A Novel Effect of Polychlorinated Biphenyls: Impairment of the Tight Junctions in the Mouse Epididymis

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Polychlorinated biphenyls (PCBs) exhibit a broad range of adverse biological effects, including reproductive toxicity. However, the mechanisms by which PCBs disrupt the epididymis remain obscure. We analyzed the gene expression profile in mice epididymis exposed to PCBs (Aroclor 1254) at doses comparable to human exposure using a cDNA microarray. Differentially expressed genes were involved in a variety of function categories and biological pathways, including GTP binding, nucleosome assembly, and ribosome and protein disulfide isomerase. The differentially expressed genes related to GTP binding were highly enriched. The abundance of GTP binding proteins related to tight junctions being reduced and the phosphorylation level of their downstream effectors were impaired after exposure to PCBs. The results of tracer studies demonstrated that the permeability of blood-epididymis barrier was increased by PCB exposure. In addition, PCB exposure also disrupted the expression of the tight junction proteins, zonula occludens-1 and occludin. We demonstrated for the first time that exposure to PCBs at doses relevant for the general population was able to affect the blood-epididymis barrier in mice through altering GTP binding and tight junction proteins. Our results provided a novel insight into the molecular mechanisms linking PCB exposure to sperm maturation.

Key Words: epididymis; GTP binding; microarray; polychlorinated biphenyls; tight junctions.

Polychlorinated biphenyls (PCBs) are a class of persistent organic chemicals that were once widely used in industrial and consumer products. Decades after their production was banned, PCBs are still listed among the top priority hazardous substances on the National Priorities List due to their frequency, toxicity, and potential for human exposure. Although temporal declines of PCB levels in the environmental and human samples are reported (Lucena et al., 2007), recent reports involving considerable human exposure observed over wide geographic variability (Consonni et al., 2012) and the presence of PCBs in food (Zhao et al., 2009) suggest a risk of chronic low-level exposure to the general population. An estimated total daily dietary intake of PCBs in residents living around large e-waste disassembly sites is reported to be as high as 12372.9 ng/day (Zhao et al., 2009).

Bush et al. (1986) first reported an inverse relationship between the human body burden of PCB congeners and semen quality. Since then, a number of human epidemiological studies associated environmental PCB exposure with male reproductive health markers, such as semen quality parameters, including sperm concentration, motility, morphology, sperm DNA integrity, and circulating reproductive hormone levels (Meeker and Hauser, 2010). Inverse associations between PCBs and sperm motility have been consistent in a wide range of reports, despite their differences in study designs, locations, and measurement methods, whereas other effects (sperm counts and morphology) are rather uncertain. The inverse relationship between PCB body burden and sperm motility suggests an underlying mechanism based on the posttesticular maturation of sperm. Elzanaty et al. note that the activity of seminal epididymal neutral-α glucosidase (NAG) is negatively associated with CB-153 exposure, a good marker for total PCB exposure. NAG is widely used as a marker of epididymal function in a clinical setting, where low NAG activity indicates a reduced epididymal function. It is suggested that posttesticular mechanisms might be partly responsible for the negative effects on sperm motility, which is observed in the same study population (Elzanaty et al., 2006).

The epididymis is essential for semen quality and sperm fertility. The testicular spermatozoa entering the epididymis are essentially immotile and infertile, and they gain the capacity for these characteristics during epididymal transit (Cornwall, 2009). The individual microenvironments of the epididymis serve to mature spermatozoa functionally. Epididymal defects, including epididymal obstruction and epididymal asthenozoosperma, are important factors for male infertility. The fact that patients with asthenozoosperma can partially address this
problem by a programmed frequent ejaculation suggests that a “hostile” epididymal microenvironment might play a significant role (De Kretser and Baker, 1999). Environmental toxicants accelerate sperm transport through the epididymis (Klinefelter and Suarez, 1997), impair epididymal function (Yan et al., 2009), and alter epididymal structure (Aruldhas et al., 2004). Given that the seminal epididymal biomarker NAG has been associated with a PCB body burden (Elzanaty et al., 2006), the negative effects of PCBs on sperm motility also might be in an epididymal factor–dependent manner.

