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The Molecular Basis and Reproductive Function(s) of Copulatory Plugs

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SUMMARY

In many animals, male ejaculates coagulate to form what has been termed a copulatory plug, a structure that varies in size and shape but often fills and seals the female's reproductive tract. The first published observation of a copulatory plug in a mammal was made more than 160 years ago, and questions about its formation and role in reproduction continue to endear evolutionary and population geneticists, behavioral ecologists, and molecular, reproductive, and developmental biologists alike. Here, we review the current knowledge of copulatory plugs, focusing on rodents and asking two main questions: how is it formed and what does it do? An evolutionary biology perspective helps us understand the latter, potentially leading to insights into the selective regimes that have shaped the diversity of this structure.

Their broad distribution among sexually reproducing animals, their diversity of form, and the multiple ways they form all argue that copulatory plugs are selectively advantageous.

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INTRODUCTION

Essentially every investigator working with laboratory mice is familiar with the copulatory plug, a product of male seminal fluid that coagulates and fills the vagina–cervical canal of the female (Fig. 1) for a number of hours after mating. Although not a guarantee of pregnancy, the copulatory plug is one indicator of successful coitus; indeed, the ''Materials and Methods'' of a voluminous literature are rife with reference to copulatory plugs. But how

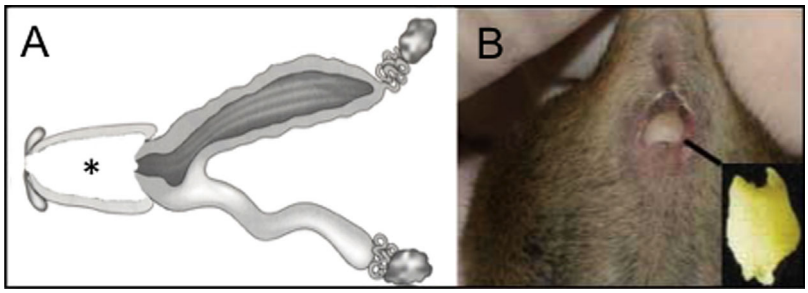

Figure 1. The mouse copulatory plug (asterisk) fills the vagina, with a slight protrusion into the cervix (**A**), and can normally be detected from external observation (**B**). Modified from Nagy et al. (2003).

widespread are these plugs, how do they form, and what is their function—aside from notifying humans that mating occurred?

Copulatory plugs or similar structures have been observed in insects (Dickinson and Rutowski, 1989; Orr and Rutowski, 1991; Duvoisin et al., 1999; Rogers et al., 2009; Avila et al., 2011, 2015); arachnids (Austad, 1984; Masumoto, 1993; Uhl et al., 2010); nematode and acanthocephalan worms (Abele and Gilchrist, 1977; O'Brien et al., 1983; Barker, 1994; Palopoli et al., 2008); reptiles (Devine, 1975, 1977; Herman, 1994; Moreira and Birkhead, 2004); rodents (Hartung and Dewsbury, 1978; Voss, 1979; Dewsbury, 1984; Michener, 1984); primates (Dixson and Anderson, 2002; Parga et al., 2006); and other mammals (Hartman, 1924, 1933; Eadie, 1948; Oh et al., 1983; Phillips and Inwards, 1985; Williams, et al., 1998; Jia et al., 2002; Paris et al., 2004; Hynes et al., 2005; Dawson, 2012; Smith, 2012). These structures exhibit great diversity in size and shape (Hartung and Dewsbury, 1978; Baumgardner et al., 1982). Here, we tap into recent advances in genetic and experimental approaches that provide a deeper understanding of the molecular basis and reproductive function(s) of copulatory plugs, focusing on rodents but with reference to additional species when appropriate.

HOW DO COPULATORY PLUGS FORM?

The first documented observation of a rodent copulatory plug is attributed to Rudolf Leuckart, who observed it in the guinea pig and suggested that it derived from dehydration of the ejaculate (Box 1). Over 40 years later, Camus and Gley (1896) showed that a coagulation reaction could be induced simply by mixing fluids from the prostate and seminal vesicles gland of a guinea pig, and thus did not require dehydration; the unknown coagulating substance(s) was given the generic term "vesiculase". Eventually, Walker understood the coagulating substance to be derived from the anterior lobe of the prostate (he originally called the anterior lobe the "coagulating gland", which was later confirmed to be of prostatic origin) (Walker, 1910a,b, 1911; Engle, 1926).

