den letzten drei posterioren Körpersegmenten über Membranen verbundene Stachelapparat aus, woran die Biene stirbt. Beim Verteidigungsstich in andere Insekten bleiben die Widerhaken aufgrund der Chitinhülle nicht in der Oberfläche stecken. Der Stachel kann zurückgezogen und erneut verwendet werden (Snodgrass, 1910, Gempe et al., 2009).

5.4 Königinnenentwicklung

Mit durchschnittlich insgesamt 16 Tagen entwickeln sich Königinnen von den drei Morphen am schnellsten. Sie verbringen 7 – 9 Tage als Larve, bevor sie sich für 4 – 6 Tage verpuppen. Als Imago sind frisch geschlüpfte Königinnen mit im Schnitt 190 mg deutlich schwerer als Arbeiterinnen (von Rhein, 1933, Dade, 1994). Ihnen steht während ihrer Entwicklung durchgehend Futter mit einem hohen Anteil an Zucker zur Verfügung (Haydak, 1970, Wheeler et al., 2006, Patel et al., 2007, Kaftanoglu et al., 2010).

Der Kopf einer Königin ist nur geringfügig kleiner als der von Arbeiterinnen und ähnelt in Form und Augengröße ebenfalls sehr dem von Arbeiterinnen (Streinzer et al., 2013), (vgl. Abb. 6A und B). Königinnen besitzen an ihren Beinen keine Werkzeuge zum Sammeln von Pollen und keine Wachsdrüsen an den ventralen Abdomensegmenten. Ihr Abdomen ist durch verlängerte Tergite und Sternite deutlich länger als das von Arbeiterinnen.

Die paarig angelegten Ovarien einer Königin bestehen aus bis zu 200 Ovariolschläuchen, die mit heranreifenden Eiern gefüllt sind. Anterior laufen ihre Enden spitz zusammen, am posterioren Ende vereinigen sich alle in breiten Schläuchen in den Oviduct. Die beiden Oviducte vereinigen sich wiederum in die Vagina (s. Abb. 6D), deren Öffnung hinter dem siebten Sternitsegment direkt neben dem Stachel liegt. Der posteriore Teil der Vagina formt eine *bursa copulatrix*, die Begattungstasche, die von einer kugelrunden Spermathek mit der Basis der sich vereinigenden Oviducte verbunden ist. In der Spermathek sammelt die Königin bei ihrem Hochzeitsflug Sperma von bis zu 20 Drohnen, welches nachreifende Eier beim Passieren befruchtet (Snodgrass, 1910). Die Königin legt über 3 bis 5 Jahre hinweg bis zu 2000 Eier am Tag (Franck et al., 2002).

Kapitel II: Manuskripte

Manuskript I

Fem and *Am*-Tra2 proteins direct female specific splicing of the *Am-dsx* pre-mRNA by activation of the female splice donor site in the honeybee *Apis mellifera*

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Abstract

Molecular analysis of the diversity of sex determination systems can contribute in our understanding of how and why pathways and gene functions evolved. Sex in the honey bee (*Apis mellifera*) is determined by heterozygosity of the *complementary sex determiner* (*csd*) gene. This sex-specific signal is realized by a cascade of genes that involves the *feminizer* (*fem*) and the *Apis mellifera-doublesex* (*Am-dsx*) gene. We have previously reported that Csd proteins control the female-specific processing of the *fem* transcript encoding the Fem protein, while Fem proteins are required to promote female specific splicing of *Am-dsx* transcripts. However, it is still unknown whether Fem protein is the direct regulator of female *Am-dsx* splicing and how this process is molecularly regulated. To analyze the molecular control of splice regulation of female and male *Am-dsx* transcripts, we have developed an *in vivo* splice assay in *Sf21*-cells by using *Am-dsx* minigene constructs to mimic sex-specific *Am-dsx* splicing. Beside Fem proteins, we co-expressed *Am*-Tra2 protein as a candidate protein that has the ability to directly interact with the pre-mRNAs and analyzed the *Am-dsx* splicing products. Here we report that both proteins, female specific Fem and non-sex-specific *Am*-Tra2 are direct regulators of alternative splicing of the *Am-dsx* minigene transcripts. In *Drosophila melanogaster* Tra and Tra2 direct splicing of female *dsx* transcripts, but none of the known exonic splice enhancers (ESEs) from *D. melanogaster* are found in the female-specific splice sites of *Am-dsx*. Here we found potential *Am*-Tra2 binding sites in the female-specific exon 5 of *Am-dsx*, that are located upstream of the female-specific splice donor site and activate the female-specific splice acceptor. This suggests that the underlying mechanism of producing the sex-specific transcription factor Dsx in these insects has diverged.

Introduction

Sex in the honeybee (*Apis mellifera*) is determined by the heteroallelic combination of the *complementary sex determiner* (*csd*) gene (Beye et al., 2003) in contrast to the well studied sex chromosome system of *Drosophila melanogaster* (Cline et al., 1996, Erickson and Quintero, 2007). About 116 – 145 allelic variants of this *complementary sex determining* gene are segregating in honeybee populations (Lechner et al., 2014, Hasselmann et al., 2008, Hasselmann and Beye, 2004). They differ at an average of approximately 6% of their amino acid residues in the putative specifying domain (Hasselmann et al., 2008, Hasselmann and Beye, 2004). Bees heterozygous at the *csd* gene are females, whereas bees homozygous or hemizygous (haploid individuals) at the *csd* locus are males. In the honeybee colonies queens lay unfertilized eggs to produce fertile males (drones) and fertilized eggs to produce females that either differentiate into workers or queens as determined by worker's differential feeding of larvae with royal jelly (Kucharski et al., 2008). Diploid males in a colony do not survive because they are eaten by worker bees shortly after they hatch from the egg. The *csd* gene translates into an SR-type protein (Beye, 2004) that in the heteroallelic constitution is required for female differentiation and directs the female splicing of *fem* pre-mRNAs (Hasselmann et al., 2008, Gempe et al., 2009), which has been shown by *fem* and *csd* gene knockdown studies. The female spliced *fem* mRNAs are translated into Fem proteins that mediate the splicing of the female *Am-dsx* mRNAs. In males, the activity of the primary signal *csd* that newly evolved in the honeybee lineage (Hasselmann et al., 2008) and the downstream target gene *fem* are not required. In the presence of homoallelic or hemiallelic Csd proteins, non-productive *fem* mRNAs are generated by introducing a premature translational stop codon into exon 3 during the splice process (Gempe et al., 2009). In the absence of active Fem protein *Am-dsx* pre-mRNAs are spliced into the male mode, lacking female exon 5. The alternative spliced *Am-dsx* transcripts in males and females translate into transcription factors that differ in a part of their C-terminal sequence (Dearden et al., 2006, Cho et al., 2007).

Here, we study the splice control of *Am-dsx* transcripts in honeybees. Dsx orthologs are transcription factors of the DM type that have an atypical zinc-finger domain in common and are key players in aspects of sexual differentiation in insects, crustacean, nematodes and mammalians (Vincent et al., 2001, Keisman and Baker,

2001, Christiansen et al., 2002, Keisman et al., 2001, Kopp et al., 2000, Sanchez et al., 2001, Williams et al., 2008, Raymond et al., 1998, Hediger et al., 2004, Suzuki et al., 2003, Kato et al., 2011, Matson et al., 2011) by integrating sex-specific information within the general developmental program. In dipteran species including *D. melanogaster*, *Ceratitis capitata*, *Anastrepha obliqua*, *Musca domestica*, *Bactrocera oleae* (Ruiz et al., 2005, Ruiz et al., 2007, Hediger et al., 2004, Saccone et al., 2008, Lagos et al., 2005, Burtis and Baker, 1989, Hoshijima et al., 1991), as well as in the lepidopteran insect *Bombyx mori* (Ohbayashi et al., 2001, Suzuki et al., 2001, Suzuki et al., 2003) and the hymenopterans *A. mellifera* and *Nasonia vitripennis* (Cho et al., 2007, Oliveira et al., 2009), *dsx* is sex-specifically regulated by an alternative splicing process. These female and male *dsx* mRNAs are translated into Dsx proteins which have the N-terminal regions in common, but have sex-specific sequences in segments of the oligomerization domain OD2 (An et al., 1996, Oliveira et al., 2009, Ruiz et al., 2007, Ruiz et al., 2005, Hediger et al., 2004, Saccone et al., 2008, Lagos et al., 2005, Cho et al., 2007, Ohbayashi et al., 2001). The sex-specific peptides of Dsx proteins control the transcription of target genes which are necessary for somatic male and female differentiation by binding to cis-regulatory elements (Burtis and Baker, 1989, Coschigano and Wensink, 1993, Sanchez et al., 2001, Keisman et al., 2001, Williams et al., 2008).

In females of *D. melanogaster*, the splicing of the *dsx* transcripts is directly regulated by the Tra and Tra2 proteins. The SR-type protein Tra together with Tra2 protein directly interacts with Exonic Splicing Enhancers (ESEs) and a purine rich element (PRE) located in exon 4 to activate a weak splice acceptor site (3´ splicing site) which leads to the inclusion of the female-specific exon 4 (Burtis and Baker, 1989, Inoue et al., 1992, Hedley and Maniatis, 1991, Lynch and Maniatis, 1996, Lynch and Maniatis, 1995, Sciabica and Hertel, 2006, Tian and Maniatis, 1993). The ESEs are located ~300 nt downstream of the splicing acceptor site and consist of six repeats of a 13-nucleotide (nt) sequence with the consensus motif (TC(T/A)(T/A)C(A/G)ATCAACA) (the so called *dsx* repeat elements (*dsx*RE)). This Tra/Tra2 binding motif has been observed in other dipteran species that suggests a canonical motif for Tra/Tra2-dependent *dsx* splice regulation (Salvemini et al., 2009, Hediger et al., 2004, Burghardt et al., 2005). The inclusion of the female *dsx* exon 4 results in the usage of a female specific polyadenylation site that causes cleavage of female transcripts ~570 nt downstream of the Tra/Tra2 binding sites. In contrast in males a

polyadenylation signal terminates transcription after the male specific exon 5 (Hedley and Maniatis, 1991). In *D. melanogaster* males, *dsx* pre-mRNAs are spliced into the male form in the absence of Tra proteins (Hoshijima et al., 1991, Burtis and Baker, 1989).

Here we study how female and male splicing of the *Am-dsx* transcripts is regulated in the honeybee in the absence of canonical *D. melanogaster* Tra/Tra2 binding sites. The Fem protein, like Tra in *D. melanogaster* is a female-specific factor that directs female splicing of *Am-dsx* pre-mRNAs. The Fem protein (403 aa) has substantially diverged in the amino acid sequence compared to the predicted *D. melanogaster* Tra ortholog (197 aa) and has an N-terminal region (212 aa) that is entirely absent in the Tra protein of the fruitfly. We have thus far no information how male *Am-dsx* mRNA processing is controlled.

In this study we report on another mechanism of *Am-dsx* splice regulation in the honeybee. The Fem/*Am*-Tra2 proteins activate the 3' acceptor site of the female exon 5 that requires a new sequence motif leading to the inclusion of the female exon and the female transcript. Male *Am-dsx* splice variants are produced in the absence of an activity provided by the sex-determination pathway (default state) implying that the sexual switch at the level of *Am-dsx* is entirely controlled by absence and presence of Fem proteins. We also report on how the *Am-dsx* splice regulation is mediated by the essential but RNA binding domain lacking SR-type protein Fem. Its co-factor *Am*-Tra2 consists of an RNA binding domain and the sequence upstream of the 5'-splice site of the female exon 5 consists of repetitive *Am*-Tra2 binding motifs. So we conclude a binding of Fem/*Am*-Tra2 proteins to these binding sites that enhance female-specific *Am-dsx* splicing.

Results

Male-specific splicing is directed in the absence of Fem and *Am*-Tra2 proteins in *Sf21* cells

Previous results showed that exon 5 of the *Am-dsx* pre-mRNA (Fig. 1A) is included in females in the presence of Fem activity and that exon 5 is excluded in its absence (Gempe et al., 2009). To study the mechanisms how the exon is selected and what trans-acting factors and binding sequence elements are required to direct sex-

specific splicing we developed a splice assay in *Sf21* cells. We used an *Am-dsx minigene* construct (Fig. 1B; cloned into plasmid pDO *Am-dsx*^{mg}) that we transfected into *Sf21* cells to study sex-specific inclusion or exclusion of exon 5. To make the *Am-dsx* gene accessible to analysis we shortened the large intron 5 to the first 225 and the last 245 nucleotides. We co-transfected cells with *pDO hGH* transcribing pre-mRNA of the *hGH* gene (Selden et al., 1986) that served as transfection and splice control in our semiquantitative RT-PCR based assay.

We observed male-specific exclusion of exon 5 in cells that were transiently transfected solely with the *Am-dsx minigene* construct (Fig 1 C, lane 3). We detected no female splicing and no inclusion of exon 5 (Fig 1 C, lane 3). Beside the most abundant fragment corresponding to the male splice variant (*Am-dsx*$^{mg\text{-}M}$) we found low amounts of two alternative splice products (*Am-dsx*$^{mg\text{-}R1}$ and *Am-dsx*$^{mg\text{-}R2}$) in *Sf21* cells that we did not detect in RT-PCR experiments from honeybee embryos. Splice product *Am-dsx*$^{mg\text{-}R1}$ includes full exon 4, the last 194 nucleotides of exon 5 and exon 6 (Fig. 1B +C; Fig. S 3c) suggesting that the female exon 5 splice donor is activated together with another splice acceptor ($SA5_2$; Fig. 1B) that is not used in honeybees. We were not able to sequence splice product *Amdsx*$^{mg\text{-}R2}$. We next amplified fragments specifically of the female transcripts that include exon 4 and the beginning of exon 5 by using oligonucleotide primer pairs #417 and #419, but did not detect any PCR fragment (Fig 1B and Fig 1C, lane 3). These results suggest that our *Am-dsx minigene* can mimic male-specific splicing of *Am-dsx* pre-mRNAs in *Sf21* cells.

Fem proteins are sufficient to direct female-specific splicing in Sf21 cells

In a previous study we showed that knockdown of *fem* mRNAs in honeybees produced a switch from female to male splicing of *Am-dsx* pre-mRNAs (Gempe et al., 2009). To test whether Fem proteins are sufficient to direct female splicing in a dose dependent manner we transfected *Sf21* cells with plasmids *pDO Am-dsx*^{mg}*, pDO fem* and *pDO hGH* that were expressing the *Am-dsx minigene*, the Fem proteins and the *hGH* splice control. We analyzed male- and female-specific splice products by RT-PCR which we semi-quantified across samples in respect of the splice product of the *hGH* control.