The reproductive toxicity of PCBs has been extensively studied, exposure to PCBs may target neuroendocrine system (Dickerson et al., 2011) and/or impair testicular functions (Cai et al., 2011). Hypothyroidism induced by exposure to dioxin-like PCB congener in early life also results in persistent decreases in adult testosterone levels and testicular damage (Xiao et al., 2011). In addition, PCBs have been shown to work as “epigenetic” toxicants to act as tumor promoters via their ability to reversibly inhibit gap junctional intercellular communication (GJIC) (Satoh et al., 2003). Gap junctions are also critical for coordinating cellular functions in the testis and epididymis (Cyr, 2011), and the PCB-induced GJIC inhibition might also play a role in male reproductive toxicology. However, the toxicology of the epididymis has received less attention. The fact that PCB exposure has been related to sperm motility in most epidemiological studies suggests that epididymal function is the key target for the adverse effects of PCBs. The aim of the present study was to screen for the sensitive effects and mechanisms of PCBs on the epididymis by analyzing the gene expression profiles and to further evaluate the putative mechanism rising from the expression profiles.

**MATERIALS AND METHODS**

**Animals and treatment.** All animal experiments were conducted according to the research protocols approved by the Xiamen University Institutional Animal Care and Use Committee. Male C57 mice, aged 21 days and weighing 12–14 g, were purchased from Fujian Medical University, China, and housed at 24 ± 1°C under a 12:12-h light-dark cycle, with free access to food and water. After a quarantine period, mice with adequate weight gain and without clinical signs were divided randomly into three experimental groups. Aroclor 1254 of analytical grade purity (lot LB38310; Supelco, Bellefonte, PA) was dissolved in dimethyl sulfoxide (DMSO) in vehicle (5 g/kg). The mice in each treatment were administered with Aroclor 1254 by oral gavage once every 3 days (either 5 or 500 μg/kg doses); control mice received an equal volume of vehicle (5 g/kg). After a quarantine period, mice with adequate weight gain and without clinical signs were divided randomly into three experimental groups. Aroclor 1254 of analytical grade purity (lot LB38310; Supelco, Bellefonte, PA) was dissolved in DMSO in vehicle (5 g/kg). The mice in each treatment were administered with Aroclor 1254 by oral gavage once every 3 days (either 5 or 500 μg/kg doses); control mice received an equal volume of vehicle (5 μl/g). The mice were sacrificed after 50 days of exposure under anesthesia. The epididymis was removed, cleared of adhering connective tissue, and stored at −80°C for further analysis or placed in 4% formaldehyde for 24 h, rinsed with prewarmed PBS. The isolated Cauda epididymis was placed in 1 ml of human tuba fluid (Chemicon, Temecula, CA), cut finely with scissors, and incubated in 5% CO2 atmosphere for 15 min at 37°C. An aliquot of the sperm suspension was placed on a clean glass slide to make a wet preparation for sperm motility evaluation. Evaluation of sperm motility was carried out as previously described (Yan et al., 2009).

**RNA preparation and microarray analysis.** Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Microarray analysis was performed in CapitalBio Corporation (Beijing, China) according to the methods previously described using mouse genome-wide oligonucleotide microarrays (Fu et al., 2012). Briefly, fluorescent dye (Cy3 and Cy5-dCTP)-labeled cDNA was produced through Eberwine’s linear RNA amplification method from 5 μg DNA-treated total RNA. The sample was then hybridized to the *Mus musculus* genome oligonucleotide set (Operon, Huntsville, AL), printed on silanized glass slides using a SMARTArray microarrayer (CapitalBio). Arrays were scanned with a confocal LuxScan TM scanner (CapitalBio), and the data of obtained images were extracted with SpotData software (CapitalBio). The raw data were normalized with a LOWESS program. Genes with a signal intensity (Cy3 or Cy5) > 800 were regarded as the expressed ones. The expression values in log2 scale were analyzed with Significant Analysis of Microarrays (Tusher et al., 2001) to identify differentially expressed genes. The list of genes twofold over- or underexpressed significantly (p < 0.05; false discovery rate (FDR) corrected) was generated.

