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Chronic testicular *Chlamydia muridarum* infection impairs mouse fertility and offspring development†

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Abstract

With approximately 131 million new genital tract infections occurring each year, *Chlamydia* is the most common sexually transmitted bacterial pathogen worldwide. Male and female infections occur at similar rates and both cause serious pathological sequelae. Despite this, the impact of chlamydial infection on male fertility has long been debated, and the effects of paternal chlamydial infection on offspring development are unknown. Using a male mouse chronic infection model, we show that chlamydial infection persists in the testes, adversely affecting the testicular environment. Infection increased leukocyte infiltration, disrupted the blood:testis barrier and reduced spermiogenic cell numbers and seminiferous tubule volume. Sperm from infected mice had decreased motility, increased abnormal morphology, decreased zona-binding capacity, and increased DNA damage. Serum anti-sperm antibodies were also increased. When both acutely and chronically infected male mice were bred with healthy female mice, 16.7% of pups displayed developmental abnormalities. Female offspring of chronically infected sires had smaller reproductive tracts than offspring of noninfected sires. The male pups of infected sires displayed delayed testicular development, with abnormalities in sperm vitality, motility, and sperm-oocyte binding evident at sexual maturity. These data suggest that chronic testicular *Chlamydia* infection can contribute to male infertility, which may have an intergenerational impact on sperm quality.

Summary sentence

Testicular *C. muridarum* infection results in tissue damage and poor sperm quality and gives rise to offspring with serious abnormalities and developmental delays.

Key words: *Chlamydia*, STI, male infertility, sperm DNA damage, offspring development.

Introduction

Chlamydia trachomatis is becoming more widely recognized as a pathogen of the male reproductive tract (MRT) and contributing to male infertility. With approximately 131 million new infections occurring worldwide each year, the potential impact of such a common sexually transmitted infection on the reproductive potential of the population is significant [1]. Many bacterial and viral infections have been found in semen and tissues of the MRT in association with male infertility, including *C. trachomatis* [2–4]. Despite male infertility accounting for 40–50% of all infertility diagnoses [5], the pathophysiology of MRT infections in causing infertility are not well characterized, other than association with sperm parameter changes [6–8]. Hypotheses for causative mechanisms include direct interactions between the infectious agent and spermatozoa, impairment of MRT tissue health and functionality, and disruption of embryogenesis [8, 9].

Testicular tissue and sperm health are important factors in clinical determination of male fertility [8, 10]. A healthy testis, as the site of sperm production (spermatogenesis), is vital to male reproduction. There are several key cell types involved in spermatogenesis in the testes; Sertoli cells (immune suppressive cells that form the blood:testis barrier and provide nutritional support for spermatogenesis), Leydig cells (interstitial androgen producing cells), spermatogenic stem cells (progenitor cells from which all sperm arise), peritubular myoid cells (basement membrane forming cells), and testicular macrophages (potently immunosuppressive cells). The blood:testis barrier and immune privilege are critical for fertility, as breakdown can result in the generation of autoimmune T and B lymphocytes, and production of anti-sperm antibodies [11]. This leads to autoimmune-mediated testicular damage, germ cell loss, and impaired sperm function [9, 11].

Some infertility or subfertility etiologies can result in dysfunctional spermatogenesis with reduced sperm count (hypospermatogenesis), rather than azoospermia, which is the absence of sperm [12]. These can result in seminal abnormalities that include low sperm count (oligozoospermia), abnormal morphology (teratozoospermia), and reduced motility (asthenozoospermia) [13]. However, combinations of such defects in the same sample are common in male factor infertility [14, 15]. In addition, sperm DNA fragmentation, which is frequently associated with oligoasthenozoospermia, and directly linked with embryogenesis failure and adverse pregnancy outcomes, can occur [14, 16].

The downstream consequence of poor spermatozoa quality is a difficulty or failure of conception. However, the impact of paternal health, including infection, on embryo and offspring health is an emerging field. While models of the impacts of paternal infection on offspring health are limited, they include *H. pylori* transmission between parents and children [17], and potential links between paternal HIV [18] and hepatitis B virus [19] infection and poor offspring health. To date, only one rat model of *Chlamydia muridarum* (a murine-specific pathogen) infection of males has been used to investigate links between infection, fecundity, and embryonic loss and found no adverse effects [20]. Conversely, models of maternal infection-induced effects on fertility include a multitude of viral [21–30], parasitic [31–34], and bacterial [35–39] pathogens. Many of these included longitudinal and mechanistic studies, which are completely lacking in the paternal field.

We have previously shown using a mouse model that *C. muridarum* colonizes the testes for up to 10 weeks post intra-penile infection [40, 41]. This study demonstrated that the viability of Sertoli and spermatogenic stem cells is compromised during acute infection. The downstream effects included decreased sperm count and motility and increased morphologically abnormal sperm [40]. This correlates with several studies of acute chlamydial infection of human semen, with findings of oligospermia [7, 42, 43], teratozoospermia [7, 44], and asthenozoospermia [7, 44]. While *Chlamydia* infections have been detected in human semen [45], baboon urethra [46], koala urethra, prostate, epididymis and testis [47] and mouse urethra, bladder, and prostate [48], the effects of a long-term (chronic) infection on sperm quality in both animal models and humans are unknown and the effects on offspring are undetermined.

This study aimed to determine whether a chronic testicular *C. muridarum* infection could be established in a mouse model and to establish whether infection affects testicular cells, spermatogenesis, fecundity, and offspring development.

Materials and methods

Ethics statement

All mice were housed under PC2 conditions and provided food and water ad libitum, in accordance with the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes. These experiments were approved by Queensland University of Technology Animal Ethics Committee (1400000250) and the University of Newcastle Animal Ethics Committee (A-2014-412).

Mice

Mice were purchased as 6 week old C57BL/6 male mice and 12 week old C57BL/6 female mice which had previously birthed one litter (fertility validation), from the Animal Resource Centre (Canning Vale, WA, AUS).

In vivo *Chlamydia* infection

Male mice were anaesthetized with 10 mg/kg xylazine (Bayer, NSW, AUS) and 100 mg/kg ketamine (Parnell Laboratory, NSW, AUS) and then infected with 1×10^6 inclusion forming units (IFU) of *C. muridarum* (Weiss strain, generously gifted by Dr. Catherine O'Connell, University of North Carolina). Mice were infected via the intra-penile route, as described previously [41, 49].

Sperm analysis

Sperm was collected from male mice 6 months post intra-penile infection by dissecting out the vas deferens and gently pushing out the compacted sperm along the tubes with closed sterile forceps into Biggers, Whitten, and Whittingham (BWW) media [50] at 290–310 mOsm (Fisk 210 Osmometer, John Morris Group, Australia). Sperm was incubated for 20 min in BWW media at 37 °C, 5%

CO_2. Immediately following swim-up, 1×10^6 sperm was moved to capacitating BWW media and incubated for 20 min, 37 °C, 5% CO_2.

Capacitated sperm was assessed in a hemocytometer to quantify the number of progressively motile sperm per milliliter. Capacitated sperm was also incubated in droplets of BWW with C57BL/6 mouse oocytes, collected as previously described [51], for 10 min. The sperm-oocyte aggregates were washed to dislodge unbound sperm, and then the remainder was viewed using light microscopy to quantify the number of bound sperm for the zona pellucida binding assay [52].