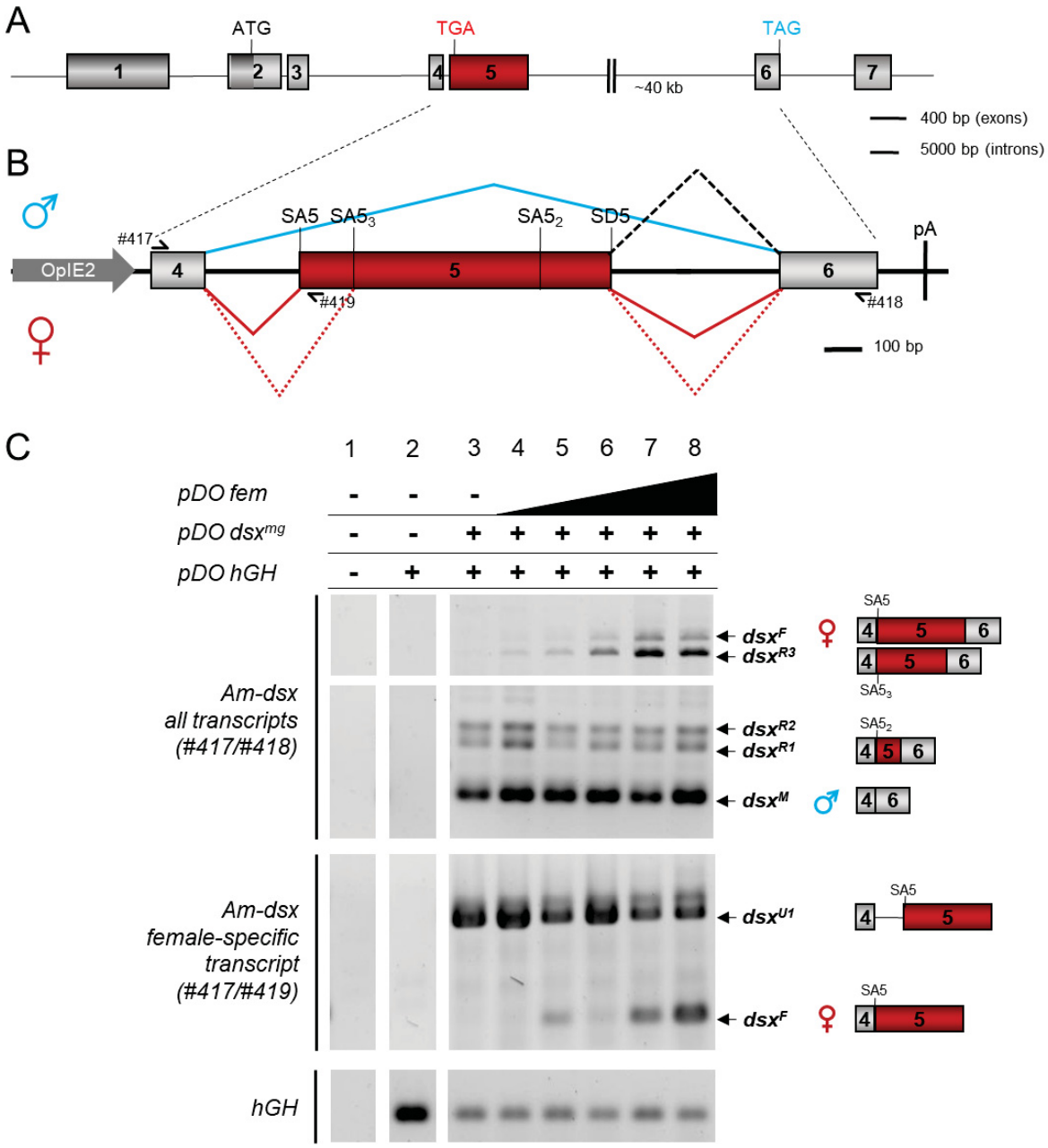

Fig. 1: RT-PCR analysis of splicing variants of the *Am-dsx mini gene* (*pDO Am-dsxmg*) derived transcripts in the presence and absence of Fem protein in *Sf21* cells. (A) Diagram of the genomic organisation of the *Am-dsx* gene. The female-specific exon 5 is shown as red box; the common exons are shown as grey boxes. The common translational start codon is shown in black and the translational stop codons are shown in red (female) and blue (male). Please note that exons and introns are shown in different scales. Intron 5 has been shortened for better illustration (marked by two vertical bars). (B) Intron and exon structure of the *Am-dsx^{mg}* that was cloned into *pDO* vector under control of the viral OpIE2 promoter. pA indicates the polyadenylation signal that we derived from *OpIE2* polyadenylation signal. The ~40 kb intron 5 of *Am-dsx* was shortened to 470 bp in the minigene. SA5 assigns the splice acceptor of the female exon and SD5 assigns the splice donor of the female exon. SA5$_2$ and SA5$_3$ indicate the alternatively used splice acceptors in the female exon that we observed when expressed in *Sf21* cells. (C) RT-PCR analysis of splicing variants of *pDO Am-dsxmg*

derived transcripts in the presence and absence of Fem protein in *Sf21* cells. 1x10^6 *Sf21* cells were incubated with ddH_20 (lane 1) or transiently transfected with 1 µg of *pDO hGH* (lane 2-8), 1 µg of *pDO Am-dsx^{mg}* (lane 3-8) and increasing amounts of *pDO fem* (0.3, 0.8, 1.5, 2.0, 2.5 µg) (lane 4-8). Male and female specific fragments were amplified by using oligonucleotide primers as indicated in (B). Conditions for PCR using oligonucleotides #417#/418 were optimized for amplification of small male *Am-dsx* fragments and large female *Am-dsx* fragments in two separate PCRs (shown in separate gel pictures). Semiquantitative RT-PCR was standardized in respect to the splice product of the cotransfected *hGH* control. Fragments were resolved in 2% agarose gels and stained with ethidium bromide. Fragments were cloned and sequenced to determine the exonic structure. The exonic structure of the amplified fragments is schematically shown to the right of the gel picture.

We detected female-specific inclusion of exon 5 with increasing amounts of Fem protein (*Am-dsx^{mg-F}*; Fig 1C, lane 4-8 upper panel). Beside the inclusion of entire female exon 5 in the presence of Fem protein we detected in the *Sf21* cells *Am-dsx^{mg-R3}* transcript (sequence, Fig. S3d) that uses a splice acceptor site ($SA5_3$) that is located 140 nucleotides downstream of the female SA5 site ($SA5_3$, figure 1B).

The inclusion of female exon 5 leads to a substantial increase in size and thus in an underrepresentation of this fragment in PCRs when compared to the smaller male fragment (855 nucleotides difference). We confirmed the female splicing with oligonucleotide primers #417 and #419 that amplifies female transcripts that include the female exon 5, but no male or *Am-dsx^{mg-R3}* transcripts. We detected again the female splice product (*Am-dsx^{mg-F}*) that increased with larger amounts of *pDO fem* plasmids that are expressing Fem proteins (Fig 1C, lane 4-8 lower panel). These results suggest that Fem proteins are sufficient to direct in a dose dependent manner female-specific splicing of *Am-dsx* transcripts.

We used in the subsequent experiments the oligonucleotide primers #417 and #419 to specifically amplify the female splice variants and the oligonucleotide primers #417 and #418 to analyze male splice products (Fig. 1B).

Expression of Am-Tra2 proteins enhances female-specific splicing in the presence of Fem proteins.

In a previous study we have identified the *tra2* ortholog gene, *Am-tra2*, in the honeybee genome (Dearden et al., 2006). Tra2 in *D. melanogaster* is not sex-

specifically regulated in the soma (Mattox et al., 1990) and associates together with Tra protein as a part of a multiprotein complex to direct the female splicing of *dsx* pre-mRNAs (Tian and Maniatis, 1993, Lynch and Maniatis, 1996). We first studied whether expression of solely the *Am*-Tra2 proteins are sufficient to direct female splicing of the *Am-dsx minigene*. Previous studies on splice regulation of Tra2 proteins in *D. melanogaster Kc* cells showed that Tra2 proteins can induce low amounts of *dsx* female-specific splicing, presumably due to the presence of endogenous expressed Tra proteins in this female cell line (Hoshijima et al., 1991).

To examine the role of *Am*-Tra2 in *Am-dsx* splicing we transfected *Sf21* cells with increasing amounts of *pDO Am-tra2* plasmids expressing the *Am*-Tra2 proteins. We observed that *Am*-Tra2 proteins were not sufficient to induce female-specific splicing in *Sf21* cells under different levels of expression (Fig. 2, lane 4-7) indicating that *Am*-Tra2 proteins cannot direct female splicing even when they are overexpressed. Next we examined whether *Am*-Tra2 protein expression can enhance female splicing when Fem proteins are expressed. We transiently cotransfected plasmids expressing Fem protein and increasing amounts of plasmids expressing *Am*-Tra2 proteins. Higher expression of *Am*-Tra2 proteins increased inclusion of exon 5 when Fem proteins are coexpressed (Fig. 2, lane 8-12), suggesting that the *Am*-Tra2 proteins enhance the female-specific splicing, but solely when Fem proteins are present.

The Fem protein interacts with the Am-Tra2 protein

To further dissect whether the enhanced splice activity of Fem proteins in the presence of *Am*-Tra2 protein involves binding between these proteins we tested the binding capabilities between these proteins. Knock down studies have shown that activity of the *fem* and *Am-tra2* genes are required for *fem* female splicing (Nissen et al., 2012).

To test binding, we fused Fem proteins to the Cerulean fluorescent protein and *Am*-Tra2 to the Yellow fluorescent protein (YFP) . We expressed the fusion proteins in *Sf21* cells and compared fluorescence life time (FRET-FLIM; Fluorescence Lifetime Imaging Microscopy (Borst and Visser, 2010, Weidtkamp-Peters et al., 2009)). If the proteins interact energy is transferred between the fluorophors Cerulean and YFP, which shortens the fluorescence lifetime of the donor fluorophore (CFP).

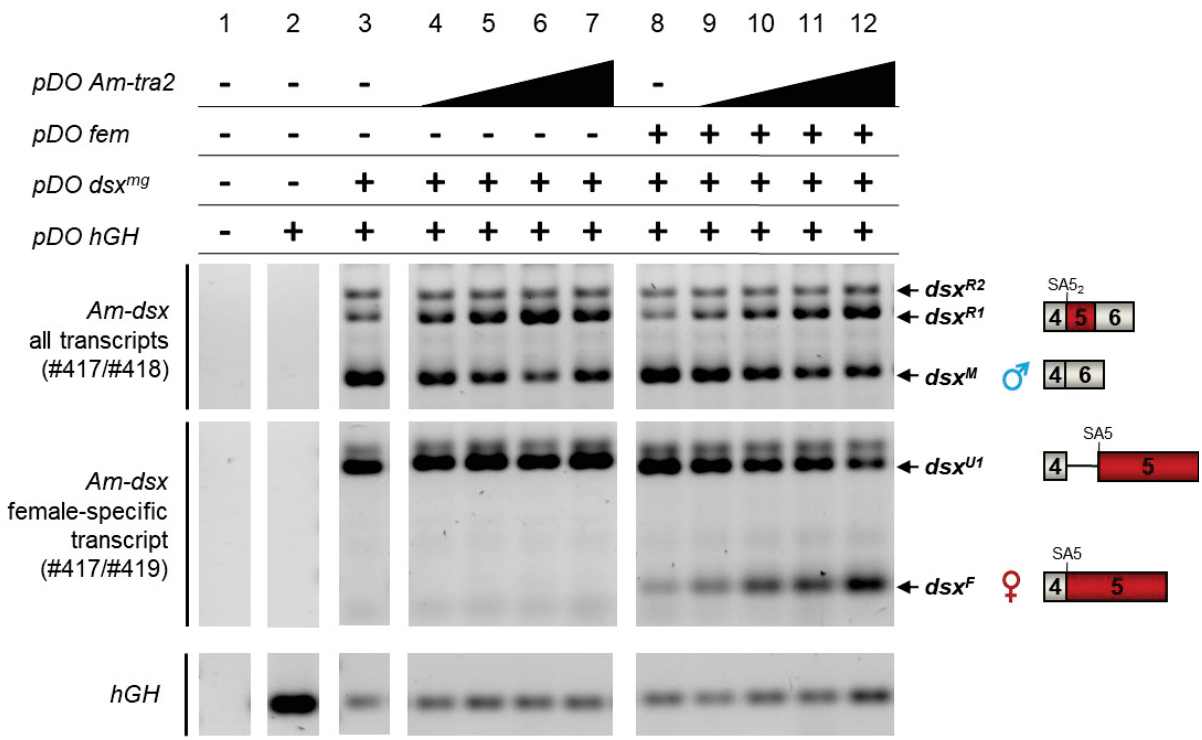

Fig. 2: RT-PCR analysis of splicing variants of *pDO Am-dsxmg* derived transcripts in the presence and absence of Fem and Am-Tra2 protein in *Sf21* cells. 1x10^6 *Sf21* cells were incubated with dd$H_2$0 (lane 1) or transiently transfected with 1 µg of *pDO hGH* (lane 2-12), 1 µg of *pDO Am-dsxmg* (lane 3-12), 2.5 µg of *pDO fem* (lane 8-12) and increasing amounts of *pDO Am-tra2* (0.3, 0.8, 1.5, 2.0 µg) (lane 4-7 and 912). Male and female specific fragments were amplified by using oligonucleotide primers as indicated in figure 1B. Semiquantitative RT-PCRs were standardized in respect to the splice product of cotransfected *hGH* control. Conditions for the PCR using oligonucleotides #417/#418 were optimized to amplify the small male *Am-dsx* fragments. Fragments were resolved in 2 % agarose gels and stained with ethidium bromide. Fragments were cloned and sequenced to determine the exonic structure. The exonic structure of the amplified fragments is schematically shown to the right of the gel picture. SA5 assigns the splice acceptor of the female exon, $SA5_2$ and $SA5_3$ those of splice acceptors that are alternatively used in *Sf21* cells.

We found that the co-expression of Fem-Cerulean and *Am*-Tra2-YFP proteins led to a significant reduction in fluorescence lifetime compared to when Fem-Cerulean proteins were expressed exclusively (Fig. 3 C) (t-test, df, $P < 0.001$). We conclude from these results that Fem and *Am*-Tra2 proteins directly bind to each other in cells.

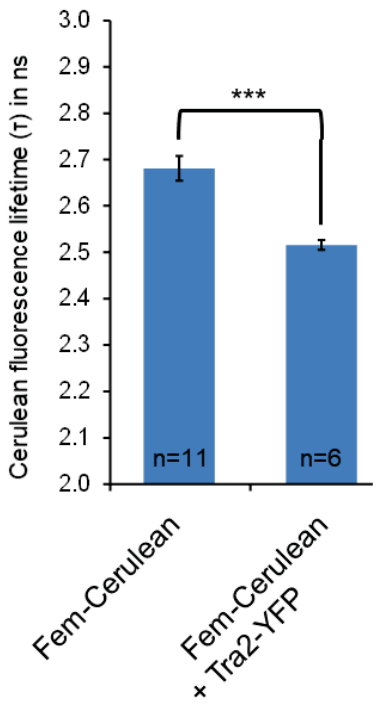

Fig. 3: Interaction studies of Fem and Am-Tra2 proteins using fluorescence life time analysis in Sf21 cells. Fem-Cerulean, Csd-Cerulean and Am-Tra2-YFP fusion proteins were expressed in Sf21 cells. The fluorescence life time was measured in the presence and absence of Am-Tra2-YFP proteins and Fem-Cerulean proteins. Fluorescence lifetimes (τ) were measured using FLIM-FRET (Fluorescence Lifetime Imaging Microscopy - Förster Resonance Energy Transfer) and compared using a 2-tailed Student t-test. n denotes the number of cells that were analyzed for each condition. *** indicates $P < 0.001$, $df = 15$.

The exon sequence upstream of the female-specific splice donor site harbors several Fem/*Am*-Tra2 protein dependent splice regulating elements

To further understand the control of exon 5 splice site activation by Fem and *Am*-Tra2 proteins, we cloned the *Am-dsx* exchange cassette (*Am-dsx ExC*) (Fig. S1a). With truncations of the region upstream of SD5 we screened for splice regulating elements (Fig. S1a-c. Based on the results of the *dsx*-ExC experiments we analysed a larger sequence upstream of the SD5 with the program HEXplorer (Erkelenz et al., 2014). This program was programmed to predict splice regularory sequences and effects of sequences on their splice enhancing and silencing properties in the vicinity of a given splice site. The HEXplorer analysis predicted four 6 nt long sequences in the analysed region to have splice enhancing effects (Fig. S2a). These four sequences have the motif GAAGAT. We then virtually mutated these motifs, which resulted in reduced HEXplorer scores and therefore the prediction that these motifs are essential for female specific splicing of *Am-dsx.* By the introduction of mutations we had to be very careful as even a point mutation can have devastating effects in splice regulation. Therefore we only used one point mutation in every four sequences, respectively (see Fig. S2b).

Defined mutations within 50 – 180 nt upstream of the female-specific slice donor site reduce splicing efficiency in presence of Fem and *Am*-Tra2

To test the importance of the predicted ESE motifs for the Fem/*Am*-Tra2-dependent inclusion of the female specific exon 5, we co-transfected the wildtype *Am-dsx*^{mg} and an *Am-dsx minigene* with 4 point mutations (*Am-dsx*^{mg}*-mut4*) that we tested with the HEXplorer, respectively, together with *hGH.* We analysed the splicing pattern with the above mentioned analysis PCRs (#417/#418, #417/#419) and an additional oligonucleotide combination, that amplifies the beginning of exon 5 to the middle of exon 6 and therefore detects female-specific usage of SA5 (#882/#418) without and with Fem/*Am*-Tra2, respectively, by analysing the observed splice products. The overall splice pattern shows that the Fem/*Am*-Tra2-dependent exon 5 inclusion, that takes place in the minigene, does not take place in *Am-dsx*^{mg}*-mut4.* We do not find any female-spliced transcript. Instead the male-specifically spliced transcript, that excludes exon 5, is still as strong as without Fem/*Am*-Tra2, indicating that default male splicing is not affected (Fig. 4a). These results show that our four mutated motifs are essential for Fem/*Am*-Tra2-dependent female exon inclusion.
To further evaluate whether SD5 or SA5 or both are affected by the mutations we examined the splice sites specifically. Thereby we observe that splicing at the SA5 is nearly prevented at the *Am-dsx*^{mg}*-mut4* as the female SA5-spliced transcript in both samples is very weak (Fig. 4b).

The SD5-analysis PCR suggests that SD5-usage is dependent from Fem/*Am*-Tra2, but independent from the tested motifs (4 point mutations), as the basic amount of SD5-spliced transcripts is reduced in *Am-dsx*^{mg}*-mut4*, while we can still observe an Fem/*Am*-Tra2-dependent increase in both transcripts (Fig. 4c).
From these results we can conclude that the mutated sequences of four times GAAGAT are essential for Fem/*Am*-Tra2-dependent exon inclusion of the female-speficic exon 5 of *Am-dsx*. These potential *Am*-Tra2 binding motifs are located about 700 bp downstream of the female-specific SA5, whose usage they directly induce.