Transglutaminase

Building from these early studies, and beginning more than 60 years ago, H. Guy Williams-Ashman and colleagues brought questions of the molecular basis of the copulatory plug into focus, using guinea pigs as a model system. The rate of seminal fluid coagulation depended on dosage of the prostate-derived "vesiculase" (Gotterer et al., 1955). The coagulation reaction was accompanied by a very specific reduction in amide-NH2 residues (Notides and Williams-Ashman, 1967), which in combination with the direct quantification of γ-glutamyl-ε-lysine dipeptide bonds, demonstrated the unknown vesiculase must be a transglutaminase (Williams-Ashman et al., 1972). This class of enzymes cross-links glutamines with (usually) lysines—specifically, an internal glutamine residue of some target protein acts as an amine acceptor, reacting with the cysteine sulfhydryl group in the active site of the transglutaminase to form a thioester bond (Williams-Ashman et al., 1980). An internal primary amine on the target protein, such as a lysine residue, then reacts with this intermediate thioester bond to form the γ-glutamyl-ε-lysine dipeptide bonds characteristic of transamidation. These bonds can form between glutamines and lysines within the same protein, or across different molecules. Thus, a copulatory plug is essentially a complex network of cross-linked molecules. Other ejaculated proteins, including spermine and spermidine, may compete with lysine residues in the thioester bond reduction step, thereby affecting the intensity of coagulation (Williams-Ashman, 1984).

The only transglutaminase known to be present in mammal ejaculates is transglutaminase IV (TGM4) (Grant et al., 1994; Pilch and Mann, 2006; Dean et al., 2009, 2011). TGM4 is a highly specialized protein that is exclusively produced in the prostate (Dubbink et al., 1998; Su et al., 2002, 2004; Dean et al., 2009, 2011). Through the years, other molecular hypotheses for the coagulation reaction emerged, including self-coagulation of seminal proteins (Tseng et al., 2011), the existence of other coagulatory plug proteins besides TGM4 (Hart and Greenstein, 1968), and even the requirement of female-derived proteins to form the copulatory plug (Hartman, 1924); however, mice missing a functional copy of *Tgm4* fail to form a copulatory

Box 1. The first description of the rodent copulatory plug by Rudolf Leuckart

Rudolf Leuckart (1822–1898), a German Zoologist with multiple interests, became a Professor first in Gießen (1850) and then in Leipzig (1869). He is best known today for his important work on parasites (The German Society of Parasitology has awarded the Rudolf Leuckart Medal for research on parasitology since 1974). Although Leuckart's 1847 publication *Zur Morphologie und Anatomie der Geschlechtsorgane* (On the morphology and anatomy of the sexual organs) is frequently mentioned as the first documented description of the rodent copulatory plug, in reality there is no mention to the plug in this work. The mistake was probably seeded by Stockard and Papanicolaou (1919), and since then taken over by other authors. In fact, as also indicated by Theodor Bischoff (1852), then a colleague of Leuckart in Gießen, the description of the copulatory plug by Leuckart appeared in 1852 in *Anatomisch-physiologische Uebersicht des Thierreichs* (Anatomical-Physiological overview of the animal kingdom), a voluminous textbook written by Leuckart and Carl Bergmann (Bergmann and Leuckart, 1855). The relevant passage can be found on page 567:

Eine eigenthümliche Funktion scheint der Inhalt von einem Paare mächtiger gewundener Schläuche zu haben, welche sich an dem männlichen Geschlechtsapparate der Meerschweinchen finden. Leuckart fand nämlich nach der Begattung die Scheide verstopft durch einen festen Pfropf, welcher genau die Form derselben angenommen hatte und sich mit einer Spitze auch in das Ostium uterus einschmiegte. Da nun der Inhalt jener Schläuche eine steife gallertartige Masse ist, so scheint es, daß dieselbe nach der Entleerung des Samens in die weiblichen Theile getrieben wird, hier durch Wasserverlust erhärtet und die Form des Pfropfes annimmt.