Capacitated and noncapacitated sperm was heat and methanol fixed onto glass microscope slides for staining purposes. Slides were stained with eosin Y and methylene blue to assess morphology. Separate slides were blocked (3% BSA v/v in PBS, 1 h at room temperature), stained with mouse anti-phosphotyrosine IgG (1:100, ab10321, Abcam, VIC, AUS), washed twice in PBS, stained with secondary anti-mouse IgG-Alexa Fluor(AF)488 (1:1000, A11059, Life Technologies, VIC), washed twice in PBS, and imaged using epifluorescence microscopy (Zeiss Axio Vert.A1, Göttingen, DE) to quantify capacitation as described by Asquith et al. [53].

Sperm was also set into low-melting point agarose on sperm chromatin dispersion assay (SCDA) slides at 4 °C for 5 min, and then slides were placed in lysis buffer for 5 min, washed in RO water for 5 min, fixed in 80% v/v ethanol for 2 min and 100% v/v ethanol for 2 min as per the manufacturer's instructions (Halotech, Spain), and then stained with SYBR Safe DNA Gel Stain (S33102, Invitrogen, AUS) mixed with VECTASHIELD mounting medium (Vector Laboratories, CA) (1:1 v/v). Stained sperm was assessed for DNA fragmentation using epifluorescence microscopy [54].

Chlamydial culture

C. muridarum for mouse infection was grown and purified by established protocol [55]. *C. muridarum* was cultured from mouse testes 6 months post-infection by homogenizing whole testes for 30 s using a tissue homogenizer with a blade attachment (TH220, DMNI International, Kennesaw, GA) into 500 µL of sucrose phosphate glutamine (SPG, 74.6 g/L sucrose, 0.512 g/L KH_2PO_4, 1.237 g/L K_2HPO_4, 5 mM L-glutamine) and quantified by infection of McCoy cell monolayers (ATCC Cat: CRL-1696) grown in complete RPMI 1640 (10% heat-inactivated fetal calf serum, 2 µg/mL gentamycin, and 100 µg/mL streptomycin sulfate, 1× Glutamax) at 37 °C, 5% CO_2, for 24 h. Monolayers were fixed with 100% methanol, washed twice with PBS and blocked for 1 h with 5% heat-inactivated fetal calf serum in PBS. Monolayers were incubated with primary sheep anti-MOMP sera (1:500 v/v, [56]) diluted in blocking solution for 1 h, washed twice with PBST, and incubated for 1 h with secondary donkey anti-sheep IgG-AF488 (2 µg/mL, Thermo Fisher Scientific, AUS). Images were captured with epifluorescence microscopy (Zeiss Axio Vert.A1).

Tissue histology and immunohistochemistry

Testes were harvested 6 months post-infection and placed into either Tissue-Tek O.C.T. Compound (Pro-Sci-Tech, AUS) and frozen at −80 °C or fixed in Bouin's solution then paraffin-embedded. Tissue blocks were sectioned (4 µm) onto hydrophilic, poly-D-lysine coated glass microscopy slides (Thermo Fisher Scientific). O.C.T tissues were fixed in 100% acetone, blocked in 5% v/v FCS in PBS, and then stained. Chlamydial MOMP staining was performed as described above. Chlamydial TC0500 staining was performed using the same method, with primary sheep anti-TC0500 IgG (Recombinant TC0500 antigen produced in house) and secondary donkey anti-sheep-IgG-AF488 (Life Technologies). Alternatively, sections were hematoxylin and eosin stained for tubule number analysis. Bouin's fixed tissues underwent antigen retrieval (all in Tris buffer pH 10, except ZO-1: sodium citrate buffer pH 6), blocking (3% BSA and 10% goat serum in TBS-0.1% tween), and staining for Claudin 11 (1:100, Thermo Fisher Scientific; 1:200 goat anti-rabbit IgG AF-555, Thermo Fisher Scientific), Zona Occludin 1 (1:100, Abcam; 1:200 goat anti-rabbit IgG-AF555, Thermo Fisher Scientific), Connexin 43 (1:50 Abcam; 1:200 goat anti-rabbit IgG-AF555, Thermo Fisher Scientific), Connexin 37 (1:50, Alpha Diagnostics International; goat anti-rabbit IgG-AF555, Thermo Fisher Scientific), and Connexin 26 (1:50, Thermo Fisher Scientific; goat anti-mouse IgG-AF555, Thermo Fisher Scientific). Images were captured using epifluorescence microscopy (Zeiss Axio Vert.A1).

Anti-sperm antibodies

Whole blood was collected via cardiac bleed and then serum collected post centrifugation. Enzyme linked immunosorbent assay (ELISA) was used to determine the titer of anti-sperm antibodies in the serum. The anti-sperm antibody (ASAB) ELISA was sourced commercially from MyBioSource (MBS2603817) and was performed as per the manufacturer's instructions.

Mouse breeding

Infected (n = 4) and noninfected (n = 4) male mice were housed for either 4 weeks, 8 weeks, or 5 months and then bred with two-four noninfected C57BL/6 females each (n = 12 per group total). Females were monitored for the presence of a vaginal plug to indicate successful mating. Pregnant females were housed individually during gestation (approximately 20 days) and then with newborn litters. At 7 days post birth, the pups were tagged for later identification, weighed, and sexed by external examination of the developing urethra, anogenital distance, and teat marks and confirmed by an experienced, skilled animal care technician. Pups were then weighed every 2–3 days until the appropriate time points (euthanasia by sodium pentobarbitone, 200 mg/kg, 47814, Virbac, NSW). The male testes and whole female reproductive tracts of the pups were investigated for morphology and function at 11–12, 21, and 42–46 days post birth. The reproductive tissues were weighed post dissection (using a dissecting microscope to remove surrounding fatty tissue) and the organ:body weight ratio calculated.

Female pup reproductive tracts were placed into Tissue-Tek O.C.T. Compound (Pro-Sci-Tech, AUS) and frozen at −80 °C. Frozen tissue blocks were cryosectioned (4 µm) onto hydrophilic, poly-D-lysine coated glass microscopy slides stained with hematoxylin and eosin and then visualized with light microscopy.

The testes of male pups were placed into Tissue-Tek O.C.T. Compound (Pro-Sci-Tech, AUS) and frozen at −80 °C. Frozen tissue blocks were cryosectioned (4 µm) onto hydrophilic, poly-D-lysine coated glass microscopy slides stained with hematoxylin and eosin and then visualized with light microscopy. The sperm from male pups was also isolated from the vas deferens when they reached sexual maturity at 42–46 days post birth. Sperm was isolated and analyzed as described previously. Additionally, the sperm count was assessed using a hemocytometer viewed under light microscopy.