Discussion

We have previously reported that the *fem* gene is required for female specific splicing of *Am-dsx* transcripts in the honeybee that implement female differentiation

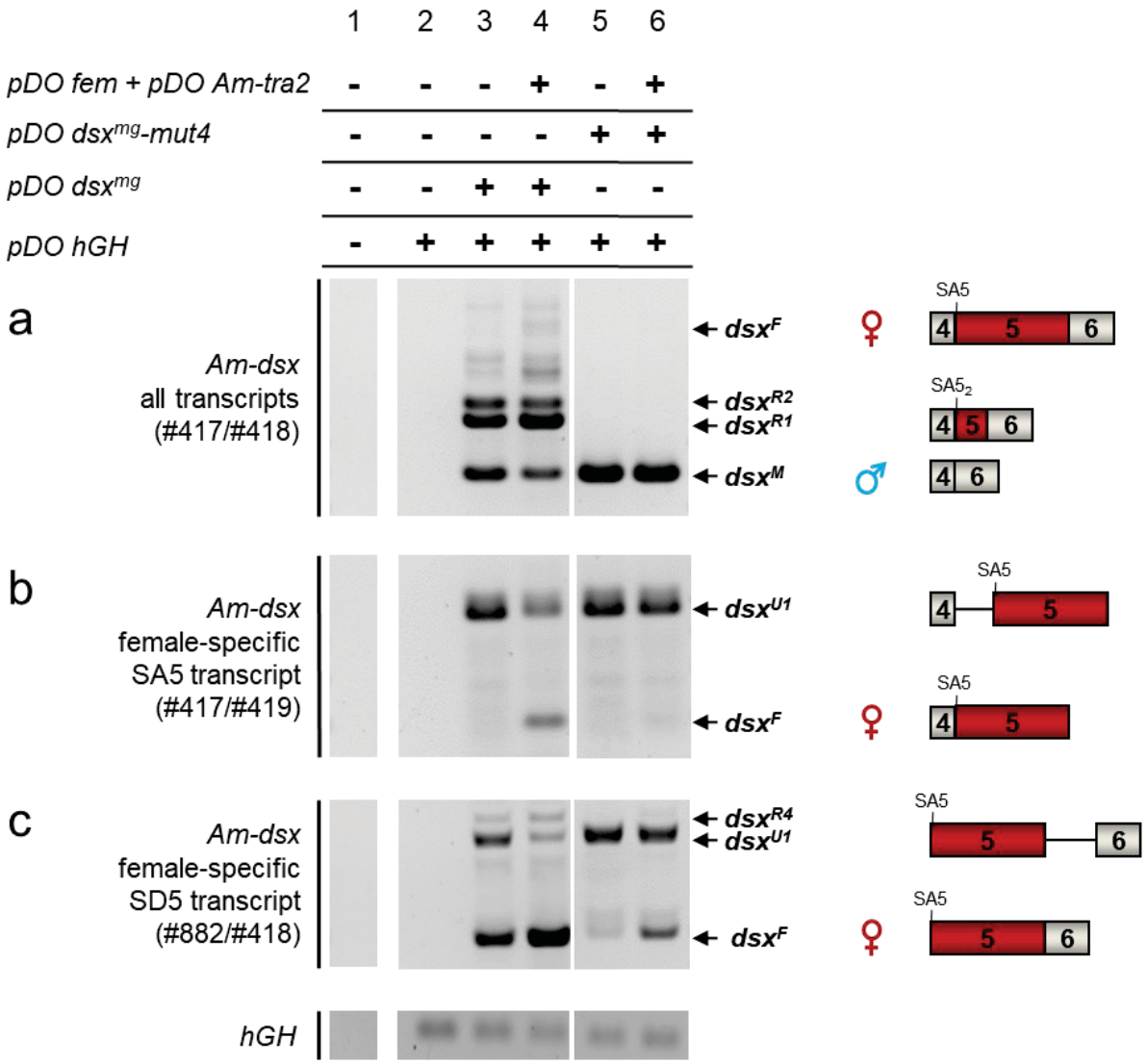

Fig. 4: RT-PCR analysis of splicing variants of *pDO Am-dsx*^{mg} derived transcripts in the presence and absence of Fem and Am-Tra2 protein in *Sf21* cells. 1x10^6 *Sf21* cells were incubated with cell culture medium (lane 1) or transiently transfected with 0.5 µg of *pDO hGH* (lane 2-9), 0.5 µg of *pDO Am-dsx*^{mg} *or pDO Am-dsx*^{mg}*-mut4*, respectively (lane 3-6) and 2 µg of *pDO fem* + *pDO Am-tra2* (lane 4 and 6). In all lanes the total amount of transfected plasmid is added up to 3 µg with the vector *pGL3*. Male and female specific fragments were amplified by using oligonucleotide primers as indicated in figure S1. Semiquantitative RT-PCRs were standardized in respect to the splice product of cotransfected *hGH* control. Fragments were resolved in 2 % agarose gels and stained with ethidium bromide. Fragments were cloned and sequenced to determine the exonic structure. We were not able to sequence fragment *dsx*^{R2} *and dsx*^{R4}. The exonic structure of the amplified fragments is schematically shown to the right of the gel picture. SA5 assigns the splice acceptor of the female exon, $SA5_2$ and $SA5_3$ those of splice acceptors that are alternatively used in *Sf21* cells.

(Hasselmann et al., 2008, Gempe et al., 2009). However, a direct involvement of the Fem protein in the splicing process has thus far not been shown. The *fem* gene is a critical component of the complementary sex determining pathway as it maintains the femaleness by a positive regulatory loop and implements female differentiation by the primary signal *csd*. In the present study we provided evidences that female Fem protein is directly regulating the splicing of *Am-dsx* transcripts. We further showed that *Am*-Tra2 protein interacts with Fem and enhances female splicing of *Am-dsx* pre-mRNAs in the presence of Fem proteins like their orthologues in *D. melanogaster*, Tra2, Tra and Dsx, do, but that the mechanism of splice regulation and the involved RNA binding sites differ from those in *D. melanogaster* (Inoue et al., 1992, Sciabica and Hertel, 2006). We established an *in vivo* splice assay in lepidopteran cells and were able to mimic sex-specific *Am-dsx* splicing using an *Am-dsx* minigene construct. In the absence of Fem protein male *Am-dsx* transcripts were generated, suggesting that male splicing is the default regulatory state in the absence of the *complementary sex determining* signal *csd*. In the presence of female Fem protein we were able to promote female specific splicing of *Am-dsx* transcripts. Taken together, our data suggest that Fem protein is sufficient to direct female *Am-dsx* splicing. *Am*-Tra2 protein further enhances female specific splicing in the presence of Fem protein, but alone it is not sufficient to direct female *Am-dsx* splicing in *Sf21* cells. This finding supports our previous study in honeybees that shows that *Am-tra2* gene is essential for female *Am-dsx* splicing in the presence of female Fem proteins (Nissen et al., 2012).

This raises the question of how the SR-type protein Fem is able to promote, in the absence of an RNA binding domain (RBD) and direct RNA binding, female *Am-dsx* splicing in *Sf21* cells. We propose four explanations for this observation: (i) endogenously expressed *Sf*-Tra2 protein in *Sf21* cells is able to replace the role of *Am*-Tra2 protein as co-factor of Fem protein in the splice process; (ii) other RNA-binding proteins are sufficient to mediate the interaction between Fem and the *Am-dsx* pre-mRNA; (iii) Fem protein has RNA binding abilities; (iv) Fem proteins when overexpressed, as it is performed here, can possibly direct the splicing. Our previous results, however, showed that *Am-tra2* gene is essential to direct female *Am-dsx* pre-mRNA splicing (Nissen et al., 2012), suggesting that either overexpression or endogenous Tra2 of *Sf21* cells is responsible.

We further studied how the Fem and *Am*-Tra2 proteins direct female-specific exon 5 inclusion. We identified the region of up to 98 nt upstream of the female SD to contain potential Fem/*Am*-Tra2-dependent splice regulatory elements by using an *Am-dsx* exchange cassette. This construct shows how truncations of *Am-dsx* exon 5 upstream of the SD affect Fem/*Am*-Tra2-dependent splicing of the *Am-dsx* minigene in *Sf21*-cells.

With the HEXplorer we identified four potential Fem/*Am*-Tra2-dependent exonic splice enhancer (ESE) sequences in the region up to 180 nt upstream of the SD. Point mutations within the four predicted sequences showed that they are essential for the activation of female-specific exon 5 inclusion by Fem and *Am*-Tra2 in *Sf21*-cells. We suggest that these motifs are utilized to recruit Fem and *Am*-Tra2 proteins to the ribonucleotide sequence and to direct the use of the SA of the female exon. Our analysis, however, cannot exclude that other sequence motifs further upstream are also involved to promote the use of the female specific splice donor or the splice acceptor site.

The role of female Fem protein as a direct component of sex-specific splice regulation suggests important implications for the control of the complementary sex determination pathway. Heteroallelic Csd protein is the primary signal that determines the female state and that directs the female processing of fem transcript that are translated into Fem proteins (Gempe et al., 2009, Hasselmann et al., 2008). Beside the role of Fem protein as a splice activator of female *Am-dsx* splicing, it is also responsible to maintain the female determined state throughout development by directing female splicing of its own pre-mRNAs into the productive female mode (Gempe et al., 2009). The Fem/*Am*-Tra2 proteins may also directly control the splicing of female *fem* transcripts. Indeed, knockdown studies of *Am-tra2* showed that *Am*-Tra2 protein is also required to promote *fem* transcript splicing into the female mode (Nissen et al., 2012). This implies that *Am*-Tra2 has an important role on two levels of the cascade of sex determination: on the level of *Am-dsx* and on the level of *fem* pre-mRNA splicing.

Although the sex-specific splicing of *dsx* transcripts is highly conserved across different insect orders (Gempe and Beye, 2011) our results suggest that molecular control of this splice process can be very different. We compared *Am-dsx* regulation with those in *D. melanogaster* in which molecular details of the splicing process have

been studied. In *D. melanogaster* the splice acceptor site is activated by Tra and Tra2 proteins (Inoue et al., 1992, Sciabica and Hertel, 2006, Tian and Maniatis, 1993). The binding of these proteins to six repeats of a 13 nt (ESE) sequence that is located ~300 nucleotides downstream of the acceptor splice site recruit the general splicing machinery (Inoue et al., 1992, Sciabica and Hertel, 2006) to promote the female splicing. This canonical 13 nt long binding motif is found at similar positions of the female exon in other dipteran species (Hediger et al., 2004, Saccone et al., 2008, Lagos et al., 2005) suggesting that an equivalent mechanism by use of the splice acceptor is operating in these dipteran insects. In *D. melanogaster*, *M. domestica*, *C. capitata* and *Bactrocera oleae* the female exon is not spliced to further downstream exons and a polyadenylation signal terminates the female transcript in this exon. In contrast, Fem/*Am*-Tra2 proteins in the honeybee promote the use of the SA5 site by involving other than the known ESEs in order to direct the inclusion of the female exon. In this study we were also able to reveal the Fem/*Am*-Tra2 binding sites that are essential to direct female specific splicing of the *Am-dsx* pre-mRNA. The four repeats of the GAAGAT and related motifs are located ~ 700 nucleotides downstream of the SA they are activating.

Taken together, our comparison of the molecular control of *dsx* splicing in different orders of holometabolous insects implicate that generation of a sex-determining female and male *dsx* mRNAs is conserved (Gempe and Beye, 2011) while the mechanisms that produce the male and female transcripts have diverged.

In the near future, we will study the direct interaction of *Am*-Tra2 and Fem proteins with the *Am-dsx* pre-mRNA sequence by RNA binding shift experiments.

Materials and Methods

Plasmid constructions

The pDO vector which was used for construction of vectors pDO Fem, pDO *Am*-Tra2, pDO *Am-dsx*^{mg} and pDO hGH is derived from the pIZ/V5-His vector (Invitrogen). We inserted in the multiple cloning site (MCS) a sequence that transcribes a Rubia-EGFP-fluorescence protein. Downstream of the second OpIE2 promoter we inserted a second multiple cloning site (MCS2) and a second OpIE2 polyA-site (for sequence of pDO vector see supplementary data Fig. S3b).

The full length *feminizer* (Fem Srev238P; GenBank: EU100941.1) open reading frame (ORF) was amplified by PCR using primers #367 and #369 from vector pGEMT Fem Srev238P excluding the last three nucleotides. The full-length *Apis mellifera transformer2* (*Am-tra2*) (splice variant *Am-tra2*^{285}; GenBank: JQ518311) ORF was amplified by PCR with primers #359 and #421. Both fragments were cloned into pDO vector using the *Bam*HI and *Mlu*I restriction sites by replacing the Rubia-eGFP fragment producing the pDO Fem and pDO *Am*-Tra2 expression plasmid.
To generate the *Am-dsx* minigene construct, a fragment containing *Am-dsx* exon 4, intron 4, exon 5 and first 225 nucleotides of intron 5, was amplified by PCR with primers #245 and #246 from genomic DNA. A second fragment comprising *Am-dsx* last 245 nucleotides of intron 5 and complete exon 6 was amplified by PCR using primers #247 and #248 from genomic DNA. These fragments were cloned into pGEMT vector (pGEMT-Kit, Promega) that produced the plasmid pGEMT dsx_Ex4-In5_225 and pGEMT dsxIn5_last245bp-Ex6. The second *Am-dsx* fragment was restricted from pGEMT dsxIn5_last245bp-Ex6 vector using *Spe*I and *Not*I restriction enzymes and cloned into the pGEMT dsx_Ex4-In5_225 plasmid using *Spe*I and *Not*I restriction sites to create the pGEMT *Am-dsx*^{mg} plasmid. *Am-dsx*^{mg} sequence was amplified by PCR using primers #317 and #320 and cloned into pDO vector using the *Avr*II and *Xho*I restriction sites, by replacing the *Avr*II/*Xho*I fragment downstream of the second OpIE2 promoter. This cloning produced the *Am-dsx* minigene plasmid pDO *Am-dsx*^{mg}. This minigene is identical from exon 4-6 to the genomic sequence (GenBank: GB18426), except for the intron 5 that we shortened from 39550 nt to about 470 nt (containing first 225 nt and last 245 nt of intron 5).
To create the pDO hGH plasmid, a fragment comprising last 10 nucleotides of exon 1 of *human Growth Hormone* (*hGH*) (GenBank: NT_010783.15) along with full intron 1, exon 2, intron 2, exon 3, intron 3, exon 4, intron 4, exon 5, and first 198 nucleotides of exon 5 was amplified from vector pXGH5 using primers #496 and #497. This amplified product was cloned into pDO vector using the *Hind*III and *Xba*I restriction sites by replacing the RubiaeGFP fragment. In *Sf21*-cells the transcribed *hGH* pre-mRNA is spliced into 313 nt long transcripts that contain exon 1, first 105 nt of Intron 1 and exon 5 (for sequence of spliced and unspliced *hGH* transcripts see supplementary data, Fig. S3).
For the FRET analysis, we generated the plasmids PIZ/V5-His Fem-Cerulean by introducing the coding sequence of *fem* gene (S2-38; (Hasselmann et al., 2008)) into

plasmid PIZ/V5-His (Invitrogen, Carlsbad, CA, USA) using the *EcoRI* and *NotI* restriction sites. In order to maintain the reading frame a spacer sequence was cloned at the end of the ORF using *NotI* and *XbaI*. The sequence of the Cerulean fluorescent protein (Rizzo et al., 2004) was inserted at the end of the spacer sequence using *XbaI* and *SacII* restriction site. The plasmid PIZ/V5-His *Am*-Tra2-YFP sequence was generated by inserting the coding sequence of *Am*-tra2 gene (Nissen et al., 2012) upstream of the spacer sequence using the *EcoRI*/*NotI* restriction sites and the yellow fluorescent protein (YFP) (Daubner et al., 1987) downstream of the spacer sequence using the *XbaI*/*SacII* restriction sites.
To create the *Am-dsx ExC* we amplified two sequences of the *Am-dsx* minigene. Sequence 1 reaches from the end of exon 4 to the first 103 bp of exon , sequence 2 reaches from the last 98 bp of exon 5 to the beginning of exon 6. Both sequences were then cloned into a pGEMT vector (pGEMT-Kit, Promega). A third PCR product containing the ORF of the *gentamycin resistance* gene was cloned into the same pGEMT vector between the two *dsx* fragments, replacing the central part of *dsx* exon 5. Truncations of the nucleotides upstream of the SD were induced by replacing *dsx* sequence 2 by shorter PCR products gained from amplifications of *Am-dsx* minigene sequences like sequence 2 but with forward primers with distances of 80, 60, 40, 20 and 3 bp upstream of the SD.

