*Pathogens and Disease*, 75, 2017, ftx045

doi: 10.1093/femspd/ftx045
Advance Access Publication Date: 18 April 2017
Research Article

RESEARCH ARTICLE

# Effect of cold water-induced stress on immune response, pathology and fertility in mice during *Chlamydia muridarum* genital infection

Tesfaye Belay[1,*], Anthony Woart[1] and Vincent Graffeo[2]

[1]Bluefield State College, Bluefield, WV 24701, USA and [2]St. Mary's Medical Center, Huntington, WV 25701, USA

[*]**Corresponding author:** Bluefield State College, School of Arts and Sciences, 219 Rock Street, Bluefield, WV 24701, USA. Tel: (304) 327 4549; E-mail: tbelay@bluefieldstate.edu

**One sentence summary:** Cold water-induced stress increases the intensity of chlamydia infection, changes pathology and reduces fertility in mice.

**Editor:** Servaas Morré

## ABSTRACT

Genital infection by *Chlamydia trachomatis* is the most common bacterial sexually transmitted disease worldwide. It causes serious reproductive health complications, including pelvic inflammatory disease and infertility. Stress is implicated as a risk factor for various infections; however, its effect on chlamydia genital infection is unknown. We previously showed that repeated exposure of mice to cold water results in increased severity of chlamydia genital infection. In this study, cold water-induced stress resulted in (i) elevated levels of norepinephrine (NE) and epinephrine in the spleen and genital tract of stressed mice; (ii) elevated IL-1$\beta$, TNF-$\alpha$, IL-6 and nitric oxide production in macrophage-rich peritoneal cells of mice; (iii) supplement of NE *in vitro* exerts an immunosuppressive effect on splenic T-cell production of cytokines; (iv) decreased *C. muridarum* shedding in the genital tract of $\beta$1Adr/$\beta$2Adr receptor KO mice; and (v) a higher rate of infertility in infected mice. These results suggest that cold water stress induces the production of catecholamines, which may play a critical role in the modulation of the immune system leading to increased intensity of *C. muridarum* genital infection.

**Keywords:** cold-induced stress; chlamydia; immune response

## INTRODUCTION

Chlamydia genital infection caused by *Chlamydia trachomatis*, the most common bacterial sexually transmitted disease worldwide, is a leading cause of pelvic inflammatory disease, fallopian tube scarring, ectopic pregnancy, infertility and neonatal conjunctivitis (Westrom *et al.* 1992; Cohen and Brunham 1999; Stamm 1999; Haggerty *et al.* 2010). Epidemiologic data from the Centers for Disease Control and Prevention and World Health Organization (WHO) indicate that *C. trachomatis* genital infection is a serious public health problem, with more than 90 million new cases occurring annually worldwide, and 4 million in the USA alone (CDC 2008). In the USA, chlamydia genital infection disproportionately affects populations of low socioeconomic status, particularly African-Americans (Ellen *et al.* 1995; Owusu-Edusei *et al.* 2013). The reasons are not well known, but stress and other unknown factors may have a role in the persistent high rate of *Chlamydia* genital infection associated with low socioeconomic conditions (Beatty, Morrison and Byne 1994; Pampel, Krueger and Denney 2010).

It is well recognized that genital infection with *C. trachomatis* leads both to the recruitment of protective immune responses to the site of infection and infiltration of inflammatory products that may also contribute to pathological damage in the host. Previous studies demonstrated that immune response to chlamydia genital infection may contribute to pathology diseases following infection (Westrom *et al.* 1992; Cohen and Brunham 1999; Stamm 1999). This has been demonstrated in patients with repeated chlamydia genital infection having a much

**Received:** 31 October 2016; **Accepted:** 14 April 2017

greater risk of tubal obstruction than those with less exposure to *C. trachomatis*.

Various animal model studies have been undertaken to identify and characterize factors that may be involved in the development of immunopathogenesis after chlamydia genital infection (Patton *et al.* 1987; Rank 1994; Kelly *et al.* 2000; Rank, Bowlin and Kelly 2000). Those murine model studies have exhibited vital aspects of human genital chlamydial infection including similar course of active infection, pathological consequences or sequelae of an infection, tubal inflammation, hydrosalpinx formation and infertility (Rank 1999).