(The content of a pair of voluminous and tortuous tubes, located on the male genital apparatus of the guinea pig, seems to have a peculiar function. Leuckart observed namely that the vagina, after mating, was packed with a stiff plug, which assumed exactly the form of the latter and snuggled itself with its tip into the ostium uterus. As the content of the abovementioned tubes is a rigid gelatinous mass, it seems that after ejaculation this mass is forced into the female parts, hardens by water loss, and assumes the form of the plug).

Only 1 year later, Leuckart provided a similar description of the copulatory plug in the article *Zeugung (Conception*), a chapter of *Handwörterbuch der Physiologie mit Rücksicht auf physiologische Pathologie* (Handbook of physiology including physiological pathology), an encyclopedic work edited by Leuckart's former supervisor Rudolf Wagner (Leuckart, 1853). Here, on page 900, Leuckart proposes a functional explanation for the copulatory plug:

So sonderbar nun aber auch dieses Manoeuver ist, so möchte es doch durch den Umstand völlig gerechtfertigt erscheinen, daß das Meerschweinchen sogleich nach der Geburtsarbeit der Weibchen den Act der Begattung vollzieht, zu einer Zeit also, in welcher der Zustand der weiblichen Genitalien sonst wohl schwerlich das ejaculirte Sperma vollständig zurückzuhalten im Stande sein möchte.

(As odd this maneuver is, it may be fully justified by the fact that the guinea pig consummates copulation immediately after the females have delivered, thus at a time point in which the condition of the female genitalia would otherwise be hardly able to completely retain the ejaculated sperm).

plug, demonstrating its necessity—at least in this species (Dean, 2013).

Targets of Transglutaminase

The target proteins cross-linked by TGM4 collectively represent the other half of the components needed to form a copulatory plug. The major target of TGM4 appeared to be a seminal vesicle-derived protein (Notides and Williams-Ashman, 1967; Moore et al., 1987), termed SVP1 in guinea pigs or SVS2 in mice, although their homology is unclear because of rapid evolutionary divergence. These proteins contain a variable number of repeats that are rich in lysines and glutamines (Moore et al., 1987). Other seminal vesicle secretions—for example, murine SVS1, SVS2, SVS3a, SVS3b, SVS4, and SVS5—were originally thought to contribute to the plug (Fawell and Higgins, 1987; Porta et al., 1990; Lundwall et al., 1997; Lin et al., 2002). If *Svs2* alone is knocked out, however, male mice fail to form a copulatory plug despite their morphologically normal seminal vesicles (Kawano et al., 2014). Thus, even if TGM4 is cross-linking other proteins in seminal vesicle secretions, their modification is not sufficient to rescue plug formation in the absence of SVS2. Modification of SVS2 may alternatively represent the primary coagulation event, followed by longer-term cross-linking of other proteins in a cascade of processes that result in the copulatory plug (Fawell and Higgins, 1987). Consistent with this hypothesis, a proteomic experiment identified 63 proteins from the copulatory plug, of which TGM4 and SVS2 together account for only 10% of all identified components (Dean et al., 2011).

The molecular foundation of copulatory plugs is analogous to epoxy, which forms as a result of a chemical reaction between two compounds. In copulatory plugs, the prostate-derived TGM4 cross-links seminal vesicle-derived SVS2, and perhaps other targets, only after they are mixed in the female's reproductive tract. Keeping TGM4 separate from its target(s) probably reflects evolutionary selection to prevent coagulation in the male reproductive tract, which would block the urethra and prevent urination in a male— with fatal consequences. For example, the murine copulatory plug forms essentially instantaneously after ejaculation, is extremely hard and durable, and takes considerable effort to experimentally re-solubilize (Dean et al., 2011).

Although rodent copulatory plugs tend to be extremely hard and robust, their consistency in primates ranges from a temporary phase of semi-coagulation (humans and gorillas) to a very robust coagulation (chimpanzees) (Huggins and Neal, 1942; Dixson and Anderson, 2002). Such variation in coagulation intensity among primates mirrors genetic variation seen in semenogelins (SEMG1 and SEMG2), which are thought to be the main targets of TGM4-mediated cross-linking. Gorillas have accumulated multiple, independent loss-of-function mutations in SEMG1 and SEMG2 whereas the number of cross-linking sites has increased in chimpanzees (Jensen-Seaman and Li, 2003; Kingan et al., 2003). In addition, gorillas have a non-functional TGM4 caused by an early termination codon (Carnahan and Jensen-Seaman, 2008). However, human TGM4 and semenogelins appear functional, even though human ejaculates do not form a copulatory plug.