To create the pDO *Am-dsx-mut4* plasmids we used site-directed mutagenesis (SDM). Therefor we ran a PCR over the *Am-dsx*^{mg} to induce point mutations and digested the resulting PCR products with *Dpn*I quick & dirty to remove methylated and unmutated DNA. The remaining mutated strands were then renaturated. First we indtroduced the two most downstream point mutations with the primers #1040 + #1041 over the template pDO *Am-dsx*^{mg} to create pDO *Am-dsx-mg Tra2-BS-SDmut_ZP1*. Second we indroduced two additional point mutations with the primers #1042 + #1043 over the template pDO *Am-dsx-mg Tra2-BS-SDmut_ZP1* to to create pDO *Am-dsx-mg Tra2-BS-SDmut_ZP2*. Third we introduced a fifth and sixth point mutation to create a plasmid we called pDO *Am-dsx*^{mg}*-mut6* with primers #1081 + #1082 over the template pDO *Am-dsx-mg Tra2-BS-SDmut_ZP2.*
To create pDO *Am-dsx*^{mg}*-mut4* we removed two of the six point mutations that we introduced into the *Am-dsx*^{mg} with the primers #1093 + #1094 over the themplate pDO *Am-dsx*^{mg}*-mut6*.

Cell culture and transfection

Sf21 cells were maintained in Spodopan medium (PAN Biotech, Aidenbach, Germany) supplemented with 20 µg/mL of gentamycin (Roth, Karlsruhe, Germany) following the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States). To assay *Am-dsx* minigene splicing we seeded 1 x 10^6 cells/ well in six-well plates. We transiently transfected the cells with different amounts of plasmid DNA using 5 µl Roti®-Insectofect reagent (Roth) per sample, following the procedure reported by manufacturer's instructions. 24 hours after transfection, we replaced the transfection reagent with Spodopan/gentamycin medium. Cells were harvested 72 hours after transfection. The transfection experiments were replicated at least three times.

RT-PCR analysis

Total RNA from *Sf21*cells was isolated using the TRIzol® protocol (GIBCO BRL Life Technologies, Germany). RNA was resolved in 11 µl of nuclease free water. The first strand cDNA from mRNA was generated by reverse transcription using 100 pmol oligo$(dT)_{18}$ primer and 200 U RevertAidTM Reverse Transcriptase following the protocol of the supplier (Fermentas GmbH, St. Leon-Rot, Germany). To analyze splicing patterns of *Am-dsx* minigene mRNAs transcribed from pDO *Am-dsx^{mg}*, we amplified male and female specific fragments corresponding to the female and male transcripts by three separate PCRs using three sets of oligonucleotides (male: #417/#418; female SA #417/#419; female SD #882/#418).

Semiquantitative RT-PCR was standardized in respect to the splice product of the cotransfected *hGH* control. All RT-PCR fragments were resolved by agarose gel electrophoresis (2%), stained with ethidium bromide and visualized with the UV-solo (Biometra, Göttingen, Germany). We show representative gel pictures of the three replicate RT-PCRs. Structure of splice products was determined by sequencing. The analysis of splice products was studied from three independent transfection assays.

FLIM-FRET analysis

FLIM-FRET (Fluorescence Lifetime Imaging Microscopy-Förster Resonance Energy Transfer) were used to study protein-protein interaction (Borst and Visser, 2010,

Weidtkamp-Peters et al., 2009). Multiparameter Fluorescence Image Spectroscopy (MFIS) was applied using a multiparameter fluorescence detection setup with the corresponding fluorescence lifetime analyses as described previously (Kudryavtsev et al., 2007, Weidtkamp-Peters et al., 2009). We used a CLSM (confocal laser scanning microscope, FV1000, IX81 inverted microscope, Olympus, Shinjuku, Tokio, Japan) equipped with a single-photon counting device with picosecond time resolution (Hydra Harp 400, PicoQuant, Berlin, Germany). The Cerulean fluorescent protein was excited at 440 nm using a linearly polarized, pulsed (40 MHz) diode laser (PicoQuant, Berlin, Germany) at 1 µW using a 60x water immersion objective (UPlanSApo NA 1.2, diffraction limited focus, Olympus, Shinjuku, Tokio, Japan). The fluorescence was detected by SPADs (single-photon avalanche diodes, PD5CTC, MPD, Bolzano, Italy) in a narrow range of the emission spectrum of the Cerulean protein (band-pass filter: HC480/40, AHF, Tübingen, Germany). The images were obtained with 10 µs pixel time and zoom10 at 256x256 pixels. A series of 60 frames was merged into one image and further analyzed (Widengren et al., 2006) as follows. The Cerulean fluorescence lifetime (τ) was fitted pixel-wise with a mono-exponential model (Weidtkamp-Peters et al., 2009) and then analyzed using the software tools “AnI-3SF” and “Margarita” developed by Prof. C.A.M Seidel group [Software Package for Multiparameter Fluorescence Spectroscopy, Full Correlation and Multiparameter Fluorescence Imaging (http://www.mpc.uni-duesseldorf.de/seidel)].

Expression of the fusion proteins in the *Sf21* cells was visually verified prior to analysis using the internal detectors of the CLSM.

Supplementary Data

Table S1: Sequences of oligonucleotides

pDO *fem*, pDO *Am-tra2*, pDO *hGH*	
#367	GATCACGCGTTTACATAGGTCCAAATCTAGGATTTGG
#369	GATCGGATCCATGAAACGGAATACAACAAATCATTC
#359	GATCGGATCCATGAGTGACATTGAGCGAAGTAGTAG
#421	TGACACGCGTTTAATATCGACGTGGTGAATAAGAGC
#496	GATCAAGCTTATGGCTACAGGTAAGCGCCCCT
#497	GATCTCTAGACTAGAAGCCACAGCTGCCCTCCACAG
***Apis mellifera dsx* minigene (Am-dsx^{mg})**	
#245	GATCGGGCCCGTGAAAATGTGGAAATTCTATTGGAGCACA
#246	GATCACTAGTTATTAGAATTATAATATTATTTAATATTTTTTTAAATATA
#247	GATCACTAGTACGTGATATTATTGACACTATGATCTATCTTATAAAA
#248	GATCGCGGCCGCTGAGGCTACGTATGTTTAGGAGGACC
#317	GATCCCTAGGGTGAAAATGTGGAAATTCTATTGGAG
#320	GATCCTCGAGTGAGGCTACGTATGTTTAGGAGGACC
Apis mellifera dsx* minigene (Am-dsx^{mg}) *exchange cassette	
#251	TAATACGACTCACTATAGGG
#318	GATCCTTAAGTGTCTTATCACGTGGAATATTTTATAT
#886	GATCTCCGGAAGAAGTAGTGAGACGTAT
#887	GATCCTTAAGCCCCCCAATTGCCCCCTCGCGAAAATCTCATAAATAAAATCAAGTAGGCA
#901	GATCCAATTGTTAGGTGGCGGTACTTGGGT
#925	CGAATGTTACGCAGCAGCAACGA
#941	GATCCAATTGTCAGGAAGATATATATCTATATGAATATATATC
#942	GATCCAATTGATGAATATATATCAGCTGTTTGTATTCAAC
#943	GATCCAATTGTGTATTCAACAATGTAAGATATAAAGAGG
#944	GATCCAATTGATAAAGAGGGATTTAAAGAGGTAAGTGTT
#945	GATCCAATTGGAGGTAAGTGTTATATTTGTAGAGATTAATT
#953	TTCTATAGTGTCACCTAAAT
***Apis mellifera dsx* minigene (*Am-dsx*^{mg}) with mutations in Tra2-binding sites**	
#1040	CATTGTAAGATAGAATTTAATCATCAGTAAGATATATATC
#1041	TCTTACTGATGATTAAATTCTATCTTACAATGAAGATATAATTG
#1042	ATTCCACACGTTAAGATTTGTTAC
#1043	TCTTAACGTGTGGAATAAATATAATAAATC
#1081	CATTTATAAATTAAGATTATCAAATGTTCACACGATTTA
#1082	GTGAACATTTGATAATCTTAATTTATAAATGATCACTTTC
#1093	CAAATGATCACACGATTTATTATATTTATTCAACACGTTAAGATTT
#1094	CGTGTTGAATAAATATAATAAATCGTGTGATCATTTGATAATCTTA
***Am-dsx* splicing**	
#417	CTATTGGAGCACAGTAGCAAACTTG
#418	GGCTACGTATGTTTAGGAGGACC
#419	GAAACAATTTTGTTCAAAATAGAATTCC
#882	CGTCACCCTTAACAATAATATCGAATC
***hGH* splicing**	
#465	GATCACGCGTCTAGAAGCCACAGCTGCCCTCC
#597	AGGTTTGGGGCTTCTGAATAG

Fig. S1: *Am-dsx^{mg} ExC*

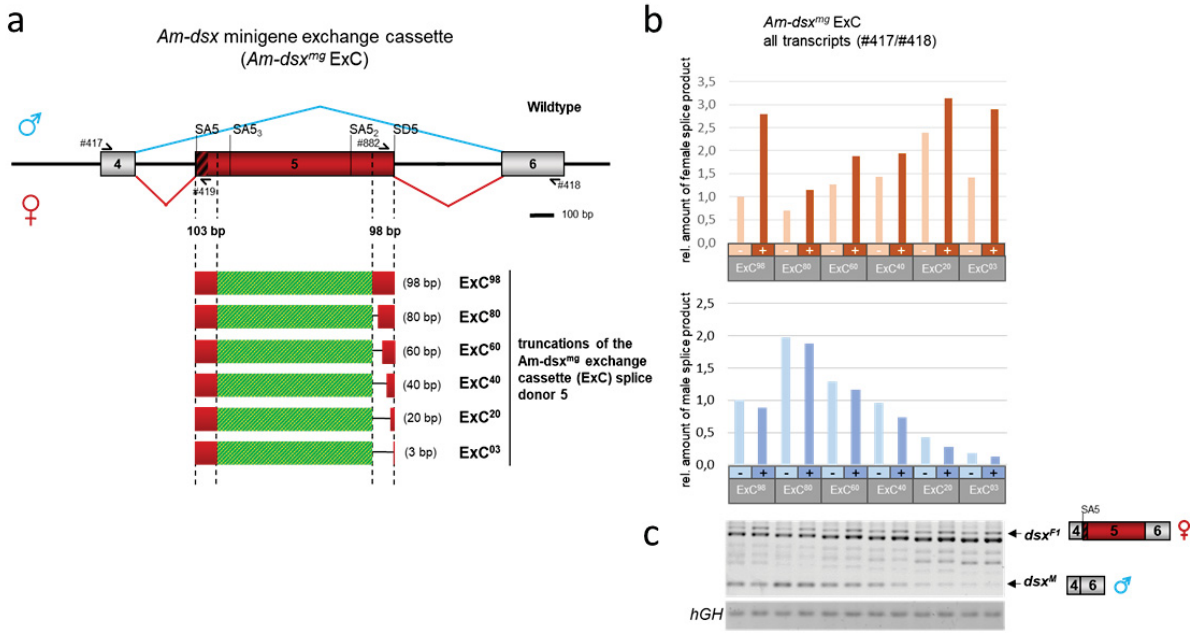

Fig. S1: (a) Schematic display of the Am-dsx^{mg} exchange cassette (Am-dsx^{mg} ExC). Central 654 bp of the female-specific exon 5 are replaced by the 534 bp coding sequence of the *gentamycin resistance* gene (yellow-green). 103 bp of the exon at the SA5 and 98 bp of the exon at the SD5 remain the *Am-dsx* sequence (*Am-dsx^{mg} ExC^{98}*). The remaining 98 bp at the SD5 are then truncated progressively in 20 bp steps (*Am-dsx^{mg} ExC^{80,} Am-dsx^{mg} ExC^{60}*, …) until there are only 3 bp of exon 5 remaining (*Am-dsx^{mg} ExC^{03}*). These clones are then analysed via semiquantitative RT-PCR for their splice products with oligonucleotide combinations, 417/#418, #417/#419 and #882/#418 as indicated in the wildtype scheme. (b) Relative amount of female (red) and male (blue) spliced product without (-) and with Fem and *Am*-Tra2 (+), based on the amount of splice product of *Am-dsx^{mg} ExC^{98}*. Values of relative amounts are arithmetic means of three transfections. (c) RT-PCR analysis of splicing variants of *pDO Am-dsx^{mg} ExC* transcripts in the presence and absence of Fem and Am-Tra2 protein in *Sf21* cells. 1x10^6 *Sf21* cells were cotransfected with 0.5 µg of *pDO hGH*, 0,5 µg of *pDO Am-dsx^{mg} ExC* and 2 µg of either *pDO fem* + *pDO Am-tra2* (+) or 2 µg of *pGL3* vector (-). Transcripts were amplified with the oligonucleotides #417/#418.

To further understand the control of exon 5 splice site activation by Fem and *Am*-Tra2 proteins, we cloned the *Am-dsx* exchange cassette (*Am-dsx^{mg} ExC*). Therefore we exchanged 654 bp in the middle of *Am-dsx* exon 5 by 534 bp of the ORF of the *gentamycin resistance* gene, whereas the first 103 bp downstream of the SA5 and the last 98 bp upstream of the SD5 remained the *Am-dsx* sequence. We then

truncated the region upstream of SD5 in steps of 20 bp to locate the splice regulating elements (*Am-dsx^{mg} ExC^{98}, ExC^{80}, ExC^{60}, ExC^{40}, ExC^{20}, ExC^{03}*), (Fig. S1a).

We co-transfected the *pDO Am-dsx^{mg} ExC* clones (*Am-dsx ExC^{98}, ExC^{80}, ExC^{60}, ExC^{40}, ExC^{20}, ExC^{03}*) in absence and presence of Fem/*Am*-Tra2, respectively, together with control plasmid *pDO hGH* into *Sf21* cells and studied the effects on SA5 and SD5 activation with respect to splicing of *hGH* control by semi-quantitative RT-PCR. To analyse the splicing pattern we used three analysis PCRs. The first PCR amplifies the region from the end of exon 4 to the beginning of exon 6 (oligonucleotides #417/#418) and therefore shows female-specifically spliced transcripts as well as male-specifically spliced ones and proofs female-specific exon inclusion. We observe that the amount of the female-specifically spliced product is increased in presence of Fem/*Am*-Tra2 in all *Am-dsx^{mg} ExC* mutants, while we observe the most prominent increase in the *Am-dsx^{mg} ExC^{98}* mutant. This increase is reduced in the mutants *Am-dsx^{mg} ExC^{80}* and further in *ExC ^{60}*. These findings suggest an Fem/*Am*-Tra2-dependent exonic splicing enhancer (ESE) element between 60 and 98 bp upstream of the SD5.
The second analysis PCR amplifies the end of exon 4 to the beginning of the female-specific exon 5 (#417/#419) and shows splice activity at the SA5. Here we also observe a Fem/*Am*-Tra2-dependent increase in the amount of the female-specifically splice transcript that is the highest in *Am-dsx^{mg} ExC^{98}*, consistent to the PCR with oligonucleotides #417/#418. Looking at the other *Am-dsx^{mg} ExC* mutants, we also observe a decrease in the Fem/*Am*-Tra2-dependent increase in the amount of the female-specifically spliced transcript at the SA5 and thus SA5-usage (*Am-dsx^{mg} ExC^{80}*).
The third analysis PCR amplifies the end of the female-specific exon 5 to the middle of exon 6 (#882/#418) and thus shows splice activity at the SD5. Here we do not observe any reduction in the Fem/*Am*-Tra2-dependent female-specific splicing, suggesting that SD5-usage is preserved by truncations of the regions next to it.

These results let us suggest that we can find splice regulating elements that enhance splicing at the female-specific SA5 in the region 60 to 98 nt upstream of SD5.