More studies in animal models have demonstrated that anti-chlamydial T-cell response in the local genital mucosa plays a role in the clearance of *C. trachomatis* from the genital tract (Igietseme and Rank 1991; Cain and Rank 1995; Morrison, Feilzer and Tumas 1995; Cotter *et al.* 1997; Johansson *et al.* 1997; Rank 1999; Igietseme, Portis and Perry 2001). It was demonstrated that immunity to *C. trachomatis* is mediated by T helper 1 cells through IFN-gamma-dependent and independent pathways (Perry, Feilzer and Caldwell 1997).

*Chlamydia trachomatis* infection of the genital tract has gained much attention because of its impact on female infertility (Tuffrey, Alexander and Taylor-Robinson 1990; Pal *et al.* 1998) and no reliable vaccine against the infection has been developed. Experimental vaccines showed that vaccine-induced immunity has the ability to prevent infection compared to immunity generated by previous infection (Igietseme, Eko and Black 2011). In that study, vaccinated mice with dendritic cells pulsed with Fc-major outer membrane protein were protected from infertility, whereas *C. muridarum* perfected mice were not, suggesting that infection-induced immunity does not prevent the pathological process leading to infertility.

It is documented that psychological or physical stress, resulting from competition, pressure and hardship of life in society, has major impacts on public health (Solomon 1987; Ziemessen *et al.* 2007). Stress may regulate the host immune response through neuroendocrine–immune system interactions (Sheridan *et al.* 1994; Madden, Sanders and Felten 1995; Borghetti *et al.* 2009). Glucocorticoids and catecholamines are hormones that serve as the major mediators of stress responses by modulating signaling events, which result in either immunosuppression or immunostimulation in the host (Solomon 1987; Ziemessen *et al.* 2007).

Norepinephrine (NE) is one of the catecholamines of the sympathetic nervous system released during stressful conditions including cold-water application. NE binds and stimulates the beta2 adrenergic receptor ($\beta$2Adr), which is predominantly expressed on $CD4^+$ and B cells (Sanders *et al.* 2003; Sanders and Virginia 2012). Studies document the existence of at least eight different alpha or beta subtypes of NE receptors (Bylund *et al.* 1994). Studies have further shown that the direct binding of NE to $\beta$2Adr modulates immune responses, including altering cytokine production, lymphocyte proliferation and antibody secretion (Elenkov *et al.* 1996; Ramer-Quinn, Baker and Sanders 1997; Kohm *et al.* 2000; Goyarts *et al.* 2008; Nakai *et al.* 2014; Lorton and Bellinger 2015).

Cold water-induced stress in mice has proven useful in defining the relationships between the central nervous and immune defense systems (Kubera *et al.* 1998; Aviles *et al.* 2004). Application of cold water as a stressor in animal models, including mice, has resulted in changes in immune responses that correlate with the activity of the neuroendocrine system of corticosteroids and catecholamines (Aviles and Monroy 2001; Rober *et al.* 2006)

The role of stress in the immunopathogenesis of chlamydia genital infection remains unknown. The objective of this study was to investigate the role of cold water-induced stress on the immune response, histopathology and fertility in a mouse model during chlamydia genital infection. The hypothesis was that exposure of mice to cold water-induced chronic stress increases the intensity of chlamydia genital infection and results in a more progressive infection and severe pathology. A better understanding of how stress contributes to the immunopathogenesis of chlamydia genital infection in a mouse model may allow us to develop more effective prevention and treatment options in humans.

## MATERIALS AND METHODS

### Chlamydia stock culture and McCoy cell line

McCoy mouse fibroblast cell line and *Chlamydia muridarum* (previously known as *Chlamydia trachomatis*) agent of mouse pneumonitis (MoPn) were kindly provided by Dr Joseph Igietseme, Morehouse School of Medicine, Atlanta, GA. Stock of *C. muridarum* was prepared by propagating elementary bodies in McCoy cells, as previously described (Belay *et al.* 2002).

### Animals

Six- to seven-week-old female BALB/c strain mice and proven fertile males for mating were purchased from Harlem-Sprague Daley (Indianapolis, IN, USA), or Hilltop Lab Animals, Inc. (Scottsdale, PA, USA). A $\beta$1Adr/$\beta$2Adr receptor knockout (KO) mouse and three wild-type mice, representing the *C57BL/6J, DBA/2J* and *FVB/NJ* background strains from which the $\beta$1Adr/$\beta$2Adr receptor KO strain was derived purchased from Jackson laboratories (Bar Harbor, ME, USA), were used in the study. Mice were housed in a vivarium at Bluefield State College (BSC) located in the Basic Science Building of the School of Arts and Sciences. Mice were given food and water ad libitum in an environmentally controlled room with equal daylight and night hours. Before starting experiments, all animal experimental protocols were approved by the BSC Institutional Animal Care and Use Committee.