Statistical analysis

Sperm parameters and anti-sperm antibodies were analyzed using GraphPad Prism (v7) software. To compare the differences in

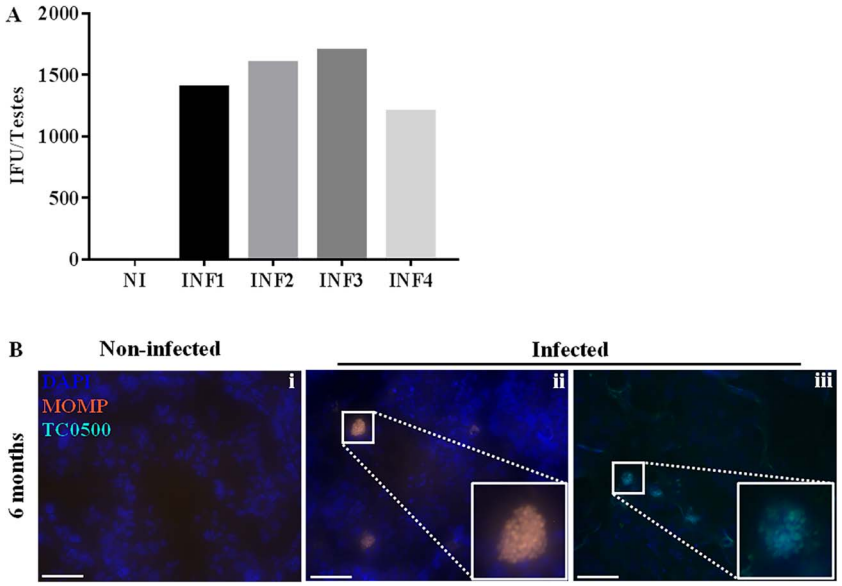

Figure 1. Testicular *Chlamydia* burden at 6 months post-infection. Six months after intra-penile infection, *C. muridarum* can be found in the testes by (A) culture of live infection and (B) immunohistochemistry. Chlamydial inclusions were detected using MOMP (Bii, orange, example highlighted by white box) and active replication marker TC0500 (Biii, aqua, example highlighted by white box), and a DAPI nuclear counterstain was also used (blue). Images were captured on 40× objective and are representative of $n = 4$, and scale bars represent approximately 50 μm.

infected versus noninfected mice at single time points, unpaired, nonparametric, Student's *t*-tests were applied. In the breeding trials, multiple comparisons two-way-ANOVAs were applied to compare sperm parameters over multiple time points, viability of pups was compared using a Chi-squared test (GraphPad Prism v8), and pup weight was analyzed using a linear mixed model accounting for "litter effects" (IBM SPSS Statistics). Histology and immunohistochemistry (IHC) parameters were analyzed using bootstrapped, independent samples, unpaired, Student's t-test (SPSS). The level of statistical significance for all tests was set at $P \leq 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Results

C. muridarum chronically colonizes mouse testes

MRT infection in mice has previously been demonstrated to persist for at least 3 months post-infection, long after infection has naturally resolved in females. To determine the effects of chronic chlamydial infection in males, mice were infected via the urethra and housed for 6 months. The mice showed no symptoms of distress or orchitis, in line with human data showing men had no history of overt testicular infection or inflammation [57]. Viable *C. muridarum* was detected 6 months post-infection by culture of testicular homogenate on McCoy cells. Chlamydial growth was further validated by IHC. Live *C. muridarum* was cultured from testis tissue ($n = 4$, Figure 1A) at titers between 1200 and 1700 IFUs/testis, giving an average of 1475 IFUs/testis. Immunohistochemical detection of chlamydial major outer membrane protein (MOMP, orange, Figure 1Bii) and early inclusion membrane protein TC0500 (CT229 homolog, green, Figure 1Biii) also showed multiple actively replicating reticulate bodies, in inclusions within testicular tissue. No *C. muridarum*-positive staining was identified in any of the noninfected testes by either culture or IHC. Taken together, these data demonstrate that viable and replicating *Chlamydia* can establish a chronic infection in the immune-privileged testes.

Chronic *C. muridarum* infection alters testicular structure

To determine if chronic testicular infection affected testicular morphology and function, H&E and IHC stainings were performed. Histological examination of H&E stained tissue showed that the luminal area of the seminiferous tubules was significantly reduced per mm^2 in infected testes compared to healthy testes ($P < 0.0001$, Figure 2A). H&E stained tissue also showed significantly increased numbers of white blood cells (WBCs) per mm^2 in infected testes compared to healthy testes ($P < 0.001$, Figure 2B). WBCs were identified in both the interstitial and epithelial/luminal spaces. Immunohistochemical detection of spermatogonial cells (marked by PCNA) showed a significant reduction in infected compared to healthy testes ($P < 0.05$, Figure 2D). Representative images of PCNA staining can be seen in Supplementary Figure S1. Immunohistochemical detection of tight and gap junctions within the blood:testis barrier (marked by Claudin 11 and ZO-1, and Connexin 43, respectively) showed substantial reduction in positive signal in infected testes. Representative images show tubules of similar stages for comparison. Similarly, Connexin 37 and 26 staining showed disruption of the basement membrane (Supplementary Figure S1). Chronic testicular chlamydial infection is associated with consolidation of the seminiferous tubule lumen, enhanced immune cell infiltration, and potentially compromised blood:testis barrier integrity.

Anti-sperm antibodies are produced by chronically infected mice

Chronic chlamydial infection of the testes was associated with pathological and morphological changes. To determine if breakdown of the blood:testis barrier and leukocyte infiltration led to the development of markers associated with male infertility, the impact of tight-junction loss in relation to the maintenance of immune privilege within the testis was investigated. Sperm isolated from the vas deferens of infected mice exhibited head-head agglutination. Representative images of sperm agglutination are shown in Figure 3B.