Fig. S2: HEXplorer

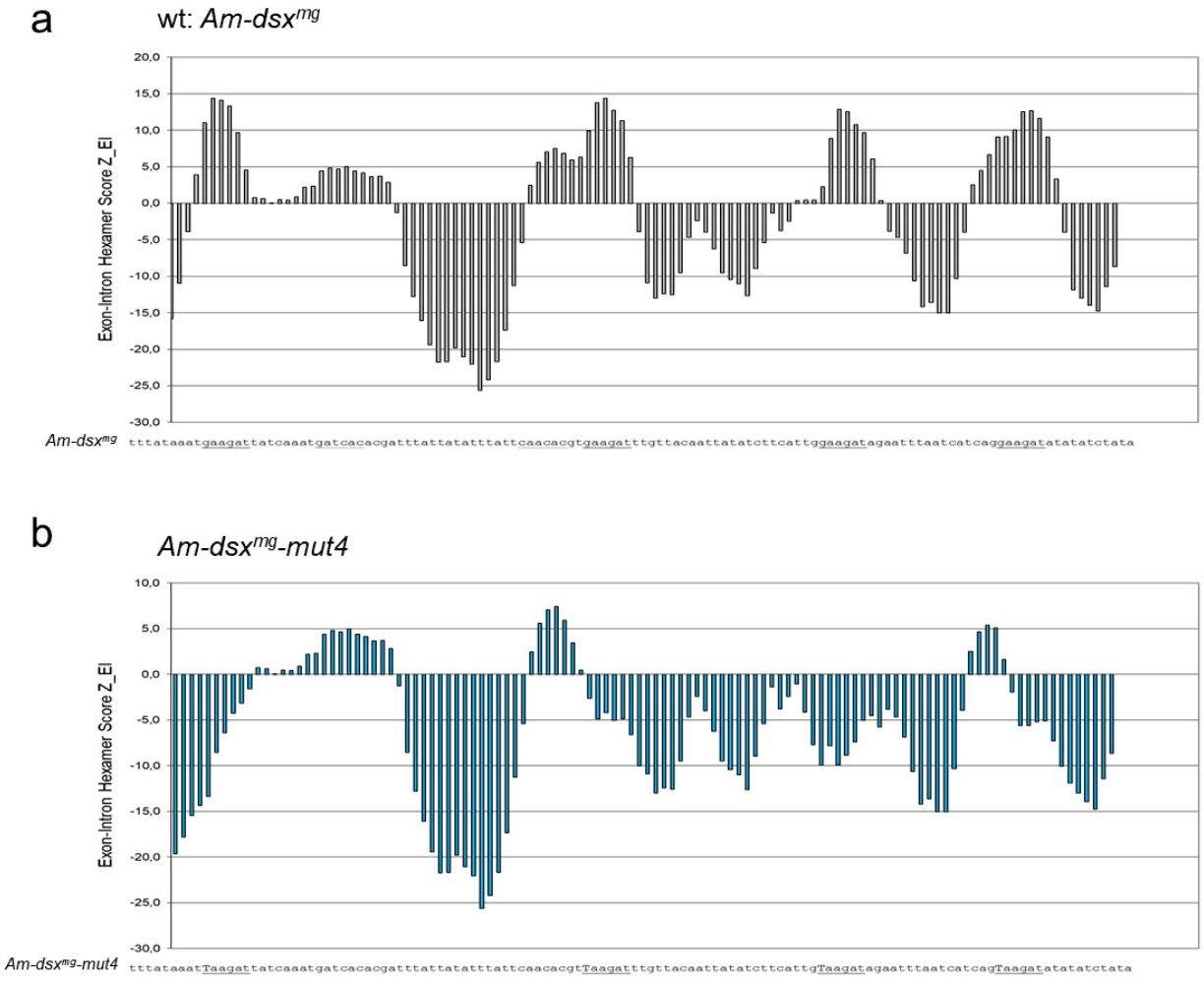

Fig. S2: Effects of sequences on their splice enhancing and silencing properties as predicted by the HEXplorer (Erkelenz et al., 2014). Sequence and HEXplorer prediction of the wildtype *Am-dsx*^{mg} (a) and *Am-dsx*^{mg}*-mut4* with four point mutations (b) of the nucleotides 54-178 upstream of the SD5. x-axis: nucleotide sequence, y-axis: HEXplorer score, every column indicates one nucleotide, underlined sequences indicate potential *Am-Tra2*-binding sites, capital lettersindicate point mutations.

Fig. S3: Sequences of *hGH, pDO* vector, OplE2 promoter, transcript *Am-dsx^{mg}-R1* and transcript *Am-dsx^{mg}-R3*.

a) Sequence of human Growth Hormon (*hGH*) fragment, cloned into *pDO* vector (underlined sequence represents intron that is spliced out in *Sf21* cells):

ATGGCTACAGGTAAGCGCCCCTAAAATCCCTTTGGGCACAATGTGTCCTGAGGGGAGA
GGCAGCGACCTGTAGATGGGACGGGGGCACTAACCCTCAGGTTTGGGGCTTCTGAATG
TGAGTATCGCCATGTAAGCCCAGTATTTGGCCAATCTCAGAAAGCTCCTGGTCCCTGGA
GGGATGGAGAGAGAAAAACAAACAGCTCCTGGAGCAGGGAGAGTGCTGGCCTCTTGCT
CTCCGGCTCCCTCTGTTGCCCTCTGGTTTCTCCCCAGGCTCCCGGACGTCCCTGCTCC
TGGCTTTTGGCCTGCTCTGCCTGCCCTGGCTTCAAGAGGGCAGTGCCTTCCCAACCATT
CCCTTATCCAGGCTTTTTGACAACGCTATGCTCCGCGCCCATCGTCTGCACCAGCTGGC
CTTTGACACCTACCAGGAGTTTGTAAGCTCTTGGGGAATGGGTGCGCATCAGGGGTGG
CAGGAAGGGGTGACTTTCCCCCGCTGGGAAATAAGAGGAGGAGACTAAGGAGCTCAG
GGTTTTTCCCGAAGCGAAAATGCAGGCAGATGAGCACACGCTGAGTGAGGTTCCCAGA
AAAGTAACAATGGGAGCTGGTCTCCAGCGTAGACCTTGGTGGGCGGTCCTTCTCCTAG
GAAGAAGCCTATATCCCAAAGGAACAGAAGTATTCATTCCTGCAGAACCCCCAGACCTC
CCTCTGTTTCTCAGAGTCTATTCCGACACCCTCCAACAGGGAGGAAACACAACAGAAAT
CCGTGAGTGGATGCCTTCTCCCCAGGCGGGGATGGGGGAGACCTGTAGTCAGAGCCC
CCGGGCAGCACAGCCAATGCCCGTCCTTCCCCTGCAGAACCTAGAGCTGCTCCGCATC
TCCCTGCTGCTCATCCAGTCGTGGCTGGAGCCCGTGCAGTTCCTCAGGAGTGTCTTCG
CCAACAGCCTGGTGTACGGCGCCTCTGACAGCAACGTCTATGACCTCCTAAAGGACCT
AGAGGAAGGCATCCAAACGCTGATGGGGGTGAGGGTGGCGCCAGGGGTCCCCAATCC
TGGAGCCCCACTGACTTTGAGAGCTGTGTTAGAGAAACACTGCTGCCCTCTTTTTAGCA
GTCAGGCCCTGACCCAAGAGAACTCACCTTATTCTTCATTTCCCCTCGTGAATCCTCCA
GGCCTTTCTCTACACCCTGAAGGGGAGGGAGGAAAATGAATGAATGAGAAAGGGAGGG
AACAGTACCCAAGCGCTTGGCCTCTCCTTCTCTTCCTTCACTTTGCAGAGGCTGGAAGA
TGGCAGCCCCCGGACTGGGCAGATCTTCAAGCAGACCTACAGCAAGTTCGACACAAAC
TCACACAACGATGACGCACTACTCAAGAACTACGGGCTGCTCTACTGCTTCAGGAAGGA
CATGGACAAGGTCGAGACATTCCTGCGCATCGTGCAGTGCCGCTCTGTGGAGGGCAGC
TGTGGCTTCTAG

b) Full sequence of *pDO* vector:

GGATCATGATGATAAACAATGTATGGTGCTAATGTTGCTTCAACAACAATTCTGTTGAAC
TGTGTTTTCATGTTTGCCAACAAGCACCTTTATACTCGGTGGCCTCCCCACCACCAACTT
TTTTGCACTGCAAAAAAACACGCTTTTGCACGCGGGCCCATACATAGTACAAACTCTAC
GTTTCGTAGACTATTTTACATAAATAGTCTACACCGTTGTATACGCTCCAAATACACTACC
ACACATTGAACCTTTTTGCAGTGCAAAAAAGTACGTGTCGGCAGTCACGTAGGCCGGCC
TTATCGGGTCGCGTCCTGTCACGTACGAATCACATTATCGGACCGGACGAGTGTTGTCT
TATCGTGACAGGACGCCAGCTTCCTGTGTTGCTAACCGCAGCCGGACGCAACTCCTTAT
CGGAACAGGACGCGCCTCCATATCAGCCGCGCGTTATCTCATGCGCGTGACCGGACAC
GAGGCGCCCGTCCCGCTTATCGCGCCTATAAATACAGCCCGCAACGATCTGGTAAACA
CAGTTGAACAGCATCTGTTCGAATTTAAAGCTTGGTACCGAGCTCGGATCCACTAGTCC
AGTGTGGTGGAATTCATGGCCTCCTCCGAGGATGTCATCAAAGAGTTTATGAGATTTAA
GGTCAAGATGGAGGGAAGCGTCAACGGACACGAGTTCGAGATTGAGGGAGAAGGAGA
AGGCCGGCCTTACGAGGGCACACAAACCGCTAAGCTCAAGGTCACAAAAGGAGGACCC
CTCCCCTTCTCCTGGGATATTCTGAGCCCTCAGTTCCAGTACGGAAGCAAAGCCTATGT
TAAACACCCTGCCGACATCCCTGACTATCTGAAGCTCTCCTTCCCTGAAGGCTTCAAGT
GGGAGAGATTCATGAACTTCGAGGACGGAGGCGTGGTGACAGTCACACAAGATAGCAC
CCTCCAGGACGGAGAGTTTATTTATAAGGTGAAACTCAGAGGAACCAACTTCCCCTCCG

ATGGCCCTGTCATGCAAAAAAAAACAATGGGATGGGAAGCCTCCACCGAGAGAATGTAT
CCTGAGGATGGCGCTCTGAAAGGCGAAATTAAAATGAGACTGAAACTCAAAGACGGAG
GACACTACGATGCCGAGGTCAAAACAACCTACAAGGCCAAGAAACAAGTGCAGCTGCC
TGGCGCCTACATGACTGATATTAAACTCGACATTATCAGCCATAATGGGGACTACACCAT
CGTGGAACAATATGAGAGAGCTGAGGGCAGACATAGCACAGGCGCTGGATCCGCGGC
CGCGAGCTCACTAGTCATATGTTCTAGAATGGTGAGCAAGGGCGAGGAGCTGTTCACC
GGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGC
GTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATC
TGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACG
GCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTC
CGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAAC
TACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA
ACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTG
AACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGA
GCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCT
GGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAACCG
CGGTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGG
TCATCATCACCATCACCATTGAGTTTATCTGACTAAATCTTAGTTTGTATTGTCATGTTTT
AATACAATATGTTATGTTTAAATATGTTTTTAATAAATTTTATAAAATAATTTCAACTTTTAT
TGTAACAACATTGTCCATTTACACACTCCTTTCAAGCGCGTGGGATCGATGCTCACTCAA
AGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGC
AAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCAT
AGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAA
ACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTC
TCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCG
TGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTA
ACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACT
GGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGT
GGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCA
GTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAG
CGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAG
ATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
ATTTTGGTCATGATGATAAACAATGTATGGTGCTAATGTTGCTTCAACAACAATTCTGTTG
AACTGTGTTTTCATGTTTGCCAACAAGCACCTTTATACTCGGTGGCCTCCCCACCACCAA
CTTTTTTGCACTGCAAAAAAACACGCTTTTGCACGCGGGCCCATACATAGTACAAACTCT
ACGTTTCGTAGACTATTTTACATAAATAGTCTACACCGTTGTATACGCTCCAAATACACTA
CCACACATTGAACCTTTTTGCAGTGCAAAAAAGTACGTGTCGGCAGTCACGTAGGCCGG
CCTTATCGGGTCGCGTCCTGTCACGTACGAATCACATTATCGGACCGGACGAGTGTTGT
CTTATCGTGACAGGACGCCAGCTTCCTGTGTTGCTAACCGCAGCCGGACGCAACTCCTT
ATCGGAACAGGACGCGCCTCCATATCAGCCGCGCGTTATCTCATGCGCGTGACCGGAC
ACGAGGCGCCCGTCCCGCTTATCGCGCCTATAAATACAGCCCGCAACGATCTGGTAAA
CACAGTTGAACAGCATCTGTTCGAATTCGTTCAGCACCTAGGAGCAGCCTTAAGAGCAG
CCTCGAGATCTTAGTTTGTATTGTCATGTTTTAATACAATATGTTATGTTTAAATATGTTTT
TAATAAATTTTATAAAATAATTTCAACTTTTATTGTAACAACATTGTCCATTTACACACTCC
TTTCAAGCGCGTGGCCGGGCTGCAGCACGTGTTGACAATTAATCATCGGCATAGTATAT
CGGCATAGTATAATACGACTCACTATAGGAGGGCCACCATGGCCAAGTTGACCAGTGC
CGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCG
GCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGA

CGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGC
CTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTC
CACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTG
GGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGA
GGAGCAGGACTGACCGACGCCGACCAACACCGCCGGTCCGACGGCGGCCCACGGGT
CCCAGGGGGGTCGACCTCGAAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGC
AATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGT
CCAAACTCATCAATGTATCTTATCATGTCT

c) Sequence of *Am-dsx* transcript *Am-dsx^{mg}-R1* (underlined and not-underlined sequence represents exon junctions)

GTGAAAATGTGGAAATTCTATTGGAGCACAGTAGCAAACTTGTAGAACTTTTCCAATATC
CTTGGGAAGCACTGTTATTGATGTACATCAATTTAAAATATGCAGGGGCTAATCCGGAAG
AAGTAGTGAGACGTATGGTTGATGAAAGTGATCATTTATAAATGAAGATTATCAAATGAT
CACACGATTTATTATATTTATTCAACACGTGAAGATTTGTTACAATTATATCTTCATTGGAA
GATAGAATTTAATCATCAGGAAGATATATATCTATATGAATATATATCAGCTGTTTGTATT
CAACAATGTAAGATATAAAGAGGGATTTAAAGAGCTAGCAACGAAATCCGTAACATGCA
CTTCTTGAAAGCAATCAGAATGTCCCAACCTAGTCGTGCGTTCCGCTGCACCGCGGCAT
GTGCCGCACCCACGGGGCCGCCAACGGGACCTCCGACATACGAGGGTGATGTGCCCT
TCATAGGGGTTGGACCACCCCCGAATCCTATCCATTTCAGGCCGTTTCTTCATCCCGAG
AATGCTCATATACCGGCTACCAGACTACCGTCCAGCCCAGATGGTCCTCCTAAACATAC
GTAGCCTCA

d) Sequence of *Am-dsx* transcript *Am-dsx^{mg}-R3* (underlined and not-underlined sequence represents exon junctions)

GTGAAAATGTGGAAATTCTATTGGAGCACAGTAGCAAACTTGTAGAACTTTTCCAATATC
CTTGGGAAGCACTGTTATTGATGTACATCAATTTAAAATATGCAGGGGCTAATCCGGAAG
AAGTAGTGAGACGTATGGTTGATGAATTATATTGAAGAAAGATTCTAATATAACTTCATTA
TTAATGAACCGATTATTCTTATATTTTTGTTGGAAAAATGACAAGAGTAAATTTAATTTTGA
CGGTTTAATGATTAAAAGATAGATTTCTTTGTCGTGAAATAGTTGGCGCATGTGGATAAC
GAAATTGGCAACAACATTCATATTTTAACAATATGTAATAAATTTTCTTTATATATCATGCA
CTTTTATTAAAGTAAAATGATGAAATTGTAGAACATTAATAAATTAAATTTTTTTGATAGTA
TATAAATCAAAAATTATATTACATATTATTCCAAAGAACTATTTCTATTATTCGGATTTTATT
TCTGATAAAACATTATAAAATACAATGGTGCACTAAGTTTCTCGAAAATAAATTTAGAGTC
GAGGACACAGGTGTTAAAACCATTACAATAAGATTTATGCTTCCAGTATATATTTGTTCAT
GTCAATTTCGTTGTATTCGTCACCCTTAACAATAATATCGAATCAAAATTGCAGAAAGTGA
TCATTTATAAATGAAGATTATCAAATGATCACACGATTTATTATATTTATTCAACACGTGAA
GATTTGTTACAATTATATCTTCATTGGAAGATAGAATTTAATCATCAGGAAGATATATATC
TATATGAATATATATCAGCTGTTTGTATTCAACAATGTAAGATATAAAGAGGGATTTAAAG
AGCTAGCAACGAAATCCGTAACATGCACTTCTTGAAAGCAATCAGAATGTCCCAACCTA
GTCGTGCGTTCCGCTGCACCGCGGCATGTGCCGCACCCACGGGGCCGCCAACGGGAC
CTCCGACATACGAGGGTGATGTGCCCTTCATAGGGGTTGGACCACCCCCGAATCCTAT
CCATTTCAGGCCGTTTCTTCATCCCGAGAATGCTCATATACCGGCTACCAGACTACCGT
CCAGCCCAGATGGTCCTCCTAAACATACGTAGCCTCA

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Fem and *Am*-Tra2 proteins direct female specific splicing of the *Am-dsx* pre-mRNA by activation of the female splice donor site in the honeybee *Apis mellifera*

Journal: Manuscript in preparation for submission to "Scientific Reports"

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Author's contribution: Manuskript I

• Vector cloning (pDO *Am-dsx^{mg} ExC^{98},* pDO *Am-dsx^{mg} ExC^{80},* pDO *Am-dsx^{mg} ExC^{40},* pDO *Am-dsx^{mg} ExC^{03}*, pDO *Am-dsx-mut4)*

• Primer design for analysis of SD5-usage

• Implementation of laboratory experiments

• Molecular evolutionary sequence analyses (comparison of sequence elements of splice products involved in female exon splicing between *Apis mellifera*, *Drosophila melanogaster, Musca domestica, Ceratitis capitata* and *Bactrocera oleae*)

• Authoring the manuscript (concerning exchange cassette, HEXplorer and *Am-dsx-mut4*; arranging of text elements, figures and supplementary figures; revision of references)

Kennzeichnung der Autorenschaft einzelner Textelemente und Abbildungen:

Textelemente:

Titel (S. 27):
Inga Nissen

Abstract (S. 28):
Inga Nissen: S. 28, Zeile 1- 18 und 23-25
Annika Roth: S. 28, Zeile 18-23

Introduction (S. 29-31)
Inga Nissen: S. 29, Zeile 1 – S. 31, Zeile 19, sprachliche und inhaltliche Änderungen von Annika Roth
Annika Roth: S. 31, Zeile 19-24

Results (S. 31-38)
Inga Nissen: S. 31, Zeile 27 – S. 35, Zeile 19

Björn Schmitt: S. 35, Zeile 21 – S. 36, Zeile 19
Annika Roth: S. 37, Zeile 17 – S. 38, Zeile 28 (Überschrift und Einleitungssatz verändert übernommen von Inga Nissen)

Discussion (S. 38-42)
Inga Nissen: S. 38, Zeile 31 – S. 40, Zeile 33 und S. 41, Zeile 16 – S. 42, Zeile 13 und S. 42, Zeile 18-23, sprachliche und inhaltliche Änderungen in allen Abschnitten von Annika Roth
Annika Roth: S. 41, Zeile 1-15 und S. 42, Zeile 14-17

Materials and Methods (S. 42-46)
Inga Nissen: S. 28, Zeile 26 – S. 43, Zeile 32 und S. 45, Zeile 1-27
Björn Schmitt: S. 43, Zeile 33 – S.44, Zeile 9 und S. 45, 29 – S. 46, Zeile 22
Annika Roth: S. 44, Zeile 10-33

Supplementary Data (S. 47-53)
Annika Roth: Text S. 48 - 49 (Kennzeichnungen zu Abbildungen, Tabellen und Bildunterschriften s.u.)