### Cold water stress protocol

Stress was induced by placing five mice in a packet filled with 2 cm of cold water (1°C–4°C) for 5 min daily for 24 days, as previously described (Aviles and Monroy 2001; Belay and Woart 2013). The water level was deep enough to cover the backs of the mice while they were swimming in the cold water. At the end of each stressing period, mice were dried with towels to avoid hypothermia. Non-stressed mice were kept at room temperature without the treatment of cold water-induced stress. In preparation for secondary infection, mice were stressed as above on day 60 of the primary infection for 10 days and re-infected on day 70 of primary infection.

### Restraint stress protocol

As an alternative to cold stress, a restraint stressing method was applied to and randomly selected group of mice as previously described (Bonneau 1996). Briefly, each mouse was placed in an adequately ventilated 60 mL conical syringe tube with holes for 5 min every day for 24 days. Mice could rotate along the horizontal axis, but could not turn head to tail.

## Peritoneal fluid harvesting protocol

After stressing mice for 19 days, at least five mice of each experimental group were injected with 2 mL thioglycollate intraperitoneally. At the conclusion of the 24-day stressing period, these mice were euthanized by $CO_2$ inhalation followed by cervical dislocation. Peritoneal cells were harvested from mice as previously described (Aviles *et al.* 2004). Briefly, 7 mL of RPMI 1640 without phenol red was gently inoculated into the lower abdominal part of each mouse using a 20-gauge needle and syringe. Cells were then harvested by low speed centrifugation and seeded at $5 \times 10^5$ cells per well in 100 $\mu$L of RPMI-1640 complete medium and stimulated with lipopolysaccharide (LPS) (5 $\mu$g mL$^{-1}$) and incubated at 37°C supplemented with 5% $CO_2$. Culture supernatant was collected at different time points for immediate nitric oxide and enzyme-linked immunosorbent assay (ELISA) protocols, respectively.

## Nitric oxide assay

Nitrite concentrations were determined using the Greiss reagent (1% sulfanilamide and 0.1% naphthyl ethylene diamine hydrochloride in 5% phosphoric acid) with reference to a standard curve for sodium nitrate as previously described (Aviles *et al.* 2004).

## T-cell isolation from mouse spleen cells

Spleens from mice in each experimental group were removed aseptically. Tissues were kept in 2 mL of complete RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin (Sigma) and 0.1% gentamicin (Sigma). The spleen tissues then were minced and teased with forceps and cells were pressed through a 70 $\mu$m size cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) and placed in an additional 5 mL of complete RPMI 1640 medium. Untouched mouse T cells from stressed and non-stressed mice were isolated using a Dynabead Magnetic Separation kit from Life Technologies (Carlsbad, CA, USA) following the manufacturer's instructions. Cell counts were determined using a TC20 Automatic Cell Counter from Bio Rad (Hercules, CA, USA) and the counts were at least $1 \times 10^7$ cells mL$^{-1}$.

## Antigen-presenting cell preparation

Antigen-presenting cells (APCs) from splenocytes of non-stressed mice were prepared by treatment with mitomycin C following the manufacturer's instructions. Briefly, splenocyte cells were washed by centrifugation at 300 × g for 10 min and freshly prepared mitomycin C at the concentration of 25 $\mu$g mL$^{-1}$ was added to $10^7$ splenocyte cells mL$^{-1}$ and incubated at 37°C for 30 min. Cells were washed four times as above and a final cell count of $50 \times 10^5$ mitomycin C-treated cells was prepared for proliferation assay.

## T-cell proliferation assay

T-cell proliferation assay was performed as described previously (Aviles *et al.* 2004) with some modifications. Briefly, cells were seeded in triplicate at a density of $5 \times 10^5$ cells mixed with $5 \times 10^5$ mitomycin C-treated cells per well in a 96-well culture plate (Becton Dickinson). The cells were stimulated with 2.5 $\mu$g mL$^{-1}$ of concanavalin A (Con A) and 5 $\mu$g mL$^{-1}$ of LPS and NE at $10^{-6}$ M or in combination and cultured for 72 h in a water-jacketed incubator at 37°C and 5% $CO_2$ (NuAire, Plymouth, MN, USA). Culture supernatants were collected after 72 h of proliferation then stored at −80°C to measure cytokine production by ELISA.