**Western blotting assay.** Protein was extracted from frozen tissue samples using homogenization, and fraction samples (40 μg proteins) were mixed with 2x coomassie brilliant blue and heated to 100°C in a water bath for 5 min. Next, 10% SDS-PAGE was performed at a constant voltage of 100V for 1.5 h. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane and blocked at room temperature for 2 h in PBS buffer containing 5% nonfat dried milk or 5% bovine serum albumin to prevent nonspecific binding of reagents and then incubated overnight at 4°C with a primary antibody. We obtained antizona occludens 1 (ZO1), anticoordinulin, anti-Kirsten rat sarcoma viral oncogene (K-ras), anti-ras homolog A (RhoA), ras-related C3 botulinum toxin substrate 1 (Rac1), and anti-phospho-myosin light chain (MLC) antibodies from Abcam (Cambridge, MA); anticoillin and antiphosphocollin antibodies from Millipore (Temecula, CA); and anti-cell division cycle 42 (Cdc42), anti-MLC, anti-LIM kinase (LIMK), and anti-phospho-LIMK antibodies from Bioworld Technology (Bioworld). After this, the membrane was washed thrice in PBST for 15 min and incubated with secondary antibody (1:10,000 dilution; Pierce) for 1 h at room temperature. Then the membrane was washed thrice in PBST, and chemiluminescence detection (Sigma, UK) was performed. The intensity of bands was quantified using Quantity One software (Bio-Rad).

**Immunohistochemistry.** Fixed tissues were embedded in paraffin and cut into 6-μm sections. The sections were deparaffinized, rehydrated, and stained following the previously described methods (Cai et al., 2011). To optimize immunohistochemical staining, an antigen retrieval protocol was carried out by immersing the sections in 10M citrate buffer (pH 6.0) and heating in a microwave. The sections were incubated with anti-ZO-1 or anti-occludin antibodies overnight at 4°C in a humidified chamber, and the negative control was incubated in the presence of irrelevant immunoglobulin G instead of the primary antibodies.

**Tracer studies.** For tracer studies, mice were anesthetized with single ip injections of 70mg/kg sodium pentobarbital (Sigma). Epididymides were fixed using vascular perfusion as described previously (Hermo et al., 2007). Briefly, the tissues were first perfused with Ringer’s lactate solution through the heart for 1 min to remove the blood, and then the solution was replaced with 1% La(OH)3 in Ringers’ lactate solution as an intercellular tracer for 2 min. Finally, the tissues continued to be perfused with 1% La(OH)3 and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer containing 0.05% CaCl2, as a precipitate preserving fixative for 10 min. After perfusion, the harvested caput epididymides were cut into 1 mm3 pieces and fixed overnight at 4°C in 0.1M sodium cacodylate buffer containing 2.5% glutaraldehyde. On the following day, the tissues were washed thrice in 0.1M sodium cacodylate buffer.
containing 0.2 M sucrose and left in buffer overnight at 4°C. The tissues were then postfixed in 1.5% potassium ferrocyanide + 1% osmium tetroxide for 1 h at 4°C and subsequently processed for routine electron microscopic analyses. Thin sections were cut with a diamond knife and stained with lead citrate. All electron micrographs were taken with a transmission electron microscope (JEM-2100 HC, JEOL, Japan).

**RESULTS**

**Sperm Motility**

In this study, the Aroclor 1254 exposure led to a significant decline in epididymal sperm motility in 500 µg/kg group (Fig. 1). A decrease in sperm count and testicular weight was also observed in this group. However, the body weights and epididymal weights were not altered. The level of serum estrogen rather than androgen responded to 500 µg/kg Aroclor 1254 exposure was significantly increased (Cai et al., 2011).

**Gene Expression Profiles**

Microarray analysis revealed large sets of differentially regulated genes in mice caput epididymides in response to PCB exposure (Fig. 2A). Using cutoffs of \( p < 0.05 \) adjusted by FDR and at least a twofold change in expression compared with the control, 209 upregulated genes and 361 downregulated genes were identified in mice caput epididymides exposed to 5 µg/kg PCBs. In those exposed to 500 µg/kg PCBs, 430 upregulated genes and 1331 downregulated genes were identified using the same cutoffs. Comparison of the expression profiles using hierarchical clustering indicated that the caput epididymal transcript responses to 5 and 500 µg/kg PCB exposure were similar (Fig. 2B). Most of the differentially regulated genes responded to PCB exposure in a dose-dependent manner.