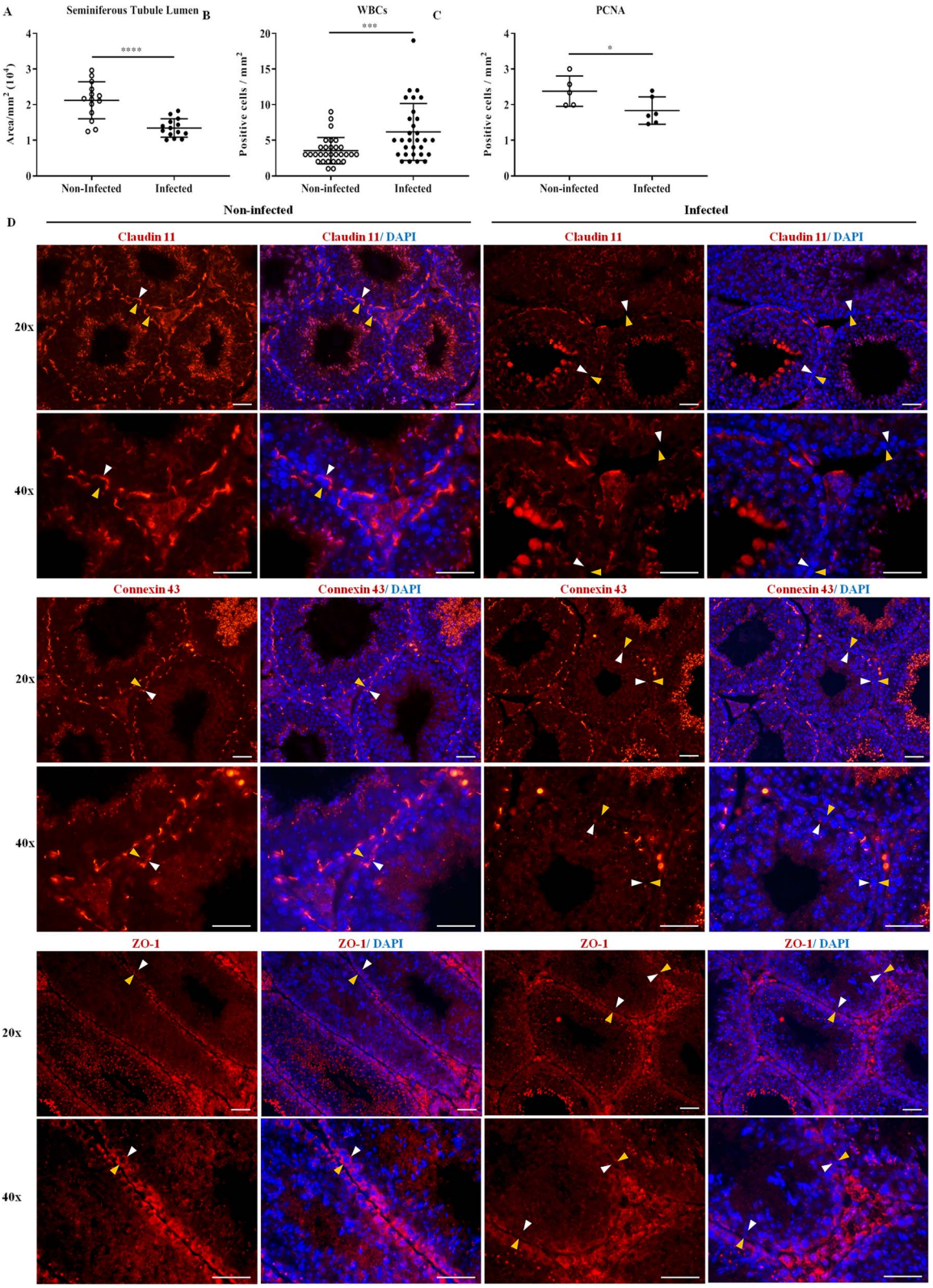

Figure 2. Testicular histology at 6 months post-infection. Six months after intra-penile infection with *C. muridarum*, testes were harvested and stained with haematoxylin and eosin, and specific markers. Significant differences were found in (A) area of the seminiferous tubule lumen (B) white blood cells (WBCs), and (C) spermatogenic cells marked by proliferating cell nuclear antigen (PCNA). Staining of the blood:testis barrier (D) marked by Claudin 11, Connexin 43, and Zona Occludin 1 (ZO-1) also showed histopathological changes (white arrows indicate presence/absence of blood:testis barrier, yellow arrows indicate Spermatogonia). Student's *t*-tests and graphs were generated with GraphPad Prism (v7), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, images representative of $n = 5$ mice, scale bars = 20 μm.

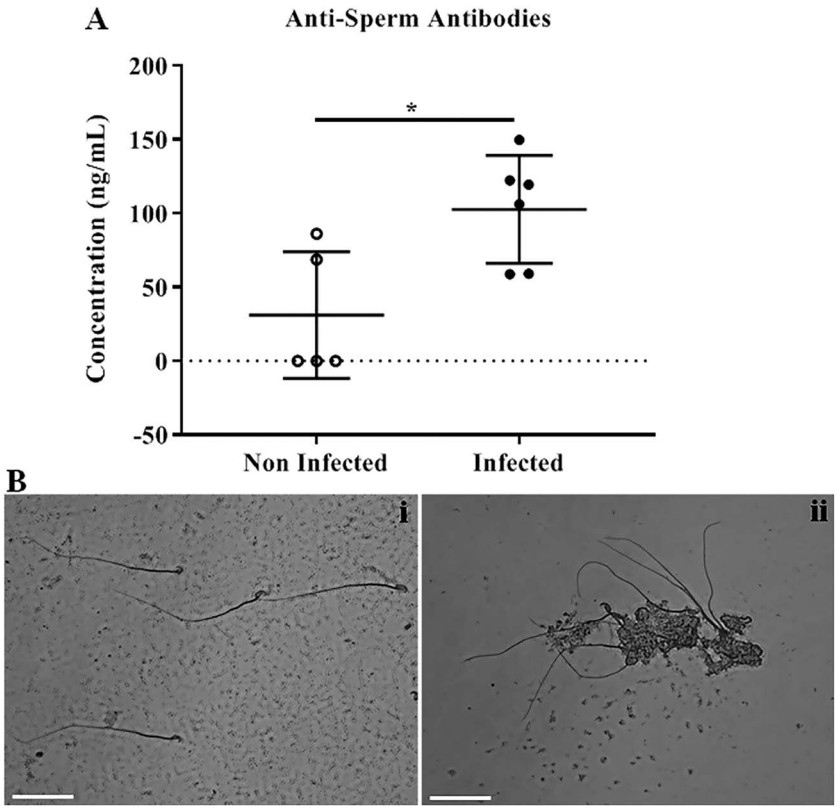

Figure 3. Titres of anti-sperm antibodies present in mouse serum at 6 months. Six months post intra-penile infection with *C. muridarum*, anti-sperm antibodies are significantly elevated compared to the noninfected control. Antibodies were measured by ELISA (A, MyBioSourse.com, CN, MBS2603817) and high titres were associated with sperm agglutination in infected mice (Bii) that were not present in healthy mice (Bi). Representative images, ×20 Mag, are of sperm isolated from the vas deferens of mice viewed by brightfield microscopy (Zeiss Axio Vert.A1, Zeiss Microscopy, DE); scale bars represent 50 µm. Student's *t*-test and graph was generated using GraphPad Prism (v7), $^*P < 0.05$, $n = 5$.

The sperm agglutination was associated with a significant ($P < 0.05$) detection of anti-sperm antibodies (ASAB) present within infected mouse serum (average 100 ng/mL, Figure 3A). Both agglutinations of sperm and ASAB were greatly reduced or absent in noninfected mice (average ASAB 25 ng/mL). Enhanced ASAB production supports breakdown in immune-privilege induced by chronic chlamydial infection.

Chronic *C. muridarum* infection decreases sperm quality

To determine the downstream effects of testicular cell and tissue changes during chronic infection, the spermatozoa produced by these testes (retrieved from the vas deferens/cauda epididymides) were assessed. Several sperm parameters were significantly impacted in infected compared to noninfected mice. The forward progressive motility was reduced from 72.7 to 48.8% ($P < 0.05$, Figure 4A). Abnormal morphology (Figure 4B) (i) including (ii) head defects, (iii) tail defects, (iv) principal piece defects, (v) head-tail connection defects, and (vi) combinations of each type of defect increased from 12 to 26.3% ($P < 0.001$). Zona-pellucida binding was reduced (Figure 4Ci–ii, $P < 0.05$). There were, on average, five sperm bound per oocyte in the noninfected sperm, compared to one sperm bound per oocyte in the infected sperm. Sperm isolated from infected mice was significantly more likely to contain fragmented DNA (24%) than the healthy counterparts (Figure 4Di, $P < 0.01$). Representative examples of intact and fragmented sperm DNA are also shown (Figure 4Dii–iii). Sperm with DNA fragmentation was detected in the noninfected samples. However, the proportion remained below ~12% and was comparable with levels of detection in healthy sperm [6]. Lastly, sperm from infected mice also displayed premature capacitation, measured by tyrosine phosphorylation (Figure 4E). When isolated from noncapacitating BWW media, 71.2% of infected group sperm was already capacitated compared to 25.1% of controls ($P < 0.05$). When transferred to capacitating BWW media, infected sperm did not undergo further capacitation (77%, $P = 0.6905$), whereas an increased proportion of noninfected control sperm did capacitate on transfer to capacitating BWW media (63.9%, $P < 0.05$). Taken together, chronic chlamydial infection of the testes is associated with attenuation of normal sperm function including aberrant sperm motility, morphology, spontaneous capacitation, DNA damage, and oocyte-binding.