## Testing the action of synthetic NE on T-cell proliferation

To investigate the influence of synthetic NE bitartrate salt (Sigma) on cytokine production, we investigated whether NE inhibits immune cell activities as measured by the amount of important cytokines produced. Spleens were harvested as above from stressed/infected (SI), non-stressed/infected (NSI) and non-stressed/non-infected (NSNI) mice. T cells were isolated using Dynabeads® Magnetic Separation following the manufacturer's instructions. Purified T cells were seeded at a density of $5 \times 10^5$ per well in 96-well plates along with the same number of APCs. Cells were proliferated for 72 h at 37°C in the presence/absence of Con A (5 $\mu$g mL$^{-1}$), (0.3 $\mu$g mL$^{-1}$) irradiated chlamydia antigen or 100 $\mu$L NE at the concentration of $10^{-4}$M. Culture supernatants were collected after 72 h of proliferation and immediately stored at –20°C to be tested for the production of selected cytokines as determined by ELISA.

## Cytokine measurement using ELISA

The amount of cytokines in supernatants collected from peritoneal fluid cells of stressed and non-stressed mice was measured by using an ELISA kit (Invitrogen, Camarillo, CA) following the manufacturer's instructions. Briefly, standard samples and test samples were diluted and added to the wells in duplicate and incubated as directed. Supernatant fluids from parallel cell cultures of spleen cell proliferation and from the peritoneal functional assays were used to determine cytokine production (Aviles *et al.* 2004). Optical densities were measured at 450 nm with an ELISA plate reader from Biotech (Winooski, VT, USA). The concentration of the cytokine in each sample was obtained by extrapolation from the standard calibration curve generated in each assay.

## Evaluation of the course of *Chlamydia muridarum* genital infection in mice

On day 17 of the 24-day stressing period, all mice were injected subcutaneously with 2.5 mg per mouse of progesterone in 100 $\mu$L of phosphate buffered saline (PBS) to regulate the mice's estrous cycles. On the final day of the stressing period, stressed and non-stressed mice were infected intravaginally with $10^3$ IFU of *C. muridarum* in a volume of 30 $\mu$L of PBS while under Ketamine-Xylazine-induced anesthesia. Infection was monitored by cervico-vaginal swabbing at 3-day intervals during primary infection as previously described (Belay and Woart 2013). To isolate *C. muridarum* shedding from genital swabs, individual wells of McCoy cell monolayer cultured in complete Dulbecco's modified Eagle's medium (DMEM) in 96-well plates were inoculated with 200 $\mu$L of vortexed solution of the genital swab from the –80°C freezer, followed by a centrifugation at 300 rpm for 1 h at room temperature. Culture plates were incubated at 37°C for 32 h, and then fixed with methanol after washing twice with PBS. Approximately 50 $\mu$L of Fluorescein isothiocyanate-labeled, genus-specific anti-chlamydial antibody from Remel (Lenexa, KS, USA) was added to each well and incubated for 2 h at room temperature. After washing and drying wells, fluorescence was observed with a fluorescence microscope to illuminate inclusions. The number of inclusions was determined by counting

inclusion bodies within 10 fields (×40), as previously described (Belay and Woart 2013).

### Detection of *Chlamydia muridarum* shedding in regions of the genital tract

Alternatively, approximate quantification of inclusion bodies was achieved by culturing homogenized tissues of the regions of the genital tract. Briefly, female mice were stressed and infected as above. The genital tracts were aseptically removed and separated into three regions, cervix, uterus, oviduct, and placed in 1 mL complete DMEM and homogenized using a QIAGEN Tissue Raptor (Valencia, CA). A volume of 200 $\mu$l of each homogenized tissue supernatant was added to monolayers of McCoy cell lines seeded in 96-well plates and incubated at 37°C supplemented with 5% $CO_2$. Isolation of *C. muridarum* was performed as described above.

### Determination of catecholamines levels in spleen and genital tract of stressed mice

The level of epinephrine (EP) or NE in the spleens and genital tracts of mice was determined as previously described (Belay and Woart 2013). Briefly, mice were euthanized and tissues were harvested and homogenized as above. The level of EP or NE was determined using a 3-cat EIA kit and following the manufacturer's instructions (Labor Diagnostika Nord GmbH and Co. KG, Germany). Absorbance was read using a microreader plate set to 450 nm. Concentrations of EP or NE were determined by extrapolation from the standard values.