Abbildungen:

Fig. 1 (S. 33)
Inga Nissen, Darstellung der Spleißprodukte verändert von Annika Roth

Fig. 2 (S. 36)
Inga Nissen, Darstellung der Spleißprodukte verändert von Annika Roth

Fig. 3 (S. 37)
Björn Schmitt

Fig. 4 (S. 39)
Annika Roth

Table S1 (S. 47)
Inga Nissen: Oligonukleotidsequenzen #367, #369, #359, #421, #496, #497, #245, #246, #247, #248, #317, #320, #417, #418, #419, #465, #597
Annika Roth: Oligonukleotidsequenzen #251, #318, #886, #887, #901, #925, #941, #942, #943, #944, #945, #953, #1040, #1041, #1042, #1043, #1081, #1082, #1093, #1094, #882

Fig. S1 (S. 48)
Annika Roth: Teil a
Oksana Netschitailo: Teil b und c

Fig. S2 (S. 50)
Annika Roth (Daten von Oksana Netschitailo)

Fig. S3 (S. 51-53)
Inga Nissen

Manuskript II

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 sizereduced reproductive organs irrespective of the sexual morphology of the organ tissue. In contrast, *feminizer* (*fem*) mutants lost the response to worker nutrition-driven size control. The first morphological worker mutants in honeybees demonstrate that the response to nutrition relies on a genetic program that is switched “ON” by the fem gene. Thus, the genetic instruction provided by the *fem* gene provides an entry point to genetically dissect the underlying processes that implement the size polyphenism.

Author summary

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

Introduction

Highly social insects are characterized by caste dimorphism, with morphologically and physiologically distinct reproductive queens and more or less sterile workers (Evans and Wheeler, 2001, Simpson et al., 2011, Trible and Kronauer, 2017). In honeybees, the development of two distinct phenotypes is controlled by different nutrition, and it is a prominent example of developmental plasticity and polyphenism (Maleszka, 2018, West-Eberhard, 2003).

One major concern for the study of caste development involves explaining how a usually sterile worker and a queen that lays up to 2,000 eggs per day develop from different diet and feeding regimens (Maleszka, 2018, Corona et al., 2016, Buttstedt et al., 2016). 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]) (Haydak, 1970, Asencot and Lensky, 1988, Asencot and Lensky, 1976, Leimar et al., 2012). WJ and RJ drive the development of female larvae in two distinct morphs. Workers have a five-day longer developmental time, lower body mass, two small ovaries containing few ovarioles, and mid- and hind-leg structures adapted for pollen collection and transport. Queens have a five-day shorter developmental time, larger body mass, and two large ovaries that contain many more ovarioles, and they lack the pollen collection structures on the legs.

Two types of models have been proposed to explain how diets and feeding regimens mediate worker/queen development. The Nutrition/Growth model suggests that queen/worker development is driven by the amount of food and balance of nutrition (Buttstedt et al., 2016, Leimar et al., 2012, Rembold and Lackner, 1981), which modulate a developmental program. Queen-destined larvae have abundant nutrition, and organ growth is only limited by the intrinsic program. Worker-destined larvae have a shortage of nutrition that restricts growth and influences metabolic parameters accordingly. In contrast, the Instruction model proposes that the RJ has a compound (or compounds) that instruct the development of queens (von Rhein, 1933, Rembold et al., 1974, Kamakura, 2011). In support of the Instruction model, research over the past decades has attempted to identify a single compound from RJ (Rembold and Lackner, 1981, Rembold et al., 1974) that can determine queen development.

A recent study provided evidence that the protein royalactin has queen-determining activity (Kamakura, 2011). However, follow-up experiments in another laboratory

were unable to repeat these results (Buttstedt et al., 2016), questioning the existence of a single determinant for queen development (Maleszka, 2018). Gradually increasing the sugar levels of WJ and altering the composition of RJ-containing diets produced workers, intercastes, and eventually queens (Asencot and Lensky, 1988, Asencot and Lensky, 1976, Leimar et al., 2012, Kaftanoglu et al., 2011), but it failed to rear only queens. The more continuous caste characteristics resulting from different feeding regimes (Nijhout, 2003) have been proposed in support of the Nutrition/Growth model. The RJ and the WJ produce different reaction norms of the general developmental program that determines the caste polyphenism. An alternative explanation is that the essential higher sugar levels for queen-destined larvae are a secondary effect and reflect the higher energy requirements for the faster and larger-growing queen organs of an otherwise instructed queen program. The rearing of larvae at day 5 in 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 (Dedej et al., 1998), suggesting two distinct states of the developmental program and the possible existence of regulatory switches (Gempe et al., 2009).

One possible mechanism by which nutrients are sensed by bee larvae is the insulin/IGF signaling (IIS) and target of rapamycin (TOR) pathways, which link the abundance of nutrition with worker and queen differential gene expression (Patel et al., 2007, Wolschin et al., 2011, Wheeler et al., 2006, Wang et al., 2013). Indeed, nutritional input can also influence growth and metabolic programs via the IIS and TOR pathways in mammals and other insects (Ikeya et al., 2002, Colombani et al., 2003, Slaidina et al., 2009). However, whether regulation of the IIS and TOR pathways drives caste differentiation or whether the regulation is a response to the activation of a queen 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 (Barchuk et al., 2007, Cameron et al., 2013). Chromatin modifications and DNA methylation analyses have indicated distinct epigenetic states in worker- and queen-destined larvae, suggesting another level of regulatory control associated with caste-specific gene expression (Wojciechowski et al., 2018, Foret et al., 2012, Kucharski et al., 2008).

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

Results

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

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

We manually reared genetic females and males on worker nutrition (Kaftanoglu et al., 2011, Kaftanoglu et al., 2010) 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 (Buttstedt et al., 2016); see Fig 4A and 4B as an example of a queen phenotype). This result indicates that our manual feeding regime mirrors the effect of a worker diet in the hive (Kaftanoglu et al., 2011, Kaftanoglu et al., 2010). To examine whether only the balance and amount of nutrition (low amount of sugar) determine small reproductive organs, we reared genetic male larvae on

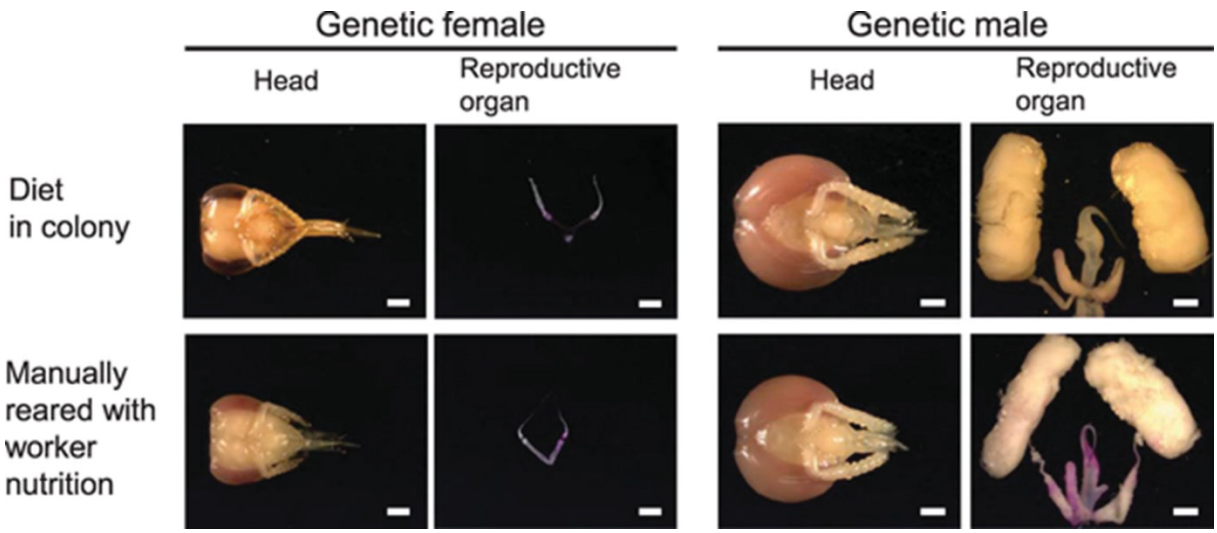


Fig 1. Reproductive organ and head phenotypes of females and males reared on worker nutrition in the laboratory and in the colony. Scale bar = 1 mm.

worker nutrition in the laboratory and compared these with males that received high amounts of sugar in the colony (Mandla and Kumar, 2016). 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 (Wiedenheft et al., 2012, Jinek et al., 2012, Kohno et al., 2016). 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

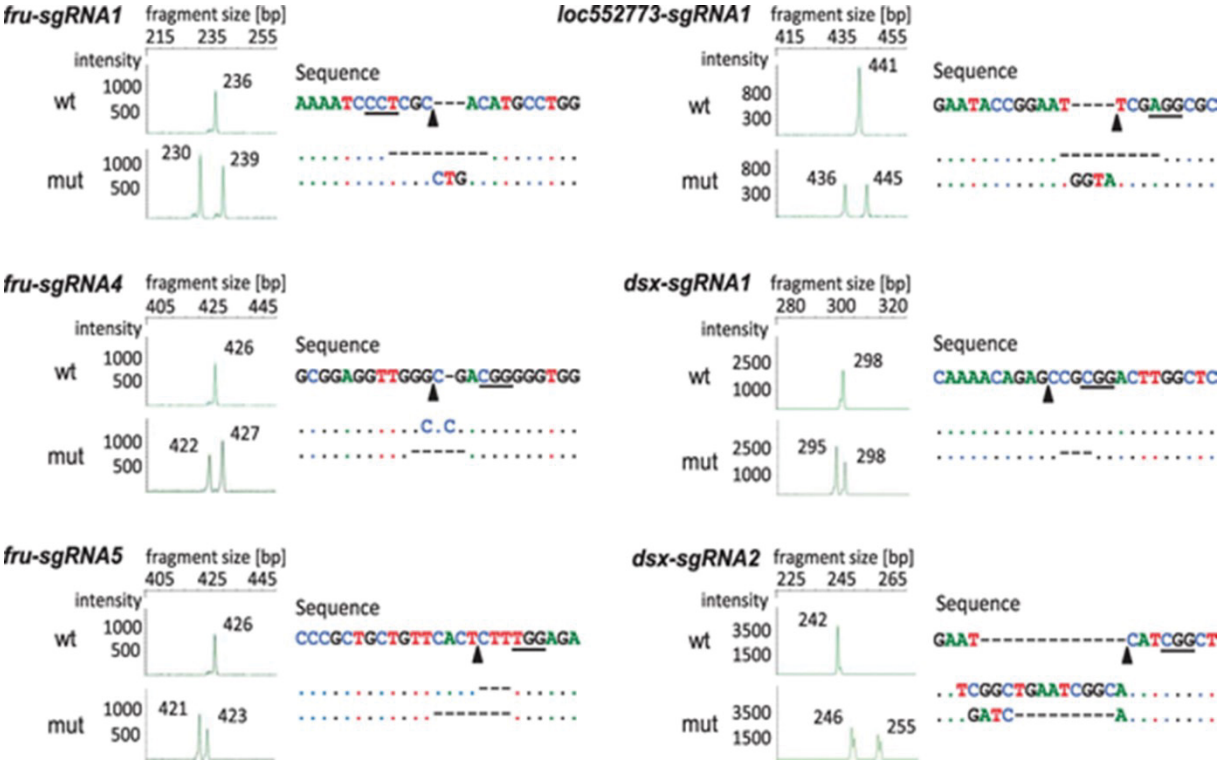


Fig 2. Examples of FL and nucleotide sequence analyses of the targeted genomic sites of single bees using the efficient CRISPR/Cas9 method. FL analysis is presented on the left, and the nucleotide sequences are presented on the right for single bees. Examples of WT alleles and mutated sequences are shown. 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.

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) (Schulte et al., 2014). 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 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

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 non-treated (wild-type) 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 protein9: FL, fragment length; pg, picogram; sgRNA, single guided RNA; WT, wild type

(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

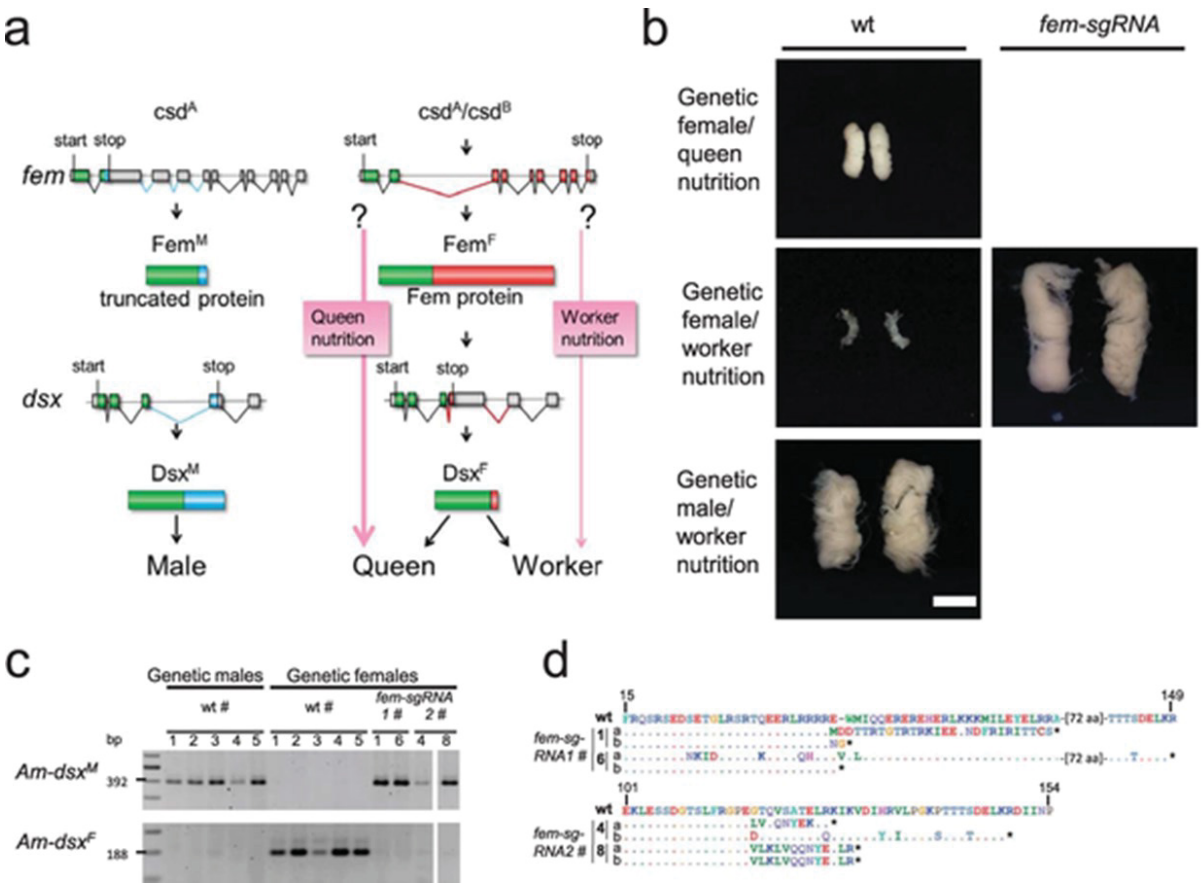


Fig 3. Size polyphenism of gonads in genetic females at larval stage 5 that were double mutants for thefemgene. (a) Model of the known components of the sexdetermining pathway in honeybees with nutritional differences in females. (b) Gonad development at larval stage 5. (Right) A pair of large gonads (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 (Nissen et al., 2012), 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.