C. muridarum infected sires produce pups with developmental delays

As male infection was associated with morphological and functional changes in spermatogenesis and male fertility indicators in vitro and ex vivo, the effect of infection on fertility was evaluated in vivo. Dams were successfully mated with either infected or noninfected sires at three time points (1, 2 and 6 months post-infection). Dams were not infected during this process [58]. There was no difference in the time to conception, the rate of vaginal plug formation, or the plug:pregnancy ratio between the breeding conditions. Two dams

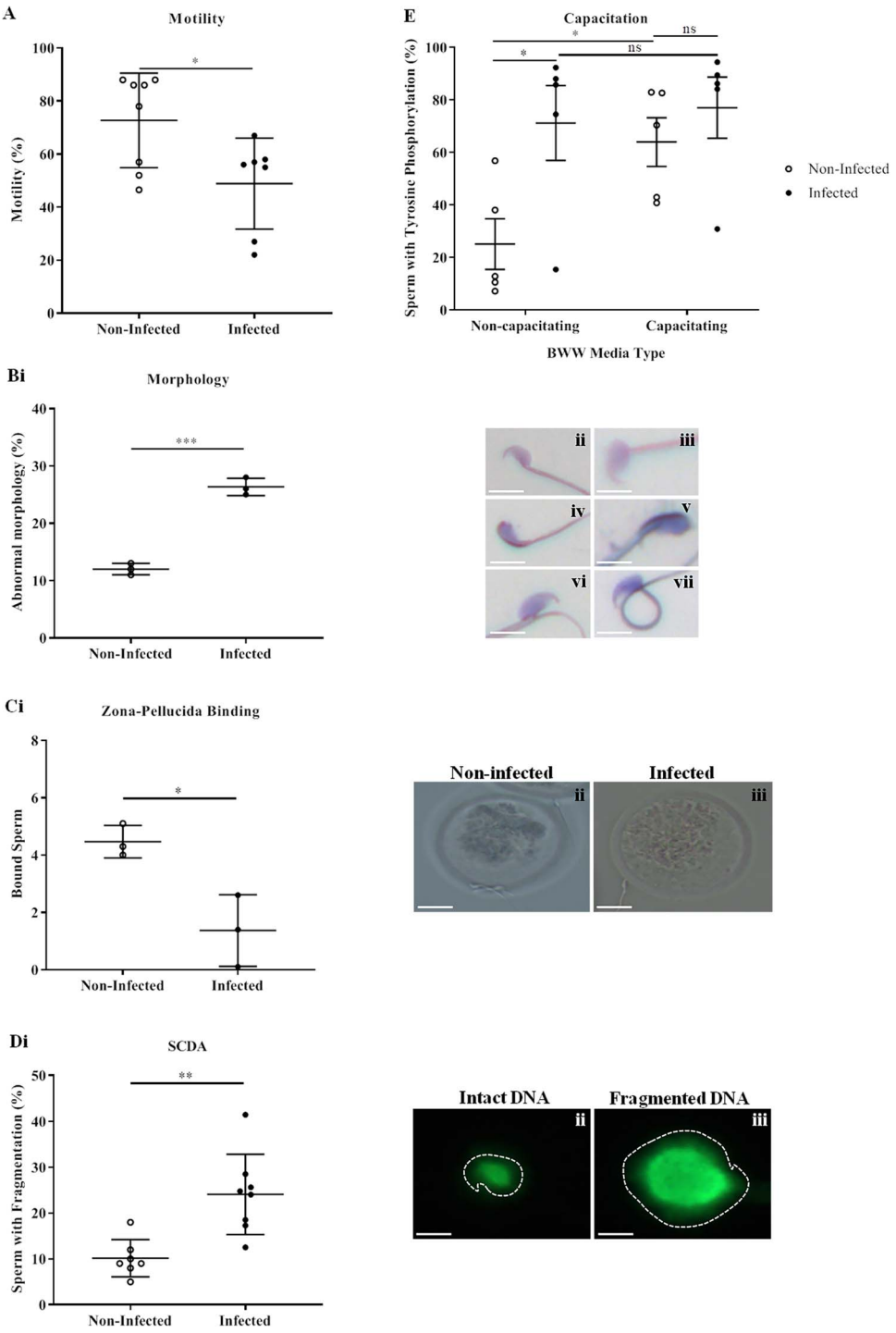

Figure 4. Sperm parameters affected by testicular *C. muridarum* infection. Six months post intra-penile infection, sperm isolated from the vas deferens of infected mice had significantly (A) reduced forward progressive motility, (Bi) increased amorphic sperm including (iii) minor and (iv) major head defects, (v) incorrect tail connections, (vi) folded principal pieces, and (vii) combinations of head and tail defects, compared to normal sperm (ii), (Ci–iii) decreased oocyte binding, and (Di–iii) elevated DNA fragmentation measured by the sperm chromatin dispersion assay (SCDA) when compared to healthy mouse sperm. The sperm was imaged at ×20 magnification (BX41 Microscope, Olympus, USA). Scale bars represent 5 μm (B and D) or 50 μm (C). Student's *t*-test was used to analyze each parameter comparing the infected to the noninfected control samples ($n = 3–7$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

housed with 1-month infected males failed to conceive over a 4 week time course. When placed with noninfected sires, they conceived within 4 days. One dam failed in the 6-month trial failed to conceive under either condition. Successful pregnancies progressed normally with no difference between the group gestation times.

On the day of birth, pup viability was checked nonintrusively so as not to cause undue stress. There were two deceased litters found with dams from the 6-month infected group. Pup viability was checked again 7 days post birth at the first available time that pups could ethically be handled (Table 1). The two failure-to-conceive and

Table 1.

Day 7/21* Viability	1 month		2 months		6 months		Total	
	Noninfected	Infected	Noninfected	Infected	Noninfected	Infected	Noninfected	Infected
Viable	12	10	12	12	11	10/8	35	32/30
Nonviable	0	2	0	0	0	2/4	0	4/6
P value#	0.17		N/A		0.15/0.05		0.05/0.05	

*Day 21 pup viability when different to day 7.
#*P* value generated in GraphPad Prism (v8) with Chi-squared test.

two deceased litters were recorded as nonviable at day 7. In the viable litters, there was no difference between the number and sex of pups per litter (6 months only, Figure 5B).

At 7 days post-birth, pups were weighed for the first time. At this time, only the 6-month group showed that sire infection caused a significant reduction in pup weight ($P < 0.05$). Pups were then weighed every 2–3 days, revealing no difference in the 1- and 2-month groups; however, there was a consistently significantly lower average pup weight in the 6-month infected group until day 21 (weaning, Figure 5A) compared to controls. At day 21, weaning occurred in all groups, and viability was reassessed (Table 1). At weaning, two litters from the 6-month infected breeding group, in line with animal ethics requirements, were euthanized. Several criteria determined this; each pup in the two litters was lighter by approximately 20% than the average noninfected breeding group pup, each pup was unable to provide food and water for themselves, and each pup exhibited significant developmental delay compared to the controls.