### Harvesting tissues for gross examination and pathology analysis in mice

To reveal histological-grade differences of the genital tracts between stressed and non-stressed mice during primary infection at several time points (2, 14, 42 days) and secondary infection (75 days), mice were euthanized during primary or at 15 day of secondary of chlamydia genital infection. The existence of hydrosalpinx fluid and any other related abnormalities of the entire genital tract harvested at 15 day of secondary infection was carried out by gross examination with naked eyes. Each genital tract was sectioned into regions containing the cervix, uterus and oviduct for pathological analysis. After photographing, excised tissues were transferred to 10% neutral formalin and processed for histology following procedures, processed and stained with H&E (Rank, Sanders and Patton 1995; Kelly *et al.* 2000). The slides were imaged and examined under a microscope digital camera for the presence of neutrophils (acute inflammation), lymphocytes and plasma cells of fibrosis (chronic inflammation) by a pathologist at St. Mary's Hospital, Huntington, WV.

### Fertility studies

After primary and secondary *C. muridarum* genital infection and resolution, the stressed, non-stressed and non-stressed/non-infected mice were mated with proven fertile males and monitored for pregnancy by daily weight-gain assessment as previously described (Pal *et al.* 1998). The mating was monitored for 19–21 days, a duration covering murine gestational and litter production. Pregnancy was determined by measuring weight gained and the mice that were considered pregnant euthanized. After the first mating, female mice that were not pregnant were mated again with a male mouse that had successfully mated with others. The percentage of pregnant mice and the number of embryos or pups in each group was compared during the 19–21 days of experimentation. The experiment was repeated three times for comparison purposes.

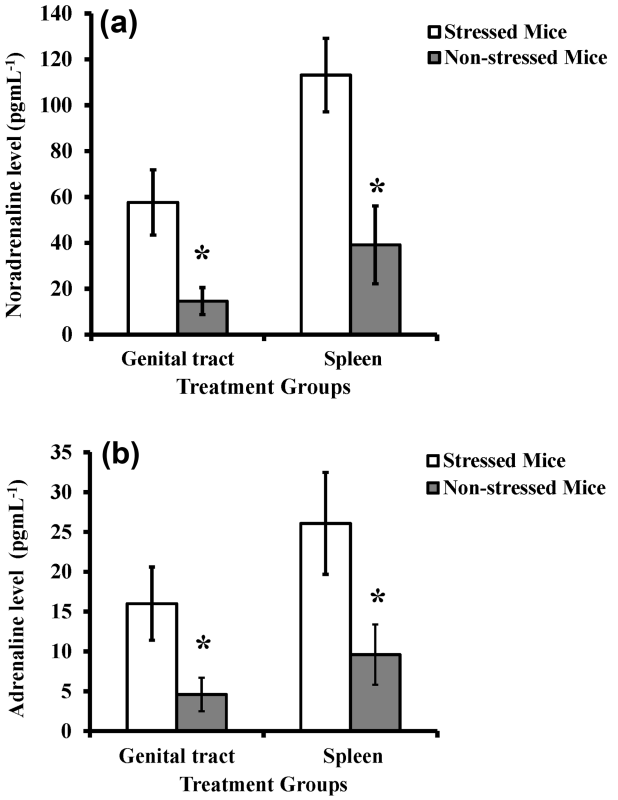


**Figure 1.** Effect of cold-induced stress on production of catecholamines in genital tract and spleen: noradrenaline (**a**) and adrenaline (**b**) measured by ELISA. Each point represents the means ± standard deviation of two experiments (n = 5 to 6 mice for each experiment). Statistical differences between treatments were evaluated by Student's *t* test. Asterisk denotes significant statistical differences between the experimental groups at the level of ($P \leq 0.05$).

### Statistical analysis

Statistical significance between any two groups was tested using Student's *t*-test and ANOVA was used to test a statistical difference between more than two groups. Level of statistical significance was at the level of $P < 0.05$.

## RESULTS

### Cold water-induced stress increases NE and EP levels in the spleen and genital tract of mice

We previously reported that cold-induced stress increases plasma levels of catecholamines (NE and EP) in mice (Belay and Woart 2013). To further assess the presence of these stress hormones in lymphoid organs and the genital tract during *Chlamydia muridarum* genital infection, we measured the levels of NE and EP in the spleens and genital tracts of cold-induced stressed and non-stressed mice using ELISA. As shown in Fig. 1a and b, mice exposed to cold stress had a statistically significant increased production of NE in the spleen ($p < 0.008$) and genital tract ($P < 0.002$), compared to that of non-stressed mice.