development, as revealed from *fem* interference RNA (RNAi) knockdown and mosaic studies using a non–worker-specific diet for bee rearing (Gempe et al., 2009,

Hasselmann et al., 2008). The Fem protein is encoded by female-specific spliced *fem* transcripts but not the male spliced variant, which harbors an early stop codon (Gempe et al., 2009) (Fig 3A). The female splicing of *fem* is directed by the c*omplementary sex determiner* (*csd*) gene when the genotype is heterozygous (Fig 3A) (Beye et al., 2003). 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 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 (Cline et al., 1996, Williams and Carroll, 2009), and it controls at least reproductive organ development in other insects that belong to different insect orders, including hymenopteran insects (Hediger et al., 2004, Suzuki et al., 2005, Shukla and Palli, 2012, Mine et al., 2017). The *dsx* transcripts in honeybees are sex-specifically

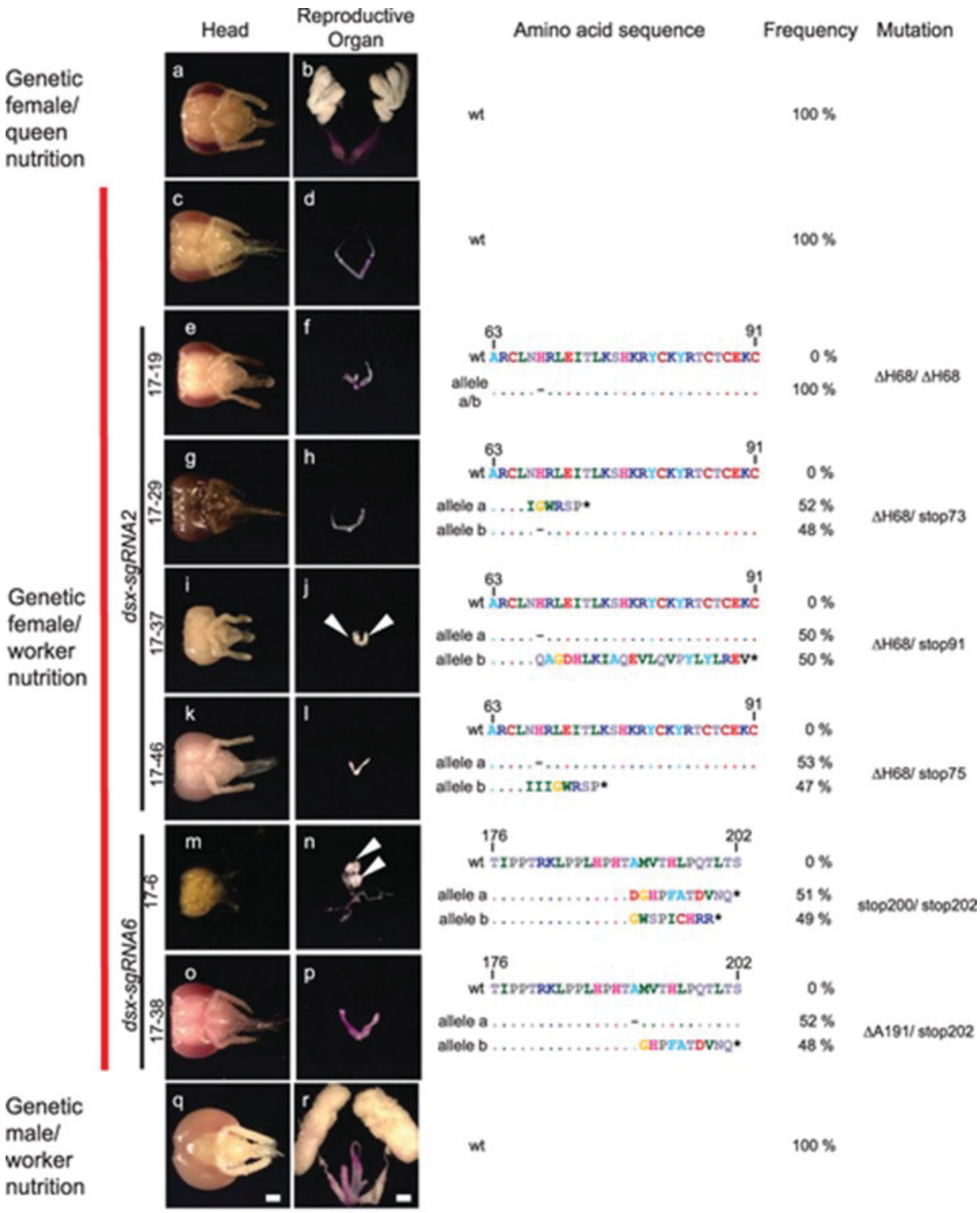


Fig 4. Size polyphenism of the reproductive organs in genetic female double mutants for the *dsx* gene. Pictures of the head and internal reproductive organs of mutated and WT control bees are shown on the left, while the genotypes at the *dsx* locus with the deduced amino acid sequences are displayed 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.

spliced by the presence of the Fem protein in females and the absence of the Fem protein in males (Gempe et al., 2009) (Fig 3A). The sexual splice variants encode a transcription factor with an intertwined zinc-containing DNA binding (DM) domain and male- and female-specific termini at the carboxyl end (Matson and Zarkower, 2012, Zhu et al., 2000, Cristino et al., 2006, Dearden et al., 2006, Cho et al., 2007). 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 (Zhu et al., 2000, Murphy et al., 2015) 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

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[1)]	
					< 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 [2)]	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%)

1) Length between the fused left and right part of the reproductive organ to its end in the sagittal plane.
Abbreviation: WT, wild type.

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 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 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 (Figs44 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 (Hediger et al., 2004, Hildreth, 1965, Xu et al., 2017). Thus, the results together suggest that the *fem* gene is required for the small size polyphenism. We conclude that the *fem* gene must be switched “ON” so that size polyphenism can be executed (Fig 5B). The essential role of the *fem* gene in small size polyphenism assigns a further key function to the *fem* gene. Previous studies demonstrated that the *fem* gene is also required to (i) induce entire female development in response to the primary signal *csd* (Gempe et al., 2009, Hasselmann et al., 2008) and to (ii) maintain the female signal during development via a positive regulatory feedback loop (Gempe et al., 2009). Whether *fem* also instructs the large size polyphenism of queens needs further functional testing once a queen-only rearing protocol has been developed for the laboratory (Buttstedt et al., 2016).

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

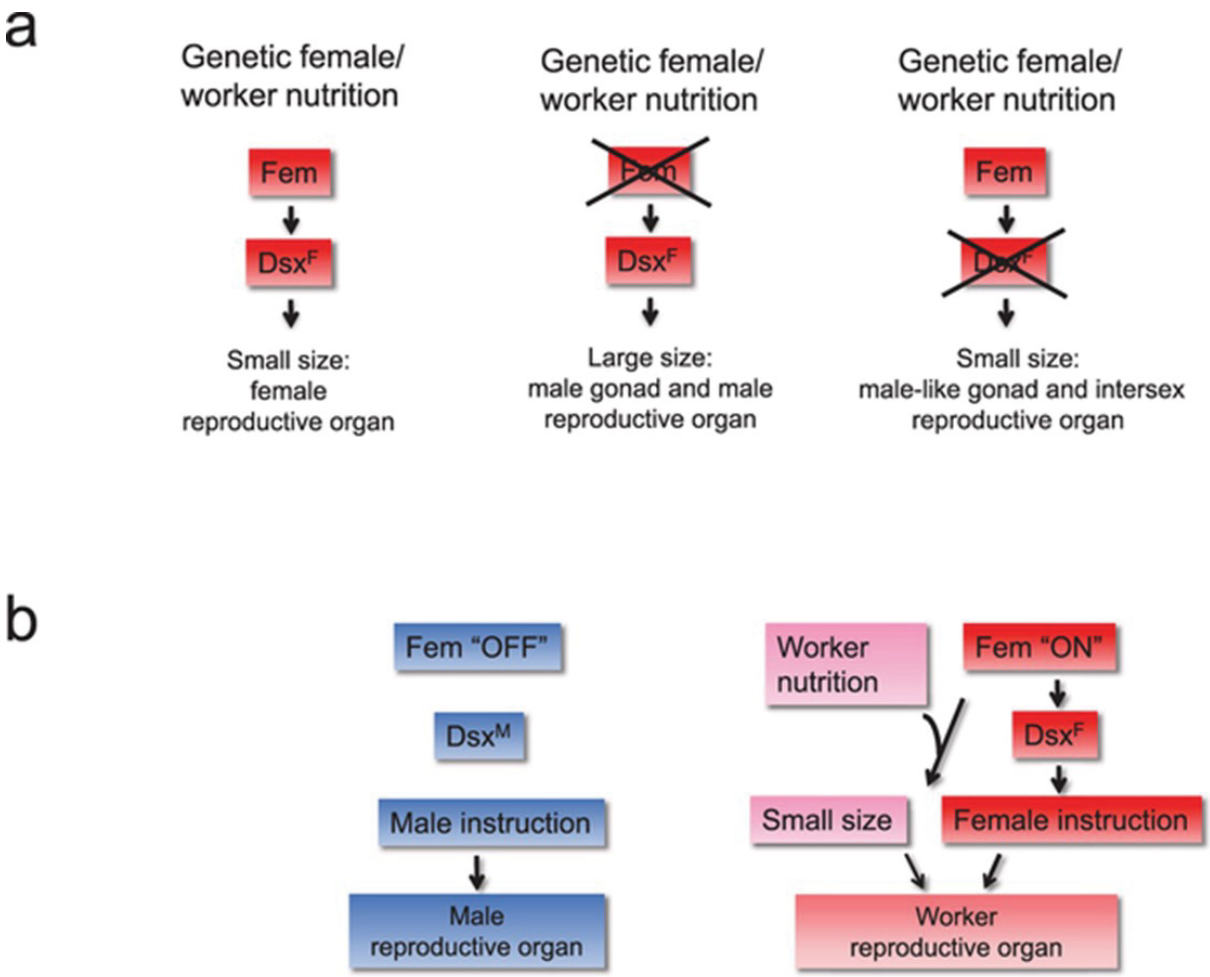


Fig 5. The role of the sex-determining genes *fem* and *dsx* in size polyphenism. (a) Schematic presentation of the mutant effects of *fem* and *dsx* gene on size polyphenism. Genetic female bees reared on worker nutrition produce only small reproductive organs. Genetic females with a mutant *fem* gene 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.

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” (Hildreth, 1965, Schüpbach, 1982, Bownes et al., 1983). The internal duct system can develop into a mixture of female/male or single poorly differentiated ducts (Hildreth, 1965). RNAi-mediated knockdown studies on the beetle *Tribolium molitor*, housefly *Musca domestica*, and sawfly *Athalia rosae*, as well as conditional expression and CRISPR/Cas9 experiments on the silkworm *Bombyx mori*, have revealed sex-related effects on internal reproductive organ development (Hediger et al., 2004, Suzuki et al., 2005, Shukla and Palli, 2012, Mine et al., 2017, Matson and Zarkower, 2012, Xu et al., 2017). Our results support a conserved role for *dsx* in the sexual development of the reproductive organ. However, in honeybees there is a nutrition-driven size control of reproductive organ development that operates upstream of or in parallel with *dsx*-regulated sexual development.

The first CRISPR/Cas9-induced morphological mutants in honeybees introduced a new genetic screening method for worker bees. We efficiently induced mutations in injected embryos using the CRISPR/Cas9 method (Wiedenheft et al., 2012, Jinek et al., 2012) and directly screened for somatic mutations in the reared honeybees (somatic mutation approach). Up to 100% of the embryos were mutated, and mosaicism among the mutated embryos was rare (<10%). The previous studies in honeybees using CRISPR/Cas9-induced mutations report on 1 out of 2 queens with only 12% and 2 out of 4 queens with only 5% and 10% mutant drone offspring, suggesting that the previously published method has a substantial lower rate and produced strong mosaicism in the queens (Kohno et al., 2016, Kohno and Kubo, 2018). These previous studies generated no worker bees that would require further crossing experiments. With very early embryonic injections (Schulte et al., 2014) and a selection step to identify the most efficient sgRNAs and Cas9 concentrations, we generated mutation rates of up to 100% and no mosaicism in worker bees directly. The rearing of the mutated embryos to worker bees was performed under controlled conditions in the laboratory (Kaftanoglu et al., 2011, Kaftanoglu et al., 2010). 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 (Figs 3 and 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 Figs 2, 3D and 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 (Zhang and Reed, 2016, Mazo-Vargas et al., 2017), the adults were genetic mosaics in which parts of the butterfly wing were WT while other parts were mutated. Enhancing the efficiency of mutagenesis can thus provide an opportunity for somatically testing gene functions in insects that are not yet genetically trackable.

Methods

sgRNA and mRNA syntheses

Cas9 mRNA was synthesized from the *Cas9* gene (Hwang et al., 2013a) (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 (Gempe et al., 2009, Schulte et al., 2014, Beye et al., 2002) using 53-mm injection pipettes (Hilgenberg, Malsfeld, Germany). Cas9 mRNA or protein (New England Biolabs, Ipswich, MA) was

applied at 400 to 2,000 ng/µl and mixed with sgRNAs using a molar ratio of 1:2 to 1:0.75. The number of injected embryos that hatch can vary greatly between experiments and sgRNAs (5% to 40%). Rearing was performed using a mass rearing technique for the worker bees (Kaftanoglu et al., 2011, Kaftanoglu et al., 2010). Freshly hatched larvae were provisioned only once with the worker larval diet (50%–53% RJ, 4% glucose, 8% fructose, 1% yeast extract, and 30%–34% water), approximately 120 to 170 mg of which was consumed (Kaftanoglu et al., 2011, Kaftanoglu et al., 2010). The larvae were incubated at 34°C and 90% humidity until the larval stage 5 or to adults. For pupal rearing we also used a slightly different diet for larvae at stage 5 (50 mg diet 2 [50% RJ, 12% fructose, 6% glucose, 2% yeast extract, and 30% water]).

DNA preparation, RNA isolation, and cDNA synthesis

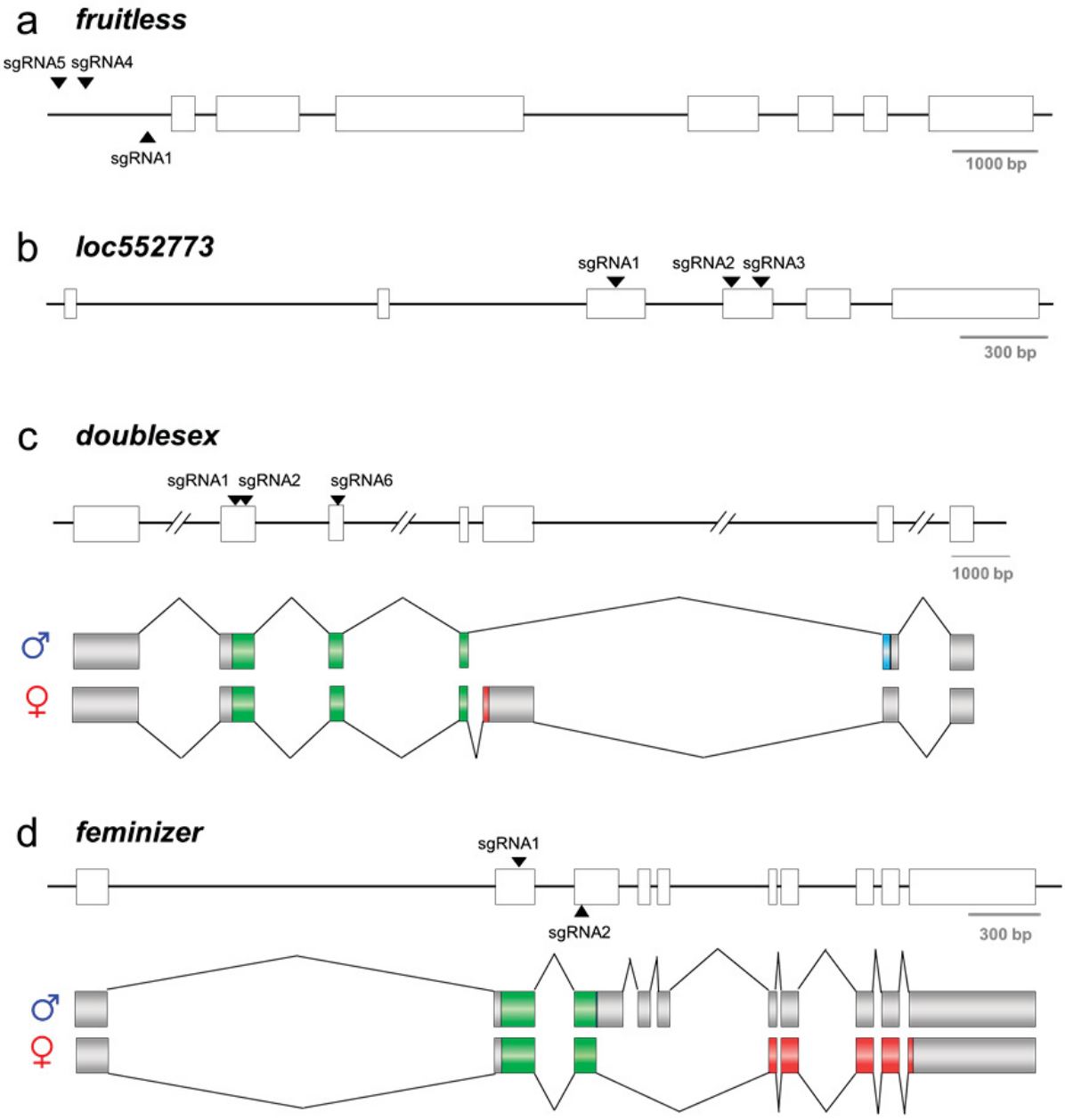
For genotyping, genomic DNA was isolated from freshly hatched L1 or L5 larvae (Hunt and Page, 1995) using the peqGOLD Tissue DNA Mini Kit (VWR, Darmstadt, Germany). RNA was isolated using the TRIZOL method (Thermo Scientific, Braunschweig, Germany), and cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Second-strand cDNA synthesis was performed by adding 10 µl of 10× DNA Polymerase Buffer, 40 U DNA Polymerase I, 0.8 U Ribonuclease H, and 65.68 µl of 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 (Hasselmann and Beye, 2004) and GoTaq polymerase (Promega). Oligonucleotide sequences were synthesized at Eurofins (Ebersberg, Germany). Amplicons were either cloned and sequenced (Sanger sequencing [Eurofins]) or sequenced via NGS. NGS index PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA), and purification of the Index PCR products was performed using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). NGS was performed on an Illumina MiSeq