Although transglutaminase-mediated copulatory plugs appear to have relatively simple genetic origins, ''copulatory plug proteins'' probably have additional functions. SVS2, for example, also binds to sperm and prevents precocious capacitation in the uterus (Carballada and Esponda, 1998, 1999; Kawano and Yoshida, 2007), and protects sperm from cytotoxic challenges in the uterine environment (Metafora et al., 2007; Kawano et al., 2014).

Plug Characteristics

The copulatory plug represents a substantial physiological investment. In house mice, copulatory plugs weighed an average 34.6 mg while the mated females from which these plugs were dissected averaged 16.4 g; thus, copulatory plugs represent 0.2% of the female body weight (plug weight is probably constrained by female body size) (Mangels et al., 2015). In mice, the first male to mate has a paternity advantage if the female re-mates with a second male (Levine, 1967). Given the cost of ejaculation, selection may favor males that avoid mating in second place (Ramm and Stockley, 2014). The very existence of male mate choice, rather than indiscriminant mating (Drickamer et al., 2003; Gowaty et al., 2003; Edward and Chapman, 2011), and evidence that males adjust their ejaculates when presented with sub-optimal mating opportunities (Pizzari et al., 2003; Delbarco-Trillo and Ferkin, 2004; Ramm and Stockley, 2007; Lüpold et al., 2011; DelBarco-Trillo, 2011; Ramm et al., 2015) together imply that ejaculates are physiologically expensive and are conserved whenever possible (Parker, 1998; Parker and Pizzari, 2010). Indeed, direct calorimetric study revealed that a single copulatory plug in garter snakes might represent 5–18% of an animal's daily energy budget (Friesen et al., 2015). To our knowledge, no equivalent study has been performed in a mammal, partly because it is currently not possible to separate the cost of seminal fluid versus spermatogenesis, as can be achieved in snakes (Friesen et al., 2015). Nevertheless, the relatively high rate of protein turnover by seminal vesicles may enable the dynamic allocation of resources by mammals (Claydon et al., 2012).

The fate of the copulatory plug varies following its formation. Murine copulatory plugs adhere strongly to the epithelium of the vagina–cervical canal, yet females generally expel the plug by 24–48 hr post-coitum (Mangels et al., 2015). Occasionally a plug cannot be found after mating (Dean, 2013). In these cases, females may have removed the plug or prevented it from forming tightly in the first place, for example, through incomplete lordosis. Interestingly, the length of time required for females to clear their reproductive tracts of copulatory plugs depends on the genotype of the male, and small plugs lasted longer than large plugs (Mangels et al., 2015). Regarding the last observation, one hypothesis is that larger plugs are easier for females to remove, given that they probably clear plugs by sloughing off epithelium and/or through proteolytic activity directed to the plug or surrounding area (Dean et al., 2011). Indeed, copulatory plugs are occasionally found in the bottom of the cage of a recently mated female, suggesting that females can clear their reproductive tracts through enzymatic degradation of the plug followed by loosening and eventual expulsion.

Other Groups

While the focus of this review is on copulatory plugs in rodents, it is useful to explore the variation in copulatory plug formation in other species. The basic chemistry seems conserved in some species, whereas novel substances are utilized by others. A transglutaminase is found in the ejaculates of the mosquito *Anopheles gambiae*, which probably cross-links the glutamine- and lysine-rich seminal protein Plugin (Rogers et al., 2009). Some genotypes of *Caenorhabditis elegans* form a plug, and this has been genetically linked to a gene called *plg-1* (Hodgkin and Doniach, 1997). In nematode genotypes that cannot form a plug, a retransposon has disrupted *plg-1* exon 3, rendering it non-functional (Palopoli et al., 2008). Interestingly, PLG-1 is a mucin rich in proline, threonine, and serine, which each serves as a substrate for post-translational glycosylation instead of cross-linking. In garter snakes, the material for copulatory plug formation derives not from male accessory glands, but from the renal sexual segment (Devine, 1975, 1977; Friesen et al., 2013). Perhaps most interesting, in some species of spiders, the sperm transfer organ breaks off and often remains embedded within the female's

reproductive tract (Suhm et al., 1996; Snow et al., 2006; Uhl et al., 2010), potentially serving a similar role as a copulatory plug.