At 14 days post-birth, developmental abnormalities were evident in pups from 6-month infected sires. In the representative photographic examples in Figure 5Ci–ii, at day 14, all noninfected group pups opened their eyes whereas all the infected group pups' eyes remained closed. Similarly, at day 14, the pup crown-rump lengths were measured, and within the 6-month infected group the pups were shorter (Figure 5Ciii–iv). These abnormal development trends were observed within all litters born to the 6-month infected group, not limited to the particularly unhealthy litters.

Pups sired by *C. muridarum* chronically infected sires have attenuated reproductive tract development

At day 42 post-birth, the female pup reproductive tracts (FRTs, whole tissue between the cervix-ovary) were weighed. There was a significant reduction ($P < 0.01$) in the 6-month infected breeding group compared to the noninfected breeding group pups (Figure 5D). When normalized to the body weight to achieve an FRT:body weight ratio, this trend was less pronounced ($P = 0.0682$, Figure 5E). The representative images show histological sections of the FRTs, after being stained with H&E (Supplementary Figure S3A). These images show the structure and vaginal glands, which are a healthy functioning indicator, in both breeding groups.

The testes of the male pups were harvested at day 11, at which time spermatogenesis and spermatogenic cell meiosis are known to occur. There was a significant difference in the weight (Figure 5F) and the testis:body weight ratio (Figure 5G) between the 6-month breeding groups, where the infected group pups had significantly smaller testes ($P < 0.001$) and a significantly reduced testis:body weight ratio ($P < 0.01$). The differences between the groups at the later time-points of day 21 and 42 were nonsignificant (Supplementary Figure S3B). However, these time-points were unable to include the euthanized abnormal pups, so they represent a sub-population of testes from mice with infected sires. Histological sections from the testes harvested at day 21 were stained with H&E (Supplementary Figure S3C) and displayed a significantly decreased number of seminiferous tubules/mm^2 in the infected group (Figure 5H, $P < 0.01$).

Pups sired by *C. muridarum* infected sires have lower quality sperm

Sperm from the pups of sires infected for 1, 2, or 6 months prior to mating was analyzed. The sperm from the male pups was analyzed at day 42 as the earliest time-point when sperm can be collected from the cauda epididymis and vas deferens. The pups of sires infected for 6 months had reduced sperm counts compared to the pups of healthy sires ($P < 0.05$, Supplementary Figure S4). Pups from 6-month sires also had the lowest sperm vitality compared to the 1- and 2-month groups, and the lowest overall vitality resulted from chronic 6-month sire infection ($P < 0.05$) compared to healthy counterparts (Figure 6A). Forward progressive motility (Figure 6B) was consistently reduced in pups from infected sires, significantly so in the 2-month group ($P < 0.01$).

When sperm from pups was used in oocyte-binding assays, noncapacitated sperm was unable to bind oocytes in significant numbers in all groups. When sperm was moved to capacitation media, positive oocyte binding was recorded for all noninfected groups, but only the one ($P < 0.0001$) and 2-month groups ($P < 0.0001$), not the 6-month group ($P > 0.05$), for infected groups. The number of sperm bound to oocytes in the 1- and 2-month groups was also significantly lower in infected conditions compared to noninfected controls. There were also significant decreases in the amount of sperm bound to oocytes as age of sires increased, between the 1- and 2-month groups ($P < 0.05$), the 1- and 6-month groups ($P < 0.0001$), and the 2- and 6-month groups ($P < 0.0001$) under both noninfected and infected conditions. Thus, chronic male infection can attenuate the normal development of offspring and has an effect on male progeny sperm function. Furthermore, spermatozoal function declines with the duration of sire infection.

Discussion

Chlamydia can be found in the testes of mice 6 months after intrapenile infection, and this exceeds the previous studies of mouse chlamydial infection chronicity [40]. The health of the testicular environment is compromised by chronic infection. Significant loss of the key blood:testis barrier structure occurred during infection and was associated with immune cell infiltration and anti-sperm antibody production. The altered testicular histology impacted