![Figure 1](https://academic.oup.com/toxsci/article-abstract/134/2/382/1631898)  
**FIG. 1.** Effects of Aroclor 1254 on epididymal sperm motility. Values (means ± SE) are representative of data obtained in four independent experiments (\( n = 4 \)). Treatments not sharing a common letter are significantly different at \( p < 0.05 \) as assessed by one-way ANOVA followed by the Duncan test.

**Functional Annotation**

To extract the biological features with the largest gene sets, functional annotation clustering using DAVID bioinformatics resources was performed. The most enriched biological annotations are presented (Figs. 2C and D). For genes responding to 5-µg/kg PCB exposure, the most overrepresented annotation clusters were nucleosome and ribosome (Fig. 2C). However, the most annotation cluster enriched in the 500 µg/kg exposed group was GTP binding (Fig. 2D). Further analysis showed that many of the differentially regulated genes belonging to the category of GTP binding were important molecular switches and multifunctional small GTPases. Several genes, such as Cdc42, K-ras, guanine nucleotide binding protein alpha inhibiting activity polypeptide 3, N-ras, and M-ras, were involved in the tight junction proteins and in the regulation of actin cytoskeleton, which suggested an alteration in the functioning of epididymal tight junctions.

**Effects of PCBs on Epididymal Tight Junctions**

To further investigate whether the epididymal tight junctions were affected by PCB exposure, mice were perfused with lanthanum nitrate as an intercellular tracer. Under the electron microscope, the tracer filled the intercellular spaces between adjacent caput epithelial cells and extended to the level of the apical tight junction complex (Fig. 3A). In the control group, the tracer does not penetrate beyond the junction complexes (curved arrow), and there is no evidence of lanthanum particles in the epididymal lumen. On the other hand, lanthanum tracer permeating the entire intercellular space, including the apical junction complexes, is observed (arrow) in PCB-exposed mice, suggesting that the tight junctions no longer prevented entry into the epididymal lumen. The distribution of lanthanum particles in the epididymal lumen is also observed (arrow head).

The tight junction protein, zonula occludens-1 (ZO-1), is a cytosolic component of tight junctions interacting with the actinmyosin cytoskeleton, and occluding is related to permeability (Van Itallie et al., 2009). The protein abundance of ZO-1 in caput epididymis decreased significantly in response to PCB exposure (Fig. 3B), and no significant changes were observed in the expression of occludin (Fig. 3B). However, the staining pattern of ZO-1 and occludin on the histological sections did not alter significantly (Fig. 3C).

**Effects of PCBs on Epididymal Actin Cytoskeleton**

As illustrated in Figure 4, PCB exposure resulted in a significant decrease in the protein abundance of Cdc42 and K-ras in mice caput epididymides, which was consistent with the transcript response of these genes. RhoA is another key GTPase in the tight junction pathway, and a decrease in RhoA protein expression was also observed in the PCB-exposed group. With all these genes being downregulated, we hypothesized that the signaling they mediated was disrupted. MLC, LIMK, and cofilin are important targets of the small GTPase signaling in...
regulation of actin cytoskeleton and key regulators of tight junctions (Popoff and Geny, 2009). Although the expression levels of these effectors were unchanged in total protein abundance after the mice treatment with PCBs, the phosphorylation level of MLC increased dramatically in a dose-dependent manner (Fig. 5). MLC phosphorylation alone is sufficient to induce tight junction regulation in the absence of any upstream stimuli (Shen et al., 2006). On the other hand, PCB exposure impaired the phosphorylation level of LIMK and cofilin in a dose-dependent manner (Fig. 5), which might have induced actin cytoskeleton rearrangement and barrier impairment (Nagumo et al., 2008).

Taken together, PCB exposure led to a decreased expression of small GTPases regulating cytoskeleton organization and tight junction function and thus altered the activity of their downstream pathway components such as MLC and LIMK-cofilin, thereby inducing changes in cytoskeleton dynamic and increased tight junction permeability. On the other hand, the impaired tight junction expression might also have contributed to the increased tight junction permeability by disrupting the barrier integrity (Fig. 6).