WHAT DO COPULATORY PLUGS DO?

Their broad distribution among sexually reproducing animals, their diversity of form, and the multiple ways they form all argue that copulatory plugs are selectively advantageous. Studying rodents, Voss (1979) proposed four hypotheses for the copulatory plug: #1) to permit a gradual release of spermatozoa within the female tract as the plug disintegrates; #2) to hinder the backflow of semen out of the vagina after ejaculation, or to otherwise enhance sperm transport through the cervix into the uterus (Voss originally considered these two functions as separate hypotheses, but we see enough similarity to combine them here); #3) to contribute to coital stimulation, which in many mammalian species is required for successful implantation of embryos into the uterus; and #4) to physically prevent the subsequent insemination of the female by other males. Evidence for each functional hypothesis exists, and it should be noted that they are not mutually exclusive. For example, the copulatory plug may contribute to copulatory stimulation (#3), which may induce contractions that promote sperm transport (#2) or make a female less receptive to additional mating (#4). Given the variation in copulatory plug biology, it is unlikely that the plug performs the same functions and to the same extent across different species.

Hypothesis #1: Sperm Release

The sperm-release hypothesis has received variable support. In guinea pigs, Martan and Shepherd (1976) removed recently deposited plugs and transferred them to estrus females that had not yet mated, observing that none of the recipient females became pregnant. Although it is true that large numbers of sperm can be observed in rodent copulatory plugs, they probably get entangled as collateral damage of the extremely rapid coagulation reaction (Asdell and Hubbs, 1964). In fact, enough sperm get entangled in copulatory plugs that they can be genotyped to determine chimaeric DNA content in transgenic males (Wilson and Sheardown, 2011). Sperm are also found throughout the copulatory plug of garter snakes, which is more gelatinous in structure than in rodents; interestingly, the sperm may be gradually released as the plug dissolves (Friesen et al., 2013) and females are more likely to remate if plugs contain little sperm (Friesen et al., 2014). At the moment, this hypothesis has not been tested with direct-transfer experiments in garter snakes as performed in the guinea pig.

Hypothesis #2: Sperm Transport

The sperm-transport hypothesis has received considerable support. When male anopheline mosquitoes were prevented from forming a plug through RNA interference, their sperm failed to reach the female's sperm storage organ and therefore did not fertilize any eggs (Rogers et al., 2009); a similar effect was observed in Drosophila (Avila et al., 2015). The sperm of male rats surgically prevented from forming a copulatory plug or who formed incomplete plugs generally failed to migrate past the vagina (Blandau, 1945a,b; Matthews and Adler, 1978; Toner et al., 1987; Cukierski et al., 1991; Carballada and Esponda, 1992). Several studies documented reduced pregnancy rates among females whose mates had their seminal vesicles and sometimes other accessory glands removed (Lawlah, 1930; Pang et al., 1979; Peitz, 1988); however, such surgery is likely to impact multiple features of the ejaculate, not simply the formation of a copulatory plug. In mice, manually removing copulatory plugs did not strongly compromise fertility in one study (Firman and Simmons, 2011) but did in another (Bloch, 1972), although it should be noted that the copulatory plugs may have already manifested their effects on sperm transport by the time they were removed. *Tgm4*-knockout male mice showed normal sperm count and sperm motility, and were normal in gross morphology, yet fewer of their sperm migrated through the cervix into the uterus (Dean, 2013). Similarly, very little of the ejaculate from *Svs2*-knockout males, who also cannot form a plug, migrate through the cervix properly (Kawano et al., 2014). Thus, a copulatory plug likely seals the vagina and propels sperm, which are ejaculated prior to plug proteins, forward through the cervix. The experiments supporting this hypothesis, however, tend to focus on extreme manipulations of the copulatory plug; future experiments should test whether natural variation correlates with variation in sperm transport.