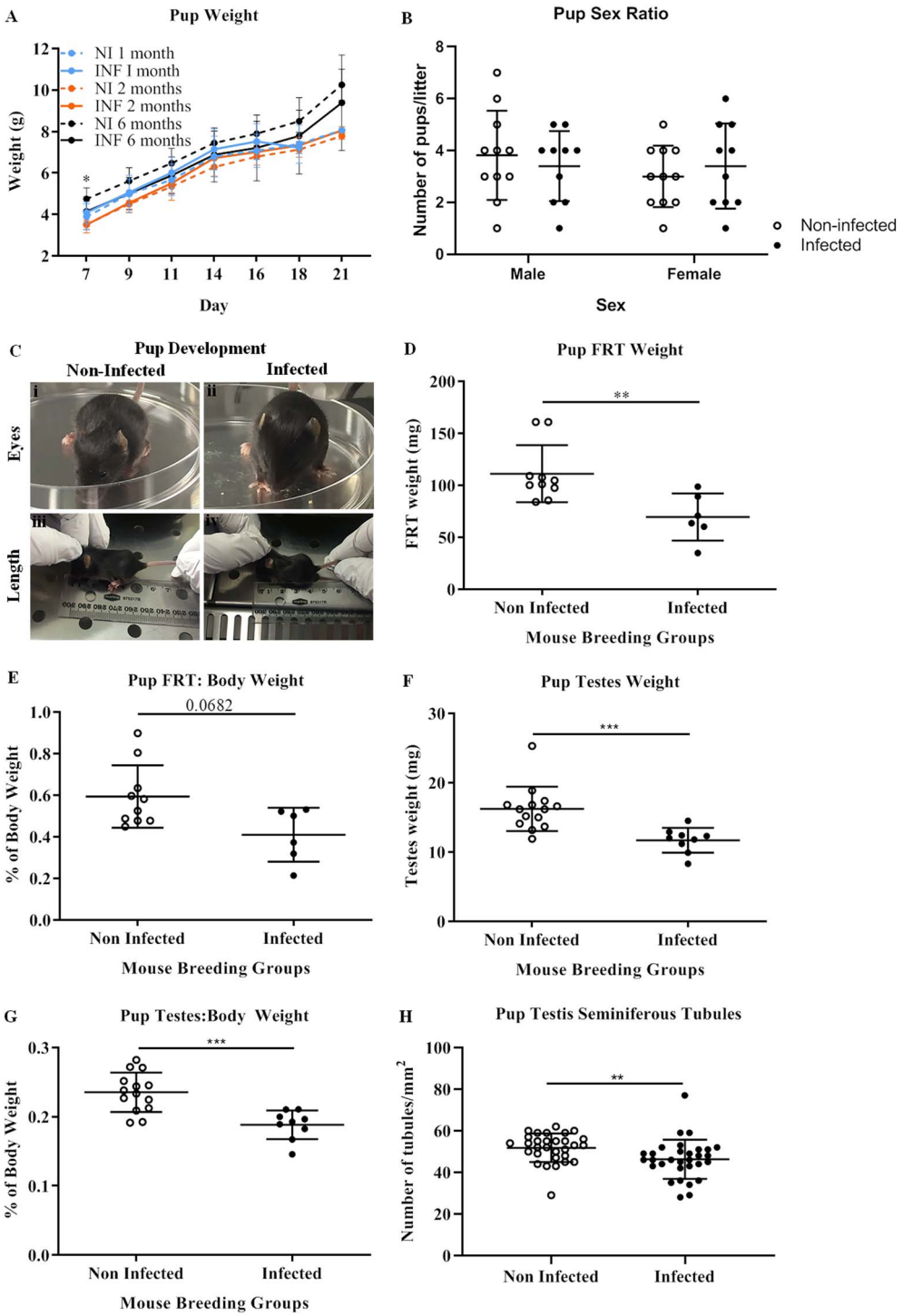

Figure 5. Pup development parameters affected by having *C. muridarum* infected sires. Infected sires gave rise to pups with (A) altered weight and weight gain (1, 2, and 6 months), (B) unaltered sex ratio, and altered (C, day 14) developmental time lines of "eye opening" and length, female reproductive tract (D, day 42) weight and (E, day 42) weight:body weight ratio, MRT (F, day 11) weight and (G, day 11) weight:body weight ratio, and (H, day 21) testes with fewer seminiferous tubules (6 months only). Student's *t*-test was used to analyze each parameter comparing the infected to the noninfected breeding groups ($n = 11–12$), $^{*}P < 0.05$ (B, corresponds to 6 months), $^{**}P < 0.01$, $^{***}P < 0.001$).

spermatogenesis, causing significantly decreased sperm quality. Sperm morphology, motility, DNA integrity, capacitation, and oocyte binding capability were all significantly adversely impacted by infection, indicating a major dysregulation of spermatogenesis. While this does not represent overt infertility, reductions correlated with decreased offspring viability and development of abnormal sperm in surviving offspring.

The chlamydial inclusions identified by immunohistochemistry contain actively replicating *Chlamydia*, demonstrated by MOMP and TC0500 staining. Additionally, viable *Chlamydia* was cultured

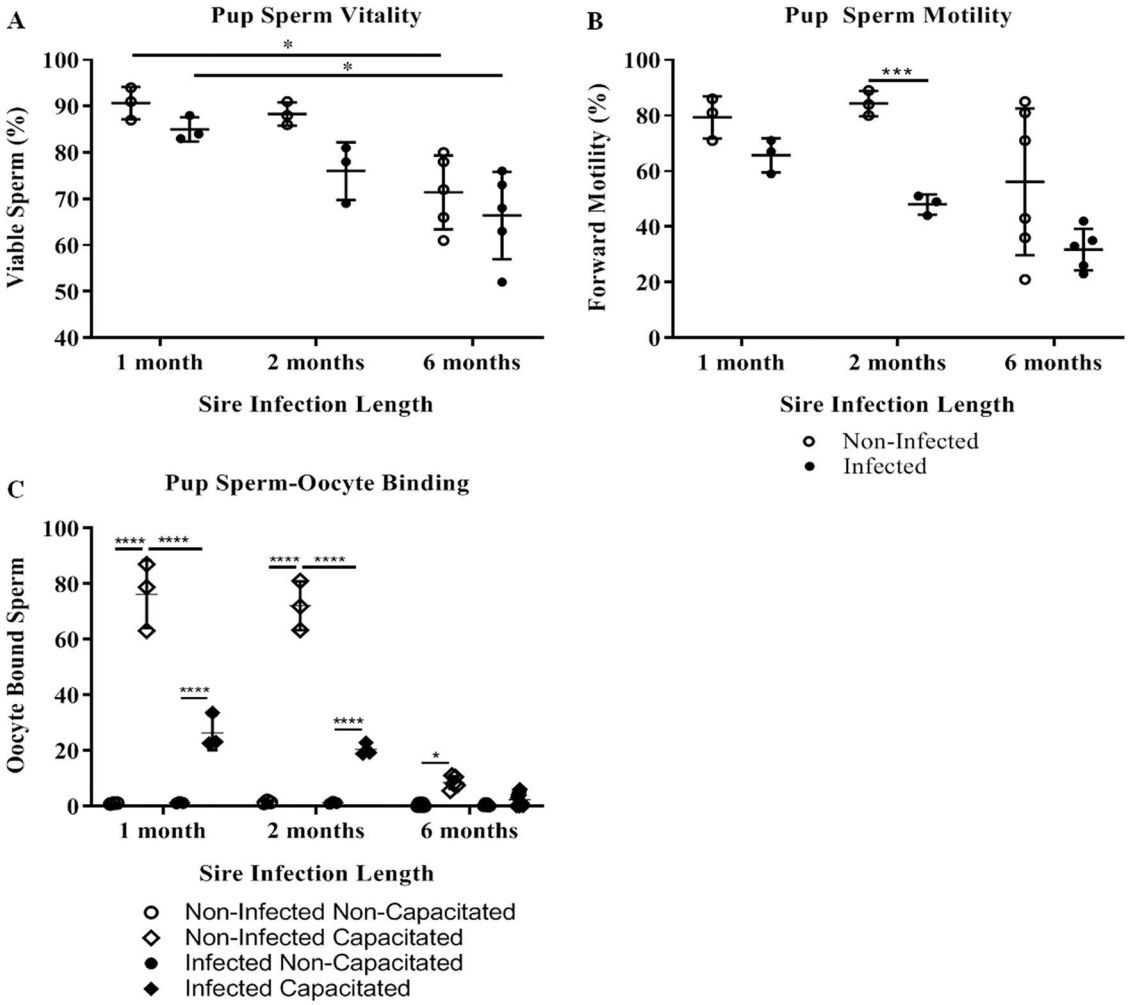

Figure 6. Pup sperm parameters affected by *C. muridarum* infected sires. (A) The oldest sires produced pups with the lowest sperm vitality and chronically infected sires produced pups with the lowest overall vitality. (B) Infected group pups had low forward progressive motility. (C) Infection group sperm had reduced oocyte binding capability. Multiple comparisons two-way-ANOVA was used to analyze each parameter comparing the infected to the noninfected control samples (n = 10) or the noncapacitated to capacitated sperm in the binding assay over time. One- and two-month studies were completed at The University of Newcastle, and the 6-month study at Queensland University of Technology, n = 3–10, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

from testicular lysates. This indicates that a low-grade but productive chronic infection was established and continued to be identifiable for at least 6 months. Productive infections are likely to remain sexually transmittable, as we have previously been able to culture viable *Chlamydia* from prostatic secretions [41]. It is unclear at this time which testicular cell types are perpetuating the chronic infection, and this requires further investigation. However, we have previously reported that Sertoli and spermatogenic cells are susceptible to infection and are apoptotic in the presence of infection and inflammation [40].

Infiltrating WBCs may induce inflammatory conditions to promote apoptosis in testicular cells. While testicular macrophages are normally able to maintain an anti-inflammatory environment, other recruited macrophages or polymorphonuclear cells may override this effect [59–61]. Alternatively, the infiltrating immune cells may be partially responsible for maintaining the infection within the testes. *Chlamydiae* are known to survive within DCs [62, 63], neutrophils [64], monocytes [65], and macrophages [66, 67].