**FIG. 2.** Effects of Aroclor 1254 on gene expression profiles in mouse caput epididymis. (A) Differentially expressed genes induced by different doses of Aroclor 1254 exposure. (B) Microarray profile showing comparison of epididymal gene response profiles between low and high doses of Aroclor 1254 treatment. The color scale of expression (log_{2} ratio) is shown. (C) Functional annotation clustering in differentially expressed gene in the epididymis from mice exposed to low doses of Aroclor 1254. (D) Functional annotation clustering in differentially expressed gene in the epididymis from mice exposed to high doses of Aroclor 1254.

**DISCUSSION**

The daily PCB intake of animals is related to estimated human daily intake in several reports (Kannan et al., 1992; Zhao et al., 2009) when the dose translation from animal to human is taken into consideration (Reagan-Shaw et al., 2008). Even the high dose which we used, 500 μg/kg, is among the lowest ever used in experimental studies (Dziennis et al., 2008; Yang et al., 2009).

PCBs are noted to decrease sperm motility in a wide range of human studies, and epididymal dysfunction might be involved. However, how PCBs affect the epididymis remains less clear. The maturation process, including acquisition of motility, occurs as spermatozoa transit through the proximal region of the epididymis and depends on the segment-specific activities of the duct (Cornwall, 2009). Characterizing global transcript responses to PCBs in the caput epididymis might yield a clue concerning the potential mechanisms for their toxicological effects. In this study, the epididymal sperm motility of exposed mice suffered a decreasing trend (Fig. 1), and the caput epididymides showed a massive global transcript response to
PCB exposure in both the low- and high-exposure groups. The most overrepresented functional annotation clusters consisted of the genes involved with the nucleosome, protein disulfide isomerase, and metabolism enzymes. These altered genes might suggest impairments in a variety of the fundamental functions of epididymal cells, such as genome transcription, protein synthesis, protein folding, and energy metabolism. The caput epididymis is the most metabolically active region, accounting for 70–80% of the total overall protein secretion in the epididymal lumen (Cornwall, 2009). The impaired fundamental functions may disrupt the capacity of the caput epididymis to maintain the luminal microenvironment required for sperm maturation.

Noticeably, a group of differentially expressed genes related to GTP binding exhibited the highest enrichment score exclusively in the high-exposure group. The group is essentially represented by small GTPases, such as Ras and Cdc42, which work as multifunctional molecular switches that cycle between an active membrane-associated GTP-bound state and an inactive cytoplasmic GDP-bound state, triggering a series of downstream events. Disrupting their signaling may affect a wide range of biological processes, including those linked to actin cytoskeleton dynamics, cell-cell adhesion, gap junction function, vesicular transport dynamics, gene transcription, cell cycle progression, and a variety of enzymatic activities. The regulatory roles that GTPases play in a diverse range of fundamental processes make them interesting candidates for further study.

In the epididymis, epithelial cell-cell interactions are mediated by adhering junctions, gap junctions, and tight junctions (Mital et al., 2011). Tight junctions and adhering junctions are response for the formation of the blood-epididymis barrier (Mital et al., 2011), and gap junctions are also present in the apical region of the epithelial cells and these are associated with the tight junctions of the blood-epididymal barrier (Cyr, 2011). The gap junctions in the epididymis might act in concert with...
PCBs CAUSE EPIDIDYMIS DYSFUNCTION

**FIG. 4.** Western blotting analysis of small GTPase expression in mice epididymis after Aroclor 1254 exposure. Intensities of protein bands were quantified using densitometry. Results were expressed as folds of optical density of the target protein and the β-actin determined in control. The mean protein expression from the control was designated as 1 in the graph. Values (mean ± SE) are representative of data obtained in three independent experiments (n = 3). Treatments not sharing a common letter are significantly different at p < 0.05 as assessed using one-way ANOVA followed by the Duncan test.