Hypothesis #3: Coital Stimulation

The coital-stimulation hypothesis posits that the copulatory plug contributes to a threshold level of stimulation required for females to engage reproductive functions. This hypothesis takes on added importance in many mammalian species (including mice), as female stimulation is required in order for implantation to proceed; without such stimulation, fertilized oocytes will pass through the uterus altogether (Lanier et al., 1975, 1979). In mouse transgenics, researchers solve this problem by transferring in vitro-fertilized embryos into surrogate mothers that have been mated to vasectomized males. The vasectomized males are obviously infertile, but provide the copulatory stimulation required for successful implantation into surrogate mothers. When mated to *Tgm4*-knockout males, which cannot form a copulatory plug, female mice gave birth to a litter 57% of the time, compared to 82% of females mated to wild-type males (Dean, 2013). Similarly, mated female mice that had plugs removed showed reduced pregnancy (Bloch, 1972).

Tgm4-knockout males still fertilize what seems to be a low, but normal number of eggs—between 3 and 11 (Dean, 2013). This reduced ability to sire litters suggests that *Tgm4*-knockout males may be less able to induce the

physiological response in females that is necessary for embryos to implant—a state called ''pseudopregnancy.'' In mice, pseudopregnancy can sometimes be artificially induced with mechanical stimulation (Ball, 1934), although such techniques are inefficient, perhaps because copulatory stimulation involves species-specific behaviors (Diamond, 1970). The natural induction of pseudopregnancy in mice depends on the transfer of ejaculate rather than just mating behavior (Yang et al., 2009), which in turn suggests that the copulatory plug may contribute to the requisite copulatory stimulation. Careful quantification of the potential contribution of the copulatory plug to induction of pseudopregnancy in mice is needed to decipher this relationship.

Hypothesis #4: Chastity Enforcement

Although protecting paternity may seem irrelevant under monogamous mating regimes employed in the laboratory, multiple lines of evidence support the chastity-enforcement hypothesis. Female mice come into estrus about once every 4 days, and can be fertilized in a window of ~12 hr (Silver, 1995). Since females rarely mate outside this window, and copulatory plugs remain secure for 24–48 hr following mating (Silver, 1995; Mangels et al., 2015), the plug could be a formidable barrier to female re-mating.

Several comparative studies support this notion of chastity enforcement. Species that do not form copulatory plugs tend to be monogamous or engage in a ''locking'' pattern of mating, whereby the male and female remain in copula for an extended period of time and over multiple bouts of mating (Lanier and Dewsbury, 1977; Hartung and Dewsbury, 1978; Voss, 1979). The locking pattern may be performed in the context of mate guarding, although it may also facilitate sperm transport (Hypothesis #2) or female stimulation (Hypothesis #3). As discussed above, gorillas do not form a copulatory plug and have accumulated multiple loss-of-function mutations in *TGM4* and its presumed targets, *SEMG1* and *SEMG2* (Jensen-Seaman and Li, 2003; Kingan et al., 2003; Carnahan and Jensen-Seaman, 2008). Gorillas probably do not experience high risk or intensity of sperm competition because a single dominant male (the ''silverback'') usually exerts his dominance over other males in the group and suppresses their mating potential. Gorillas may thus invest disproportionately in pre-copulatory phenotypes (large body size, aggressive interactions) while relaxing selection on post-copulatory phenotypes (copulatory plugs). In contrast, female chimpanzees generally mate with many males, and male ejaculates form firm, compact copulatory plugs (Dixson and Anderson, 2002). An overall correlation between the relative promiscuity of females and the intensity of the coagulation among copulatory plugs exists in primates (Dixson, 1998; Dixson and Anderson, 2001, 2002). Yet, a recent game-theory model made the opposite prediction: copulatory plugs will be less effective when females mate with many males, due to selection for males to remove competitor plugs (Fromhage, 2011).