Immune cell infiltration and loss of immune privilege, indicated by the reduced abundance of ZO-1/Claudin 11^{+} tight junctions and Connexin26/37/43^{+} gap junctions, in infected testes likely lead to production of ASABs [68]. These ASABs may be responsible for the frequent agglutination of sperm heads that occurred within infected sperm samples [69]. Several alternatives may be possible for the cause of the ASAB production, including breakdown of the epididymal partial immune-privilege [70, 71]. The sperm agglutination may also be *Chlamydia*-mediated, independent of ASAB presence, via ligand binding [72, 73]. Regardless, sperm agglutination could be inhibitory in the setting of natural human conception, as it would impede forward progressive motility though an overt impact was not observed in this mouse model.

In this study, multiple sperm parameters were altered during infection. Interestingly, infected mice had significantly greater numbers of sperm with DNA fragmentation than healthy mice. Sperm DNA integrity is important for fertility [74]. Mature sperm does not have DNA repair capability [75], instead the oocyte carries out DNA repair after fertilization [75]. However, in some cases, the excessive damage requiring repair causes a delay in the time for the first oocyte cleavage [76]. This cleavage delay is associated with

embryogenic failure and adverse pregnancy outcomes, for example, miscarriage [75, 76].

Asthenozoospermia is also associated with DNA fragmentation. This is hypothesized to be the result of metabolic deregulation as a downstream effect of functional gene fragmentation [14]. This trend was observed here; infected mice produced significantly fewer motile sperm. Sperm that lack forward motility will have reduced capability to traverse the FRT and compete for binding of an oocyte.

Sperm morphology will also influence the motility and oocyte binding capacity [77], although humans have remarkably few morphologically normal sperm so conclusions drawn from this result are limited [13]. The sperm produced by infected mice in this study had significantly higher amounts of amorphic sperm compared to the healthy mice. This correlates with a study that also found that *C. trachomatis* infections in men resulted in higher percentage of amorphic sperm present in semen [44].

Capacitation is also an important factor in oocyte binding, as sperm lacking or having prematurely undergone capacitation cannot complete an acrosome reaction and achieve fertilization [78]. The sperm isolated from infected mice displayed premature capacitation, marked by significantly increased levels of tyrosine phosphorylation. The mechanism for *Chlamydia*-mediated induction of premature sperm capacitation in this study requires further investigation. Some studies suggest that *Chlamydia* adheres to the outside of sperm, causing molecular changes that induce capacitation [79, 80]. In vitro tyrosine phosphorylation (TP) in human spermatozoa was induced by co-incubation with *C. trachomatis* [80]. TP is intrinsically involved with sperm capacitation, making it a useful marker in establishing sperm capacitation status [81]. Alternatively, some studies show that *Chlamydia* spp. can alter the redox balance within host cells as a result of ROS production [82, 83]. Redox-mediated regulation of sperm capacitation is also well characterized [78].

Regardless of the mechanism of chlamydial infection-induced impairment of multiple sperm parameters, the effect was reduced oocyte binding. Only noninfected, capacitated mouse sperm were able to bind oocytes in significant numbers. This indicates a significant likelihood of infection-induced sub-fertility in vivo. In a human IVF setting, this suggests that sperm from an infected individual would be less likely to bind an oocyte. Combined with the DNA fragmentation present in infected sperm, intra-cytoplasmic sperm injection which bypasses oocyte binding may also be less successful [84].

To study the functional outcomes of the sperm abnormalities, sires were infected for 1, 2, or 6 months and then mated with noninfected, healthy dams. The offspring born to chronically 6-month infected sires rather than the 1- and 2-month infected sires displayed low birth weight, developmental delays, and abnormalities of reproductive tract development. Female offspring had smaller, though histologically normal appearing, reproductive tracts, while males had smaller testes.

A commonality between the 1-, 2- and 6-month infected groups was that male pups had poor sperm quality (reduced sperm count, motility, and capacitation/oocyte binding ability). Low sperm quality may be due to testicular abnormalities, as the number of seminiferous tubules in the testes of pups from 6 month infected sires was significantly reduced. The sperm quality declined in direct correlation to the length of paternal infection, i.e., 6-month infected sires gave rise to the pups with the worst sperm quality.

There was no difference in conception rate between the infected and noninfected breeding groups (at any time point). In mice, acute depression of sperm function would be required to cause infertility, which was not the case here. Sufficient functional sperm is still present, and this sperm will generate normal conception rates and numbers of offspring. This hypothesis accounts for the proportion of pups from the infected breeding groups with normal weight and reproductive tract development. However, of more immediate biological importance were the nonviable litters produced at two time points.

Sperm with single or nondeleterious abnormalities may also be present, for example, varying amounts of DNA fragmentation. The variability could explain the presence of both viable and nonviable litters within the infected group. Unfortunately, whether nonviable litters were stillborn or if they died after birth could not be determined. Dams have altered maternal behavior, including cannibalizing neonates if stillborn, particularly unhealthy, or in stressful environments [85, 86]. Multiple other healthy litters were born to both groups on the same day as the nonviable litters, suggesting that pups were stillborn or unhealthy. Two out of twelve chronic 6-month infected breeding group litters also had very low birth-weights; they were not cannibalized by the dams but were euthanized before weaning. Four nonviable chronic infection group litters combined with two failure-to-conceive litters from the acute infection groups resulted in six out of thirty-five nonviable pregnancies.

The abnormalities present in at least two of the chronically infected breeding group litters could have been the product of several factors. Firstly, improper repair and/or replication of fragmented DNA present within sire sperm may contribute to offspring abnormalities, as previously discussed [87, 88]. Sperm DNA fragmentation, although strongly correlated with adverse pregnancy outcomes, can also lead to fetal malformation [76]. This has been studied extensively in humans with sperm apoptosis and ROS as the etiological agents [89]. This is usually in the context of paternal transmission of genetic mutations/diseases including Y chromosome deletion, achondroplasia, and the predisposition of childhood cancer development [89]. Genetic diseases such as achondroplasia found in offspring of older males are particularly relevant to the two abnormal litters produced from breeding infected sires as these litters showed delayed development initially and then by weaning, development was significantly stunted [90]. This resulted in the litters containing small pups around half the size of the average healthy pup.

An additional factor that may contribute to the abnormal litters is epigenetic changes (epimutation) within the germ line and the sperm itself. It is now accepted that epimutation including to micro and noncoding RNA within the germline is potentially carried within gametes leading to epigenetic inheritance [91–94] and that *Chlamydia* can induce methylation mutation on such sites [95–97]. From a reproduction-specific viewpoint, there is some evidence that men conceived via IVF due to male factor infertility had infertility problems of their own later in life [98, 99] and in murine models of maternal cigarette smoking and paternal DDT exposure that male infertility can be transgenerational up to F3 generations [52, 100]. The effects of *Chlamydia* infection may be due to epimutation and as such the current intergeneration effect may also be transgenerational; however, this is hypothetical as no mechanism has been yet been identified. A combination of DNA fragmentation and epimutation in the male germ line could be a key determinant in the role of *Chlamydia* in offspring mortality and morbidity.

Given the frequency of the diagnosed *Chlamydia* infections in the human population, these results indicate that there is cause for concern past those immediate issues normally associated with infection. When the asymptomatic infections are taken into consideration,

there is an alarming potential for chlamydial infections to impact the fertility of a large portion of the human male population, either directly, through decreasing the quality of the father's sperm, or indirectly, through decreasing the quality of the offspring's sperm.

Supplementary data

Supplementary data is available at *BIOLRE* online.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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