**FIG. 5.** Western blotting analysis of MLC, LIMK, and coflin and their phosphorylation status in mice epididymis after Aroclor 1254 exposure. Intensities of protein bands were quantified using densitometry. Results were expressed as folds of optical density of target protein and the β-actin determined in control. The mean protein expression from the control was designated as 1 in the graph. Values (mean ± SE) are representative of data obtained in three independent experiments (n = 3). Treatments not sharing a common letter are significantly different at p < 0.05 as assessed using one-way ANOVA followed by the Duncan test.
with the blood-epididymis barrier in regulating the epididymal luminal milieu (Cyr, 2011). The blood-epididymis barrier contributes to creating a unique luminal microenvironment for sperm maturation by restricting the passage of molecules and cells from entering or exiting the epididymal lumen and thus is critical for male fertility. Collaborating with the selective transport machinery of ions and solutes across the epithelium and the secretive and endocytic functions of the principal cells, the barrier allows the epididymis to concentrate certain inorganic ions and small organic molecules. Changes in the distinct luminal microenvironment might disrupt sperm maturation. In this study, lanthanum nitrate, a well-known intercellular ionic tracer, penetrated some of the apical junctions of epididymal epithelium cells after PCB exposure. This suggested that the regulation of material movement across the epididymal epithelial cells by the blood epididymis barrier was impaired.

Tight junctions between adjacent principal cells are essential structures for the formation and maintenance of the blood-epididymal barrier (Mital et al., 2011). PCBs can disrupt tight junction integrity and permeability in other systems such as the intestinal epithelium (Choi et al., 2010, 2012) and brain capillary endothelium (Seelbach et al., 2010). However, there is little information concerning the PCB impairment proteins comprising epididymal tight junctions. In the epithelium of the caput epididymidis, the expression of occludin and ZO-1 is noted over the apical cell surface of principal cells and might contribute to the function of tight junction (Mital et al., 2011). However, the expression of occludin might not be always essential for forming tight junction in all epididymal regiments (Cyr et al., 1999), whereas ZO-1 is critical for tight junction formation and function (Furuse, 2010). In this study, the decreased expression of ZO-1 might contribute to the increased junctional permeability (Fig. 3).

The regulation of tight junctions is thought to represent a key determinant in material movement through the paracellular pathway across epithelia. It is well recognized that certain small GTPases play a central role in regulating tight junctions (Popoff and Geny, 2009). Disrupting activity of Rho GTPase such as Rho, Rac, and Cdc42 results in selective modulation of tight junction proteins including alternated localization at cell junctions (Bruewer et al., 2004; Gopalakrishnan et al., 1998), aberrant phosphorylation (Gopalakrishnan et al., 1998), and changes in detergent solubility (Jou et al., 1998), and different Rho GTPases appear to exert distinct, even opposing, effects in the process. However, activation and inactivation on each of the GTPases increases paracellular permeability (Bruewer et al., 2004). Hyperactivation of Ras also contributes to changes in distribution and phosphorylation of tight junction proteins (Chen et al., 2000). A subtle balance of Rho/Ras-GTPase activity and interplay between them is required to maintain normal functioning of the epithelium barrier (Popoff and Geny, 2009). The impaired expression of small GTPases and the phosphorylation status of their effectors in caput epididymis after PCB exposure might be the possible cause of the increased blood-epididymis barrier permeability.

The consequence of PCB-induced disruption of blood-epididymis barrier integrity might contribute to the impaired male fertility. The hypothesis that the functional blood-epididymis barrier is critical for male fertility is partially supported by observations of infertile patients. In the caput epididymides of infertile patients with nonobstructive azoospermia (Dubé et al., 2008) or obstructive azoospermia
(Dube et al., 2010), the expression of tight junction components including ZO-1 and claudin-10 is disrupted. Epithelial cell lines derived from the caput epididymis of the obstructive azoospermia infertile patient are unable to form tight junctions (Dube et al., 2010). The expression of tight junction proteins and the members of the Ras protein family, including Ras, RhoA, and Cdc42, is dysregulated in the cells. These defects make the epididymal luminal environment of the patients unlikely to be optimal for sperm maturation and survival even after surgical reconstruction. It might explain why fertility is not restored in all patients after epididymovasostomy. A chronic accumulative exposure to PCBs leading to blood-epididymis barrier impairment might also contribute to the male reproductive health problem in a similar way.

This study has revealed a global transcript response to PCBs at doses relevant to the general population, providing insights into the mechanisms by which PCBs disrupt the epididymis. One of the mechanistic hypotheses generated from the expression data is that PCBs might affect the epididymal function through regulating tight junctions. Our observations indicated that the integrity and permeability of tight junctions were altered after PCB exposure, and this might contribute to the adverse effects of PCBs on male reproduction.

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