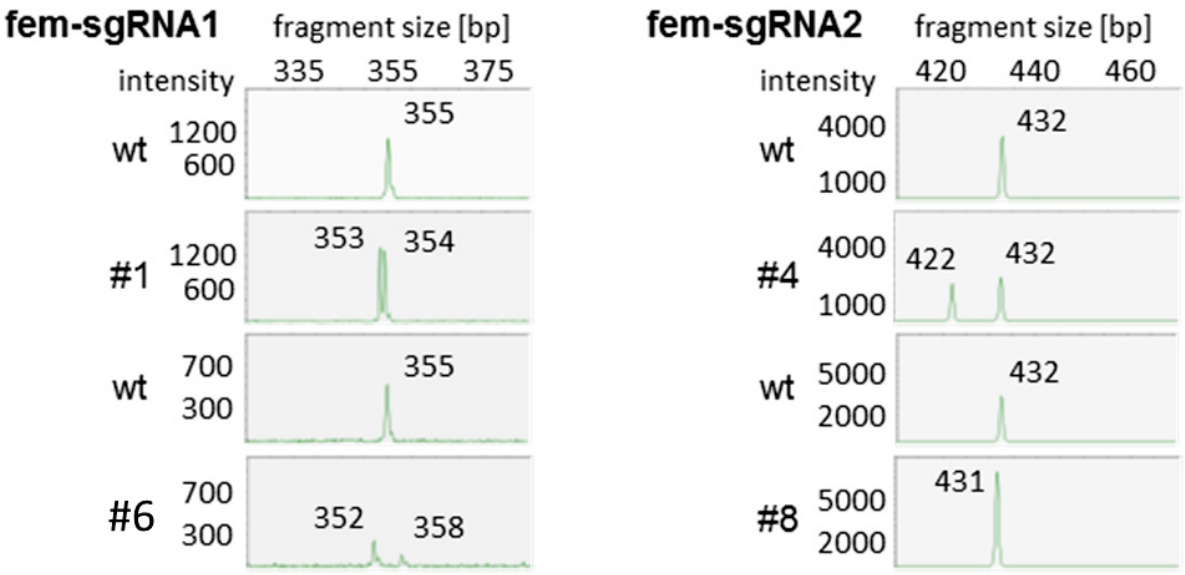
system using the MiSeq Reagent Kit version 2 (500 cycles; Illumina), generating 800,000 paired-end reads with a read length of 2 × 250 bp, resulting in approximately 15,000 paired-end reads per sample. We removed contamination by removing sequences that were less frequent than 5%. The FLs of hexachlorofluorescein (HEX)-labeled amplicons were determined using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and Peak Scanner software (Thermo Scientific). For the *fem* mutants, we conducted fragment and sequence analysis on the amplicons of the cDNAs to ensure that the many *fem*-related sequences observed at the genomic *fem* locus (derived from duplication events) (Koch et al., 2014) were not amplified.

Supporting Information



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

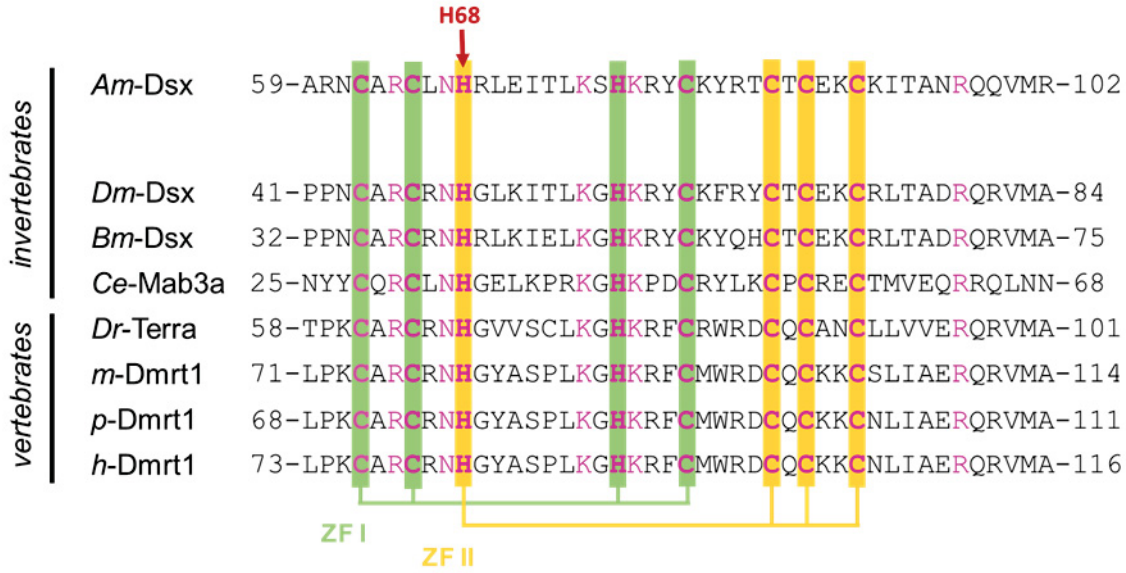
		ACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCCAGGAAAACCAACAACAACA ACGAAAAATTAAGGTAGATATTCATAGAGTTTTGCTAGGAAAACCAACAACAACA ACAAAAAATTAAAGTAGATATTTATAGGATTTTGCCAGGAAAATCAACAACAACA TCTGATGAACTTAAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG TCTGATGAACTTAAACGGGATATTATCAATCCTGAAGATGTGATGCTCAAAAG ACTGATGAACTTAAATGAGATATTATCAATCCTGAAGATGTGATGCTCAAAAG
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 [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.

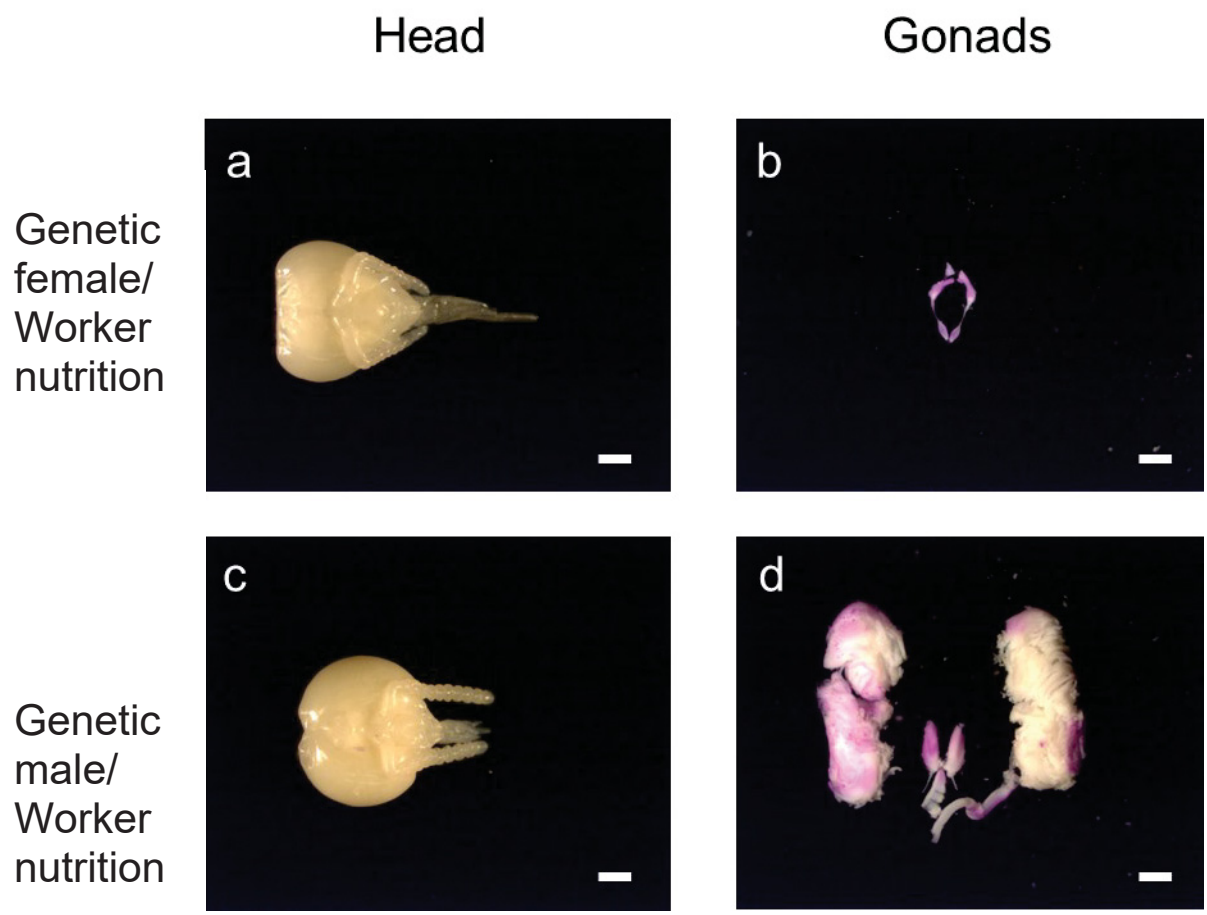
Injected sgRNA	Larva No.	Alignment of *dsx* sequences
dsx-sgRNA2	17-19	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele .....---.....
dsx-sgRNA2	17-29	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele a .....----..... Allele b .....---.....
dsx-sgRNA2	17-37	Wildtype CGATGTCTGAATCATCGGCTGGAGATCACCT Allele 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 (Leimar et al., 2012)

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 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 ---------- GCGGGAGTGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG GCGGGAGTGGTGGCGGCGGCGGCGGCGGCGGCGGCGAACATAAAATCCCTCG ----------CGAACATAAAATCCCTCG CACAT CACAT CACAT
fru-sgRNA4	3	Wildtype AGTGGCGGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele a AGTGGCGGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA Allele b AGTGGCGGGGGGAGGAGGGTCGGAGGGGACGGGTGGAAGCTGGCGGA GGTTGGGC-GACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTGGCCCGACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG GGTTG-----ACGGGGGTGGCGGCCGATTCTCGGTTGGTGGTAGTAGTGGCGGAG
fru-sgRNA5	2	Wildtype GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACTC Allele a GGCTTCAACGCGGCTCGGTTGGGTGGTGACCCGCTGCTATTCCCT- Allele b GGCTTCAACGCGGCTCGGTTGGGTGGTGGCCCGCTGCTGTTCACT- TTTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA -TTGGAGAGGAAAGGGTTGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGA
fru-sgRNA5	4	Wildtype GTTGGGTGGTGGCCCGCTGCTGTTCACTCTTTGGAGAGGAAAGGGT Allele a GTTGGGTGGTGGCCCGCTGCTGTTC---CTTTGGAGAGGAAAGGGT Allele b GTTGGGTGGTGGCCCGCTGCTGTTCA--CTTTGGAGAGGAAAGGGT TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG TGCGCGAGGAGCGACGGGGACAGGGTGGGAAAAAGAGAGGGATGCGTGAAGGAGG AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAGAAGGAGGA AAGGGTGAAGAACGAGGGAAAGGAGGAAGAGGAGGAGGACGGAGGAG---GAGGA 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

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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

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Data Availability

All relevant data are within the paper.

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A genetic switch for worker nutrition-mediated traits in honeybees

Journal: „PLOS Biology“

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Author's contribution: Manuskrip II

- Experimental design
- Design and synthesis of *dsx*-RNAs
- Implementation of *dsx*-injection experiments
- *in vitro*-rearing and dissections of individuals of *dsx*-experiments
- Implementation of *fru*-, *loc*-, *fem*- and *dsx*-molecular analyses
- Primer design for *dsx*-experiments
- Data analyses
- Authoring the manuscript

Kapitel III

Zusammenfassung

In der Geschlechtsbestimmungskaskade der Honigbiene *Apis mellifera* ist dem initialen Signal *csd* direkt *fem* untergeordnet. Durch geschlechtsspezifisches Spleißen entstehen in Männchen und Weibchen unterschiedliche *fem*-Transkripte. Das ausschließlich in Weibchen vorhandene Fem-Protein induziert das weibchenspezifische Spleißen von *Am-dsx*. Für die Bildung des weiblichen *Am-dsx*-Transkripts ist Fem-Protein notwendig. *Am*-Tra2-Protein hat in Kombination mit Fem eine steigernde Wirkung auf die Bildung weibchenspezifischer *Am-dsx*-Transkripte. *Am*-Tra2 alleine ist dazu nicht ausreichend, weshalb Fem und *Am*-Tra2 miteinander interagieren, um das weibchenspezifische Spleißen von *Am-dsx* zu induzieren. Das geschlechtsspezifische Spleißen von *Am-dsx* führt zur Bildung männchen- und weibchenspezifischer *Am*-Dsx-Proteine, die als Transkriptionsfaktoren wirken und durch die Erzeugung unterschiedlicher Genaktivitäten strangabwärts gelegener Gene den männlichen oder weiblichen Entwicklungsweg einleiten. Das weibliche *Am-dsx*-Transkript entsteht durch Exoninklusion des weibchenspezifischen *Am-dsx* Exon 5. Zur Induktion dieser Exoninklusion sind vier Sequenzmotive aus sechs Nukleotiden zwischen 90 und 180 bp strangaufwärts des Exon 5-Spleißdonors essentiell. Diese Sequenzmotive aktivieren Fem/*Am*-Tra2-abhängig das Spleißen am Exon 5-Spleißakzeptor, der sich ca. 700 bp strangaufwärts befindet. Diese molekularen Mechanismen konnten mittels eines *in vivo* Spleißassays in Schmetterlingszellen (*Sf21*-Zellen) und mit Hilfe des FLIM-FRET-Verfahrens nachgestellt beziehungsweise nachgewiesen werden.

Der Stellenwert von *fem* und *Am-dsx* bei der nahrungsabhängigen Kastendifferenzierung in der Honigbiene wurde mit Hilfe der CRISPR/Cas9-Methode nachgewiesen. Die Reproduktionsorgane von Honigbienenarbeiterinnen sind im Vergleich zu denen von Königinnen verkleinert. Diese Größenreduktion wird durch Arbeiterinnen-spezifisches Futter vermittelt und findet nur in Weibchen statt. Die durch Futter vermittelte Kastendifferenzierung wird durch das Fem-Protein in das genetische Geschlechtsbestimmungssignal integriert. Weibchenspezifisches *Am*-Dsx-Protein ist essentiell für die Differenzierung der weiblichen Reproduktionsorgane, nicht jedoch für die Bildung sekundärer Geschlechtsmerkmale am Kopf. Im Gegensatz zu Fem hat *Am*-Dsx keinen Einfluss auf die durch das Arbeiterinnenfutter vermittelte Größenreduktion der weiblichen Reproduktionsorgane. Daher kann hier gezeigt werden, dass *Am-dsx* die weibliche Entwicklung untergeordnet oder parallel zur Größenentwicklung der weiblichen Reproduktionsorgane steuert.

Die hier verwendete somatische Mutationsmethode erlaubt die Darstellung durch Genmanipulation erzeugter morphologischer Merkmale in hocheffizienter Weise direkt in der injizierten Generation. Da keine Mosaike erzeugt werden, ermöglicht sie genetische Screens am Individuum ohne die Notwendigkeit weiterer Kreuzungsexperimente oder großformatiger Laborkolonien.

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