In many different species, a positive correlation exists between the risk and intensity of sperm competition (one female mating with multiple males during a single fertile period) and the size of the testes relative to the body (Harcourt et al., 1981; Møller, 1989; Gage, 1994; Byrne et al., 2002; Pitcher et al., 2005; Ramm et al., 2005; Firman and Simmons, 2008a). The dominant interpretation of this relationship is that, under the pressures of sperm competition, the reproductive payoff for males lies with increased investment in spermatogenesis (Parker, 1990). If copulatory plugs function in the context of sperm competition, then we might also predict a correlation between features of copulatory plugs and relative testis mass. Indeed, the relative size of the seminal vesicles, a primary source of copulatory plug protein in mammals, is positively correlated with relative testis mass (Dixson, 1998; Dixson and Anderson, 2001). Seminal vesicle size as well as copulatory plug size is positively correlated with the inferred level of multiple mating in rodents (Ramm et al., 2005), while seminal vesicles are a significant predictor of paternity success in a semi-natural population of house mice (Stockley et al., 2013). Considering the correlation between mating ecology and coagulation intensity, larger seminal vesicles and/or a more solidified copulatory plug may have an adaptive advantage in the context of multiple mating. Other species do not align with these general comparative results, however. Two rodent species, *Peromyscus polionotus* and *P. californicus*, are both behaviorally and genetically monogamous (Foltz, 1981; Ribble and Millar, 1992), yet the males of both species form plugs (Baumgardner et al., 1982; Gubernick, 1988). Therefore, the plugs could play a different functional role in these species, or perhaps monogamy was adopted recently, leaving little evolutionary time for copulatory plug proteins to accumulate loss-of-function mutations, as observed in gorillas (Jensen-Seaman and Li, 2003; Kingan et al., 2003; Carnahan and Jensen-Seaman, 2008).

In addition to these comparative patterns across species and mating ecology—which will always be indirect—several direct experiments have demonstrated a role of the copulatory plug in inhibiting female re-mating. Mangels et al. (2016) used *Tgm4*-knockout males in a one-female-two-male mating regime, showing that if the first male to mate could not form a plug, the second male (which could form a plug) sired almost all the offspring; conversely, the first male sired almost all the offspring if he could form a plug (Mangels et al., 2016). They repeated the experiment after vasectomizing all first-to-mate males to demonstrate this difference in paternity was due to the plug itself, rather than differences in the timing of fertilization or defects in spermatogenesis. If first-to-mate males had their plugs experimentally removed following mating, they lost paternity to second-to-mate males, compared to control matings where first male plugs were left intact (Sutter and Lindholm, 2016). Furthermore, if first-to-mate males had recently mated, their copulatory plugs were smaller and they too lost paternity to second-to-mate males, compared to matings where first males were sexually rested (Sutter et al., 2016).

Other studies demonstrated that the copulatory plug is *not* effective at preventing insemination by second males, including studies in rats (Lanier et al., 1979), Syrian hamsters (Oglesby et al., 1981), and deer mice (Dewsbury, 1988). Furthermore, both females and males of some species can remove copulatory plugs with apparent ease (Mosig and Dewsbury, 1970; Milligan, 1979; Wallach and Hart, 1983; Fenton, 1984; Koprowski, 1992; Parga, 2003), further complicating the interpretation of its function as a means to enforce chastity.

Sexual Conflict

The mixed experimental results of copulatory plug function may be reconciled by considering plug dynamics through the lens of sexual conflict. Males obviously benefit from preventing female re-mating, but females would lose out on the many hypothesized benefits of mating with more than one male during a single fertile period (Jennions and Petrie, 2000; Zeh and Zeh, 2001; Fedorka and Mousseau, 2002; Tregenza and Wedell, 2002; Firman and Simmons, 2008b; Slatyer et al., 2012). Therefore, selection may favor males that make ever more effective plugs and favor females that reduce the efficacy of the plugs, for example, by removing them mechanically or enzymatically. Importantly, such conflict will never reach equilibrium, and so is analogous to a host/pathogen evolutionary arms race. Differences among studies may simply represent snapshots of species at different evolutionary time points: males are currently ''winning'' in some species, while females are in others. The sexual conflict hypothesis may also help explain why, even in species that form copulatory plugs, pregnant females often carry embryos sired by different fathers (Birdsall and Nash, 1973; Schwartz et al., 1989; Searle, 1990; Eberle and Kappeler, 2004; Dean et al., 2006; Firman and Simmons, 2008a). In other words, selection may favor females who can overcome the inhibitory effects of copulatory plugs. The more relevant question is therefore not whether plugs absolutely prevent female re-mating, but whether they inhibit female re-mating, even imperfectly.

Several molecular patterns support the sexual conflict hypothesis. Copulatory plug proteins are often the targets of recurrent adaptive evolution, whereby functional changes, like nonsynonymous mutations or protein length variation, are repeatedly driven to fixation by selection (Dorus et al., 2004; Clark and Swanson, 2005; Karn et al., 2008; Ramm et al., 2008, 2009; Dean et al., 2009, 2011; Carnahan-Craig and Jensen-Seaman, 2014). Across primate species, the rate of divergence of SVS2 is positively correlated with the inferred intensity of sexual conflict (Dorus et al., 2004). Such recurrent functional divergence over time can only occur if the selective optimum is constantly shifting—for example in host–pathogen or male–female evolutionary arms races. The correlation to inferred mating ecology strengthens the case that copulatory plug proteins evolve under the pressures of sexual conflict. The length (and thus the molecular mass) of transglutaminase-targeted copulatory plug proteins is positively correlated with relative testis mass, suggesting that selection favors copulatory plug proteins that contain more cross-linking sites (Ramm et al., 2009).

Divergence in copulatory plug proteins may be advantageous if it allows them to evade or resist female degradation machinery, but then female-derived degradation machinery will be selected to regain recognition of these divergent proteins. Female mice add two rapidly evolving proteases (Lactotransferrin and Kallikrein-related peptidase 14) in or around the vicinity of the copulatory plug shortly after mating; in response male mice transfer several rapidly evolving protease inhibitors to females in their ejaculates (Dean et al., 2011). Males knocked out for one of these, Protease nexin 1 (now called Serpin 2), had ejaculates with higher-than-normal proteolytic activity, leading to malformed plugs (Murer et al., 2001). A negative correlation exists between protease activity assayed from a plug and its size among wild-derived, inbred strains of mice (Mangels et al., 2015). Taken together, these patterns hint at a molecular basis of sexual conflict, with females producing proteases to loosen or degrade the plug versus males contributing protease inhibitors and rapidly evolving copulatory plug proteins in order to evade that degradation. More specific tests of potential interactions between female-derived proteases and male-derived protease inhibitors, and their effects on each other, are needed to further evaluate the sexual conflict hypothesis. However, males also contribute proteases in their ejaculates, and proteases and protease inhibitors almost certainly have additional functions outside the context of copulatory plugs (Wolfner, 2002; Kawano et al., 2010).

Another prediction of sexual conflict is that the fate of the copulatory plug, in terms of its survival and efficacy, should depend on the *interaction* between male and female genotypes rather than male or female genotype alone. This interaction would exist because conflict may be less intense if females mate with a preferred male compared to a non-preferred male. Variation in the degree of sexual conflict could result in a male–female genotype interaction term that is correlated with patterns of female mating preferences, yet such an interaction term was not detected when males of eight different inbred strains of mice (six of which were wild-derived) were crossed to two different female genotypes (Mangels et al., 2015). In a follow-up experiment, females were exposed to the odors of both a preferred and non-preferred male, then mated to one of these; still, no evidence was found that females adjusted their clearance of copulatory plugs based on presumed mate preferences (Mangels, 2016). Thus, a male–female interaction term does not appear to affect copulatory plug dynamics, although these experiments may still be underpowered to detect such interactions.

Copulatory plugs are predicted to show some kind of dysgenesis when individuals from different species, or even different populations, interbreed. The reason is that if coevolutionary arms races are occurring in independently evolving lineages, then gene flow between them will bring together mismatched copulatory plug ''offenses'' and ''defenses''. Support for this model comes from species crossing studies: Mating *Drosophila mojavensis* and

Drosophila arizonae results in a larger and longer-lasting insemination reaction (which can be thought of as a copulatory plug) compared to intraspecific crosses (Knowles and Markow, 2001). Similarly, when two different species of mice (*Mus domesticus* and *Mus musculus*) were hybridized in the laboratory, copulatory plugs were larger than in control intraspecific crosses (Dean and Nachman, 2009).

CONCLUSIONS

Copulatory plugs are an important feature of reproduction, but their biochemical basis and physiological roles remain incompletely understood. This structure appears to play multiple roles that may not be equally important among species, including the slow release of sperm, the promotion of sperm transport, the contribution to female stimulation, and the inhibition of female re-mating. Surgical, experimental, and genetic modification has enabled many insights, and should continue to shed light on the biology and evolution of this remarkable structure.

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