Similarly, the EP level in the spleen ($P < 0.001$) and genital tract ($P < 0.002$) of stressed mice was higher than that of non-stressed mice. The secretion level of stress hormones by cold water-treated and restraint-stressed mice were almost identical, suggesting application of cold water as a physical stressor and restraint stress as a psychological stressor in mice resulted in the same changes in the neuroendocrine system of corticosteroids and catecholamines. This elevated level of catecholamines in the spleen and the genital tract as a result of cold stress treatment may suggest that these stress hormones contribute to impairment of the immune system during chlamydia genital infection.

## Cold water-induced stress increases production of proinflammatory cytokines and nitric oxide in peritoneal macrophages

This study was to examine the effect of stress on the secretion of proinflammatory cytokines and production of reactive NO by peritoneal cells. We tested the hypothesis that stress would alter the pattern of secretion of proinflammatory cytokines and NO. As shown in Fig. 2a–c, cold water-induced stress or restraint stress markedly increased production of proinflammatory cytokines of interleukins (IL) 1$\beta$ and 6, and tumor necrosis factor-alpha (TNF-$\alpha$) in culture supernatant of LPS-treated peritoneal fluid cells compared to that of non-stressed mice. Similarly, high level of NO was produced in culture supernatant of stressed mice compared to that of non-stressed mice (Fig. 2d).

## Exposure of naive splenic T cells to NE leads to a reduced production of cytokines

The purpose of this experiment was to examine whether synthetic NE contributes to decreased *in vitro* cytokine production by T cells. Similar to our previous studies, exposure of mice to cold water-induced stress resulted in an increase in the secretion of IL-10 and TNF-$\alpha$ (Belay and Woart 2013), while addition of synthetic NE to naïve T cells resulted in a reduced production of IL-10 (Fig. 2e), TNF-$\alpha$ (Fig. 2f) and IL-2 (Fig. 2g). T cells isolated from *C. muridarum*-infected stressed mice showed increased production of IL-2 (Fig. 2g) and INF-$\gamma$ (Fig. 2h) compared to controls.

## Cold water-induced stress increases the intensity of *Chlamydia muridarum* genital infection during primary infection

To determine the level of infection in stressed and non-stressed mice, we measured the number of viable *C. muridarum* present on vaginal swabs collected every 3 days post infection. As shown in Fig. 3a, there are greater numbers of chlamydia inclusion counts in the swabs of stressed mice compared to non-stressed mice (Fig. 3b), indicating that cold-induced stress increases the intensity of *C. muridarum* genital infection in mice.

## Increased *Chlamydia muridarum* shedding in regions of the genital tract of stressed mice

Since cervical-vaginal swabs do not show the amount of *chlamydiæ* burden in each region of the genital tract, we also measured number of *C. muridarum* in tissue homogenates of different genital regions: cervix, uterine horn and oviduct. Similar to Fig. 3a, there are greater numbers of IFUs in the homogenates of stressed mice compared to homogenates of non-stressed mice (Fig. 3c). Although the deposition of *C. muridarum* was at the cervix, high IFU counts were detected in the oviduct of stressed mice compared to non-stressed mice ($P < 0.05$). This significant difference in chlamydia shedding shows a rapid spread of *C. muridarum* to the upper region of the genital tract, suggesting the existence of differential distribution of *C. muridarum* and disease severity in mouse genital tract.

## Deficiency in $\beta$1Adr/$\beta$2Adr receptors results in reduced shedding of *Chlamydia muridarum* in the genital tract of stressed mice

To assess the relative role of NE receptors in stress-induced responsiveness to chlamydia genital infection, we examined the susceptibility of a $\beta$1Adr/$\beta$2Adr receptor KO mouse exposed to cold water-induced stress. We observed that $\beta$1Adr/$\beta$2Adr receptor-deficient mice shed less *C. muridarum* IFUs in the genital tract compared to the control wild-type mice (representing the C57BL/6J, DBA/2J and FVB/NJ background strains from which the $\beta$1Adr/$\beta$2Adr receptor KO strain was derived). The decrease in *C. muridarum* burden in the genital tract of $\beta$1Adr/$\beta$2Adr receptor KO mice exposed to cold water-induced stress, as compared to cold water-induced stressed wild type mice, provides an initial indication that an absence of NE receptors reduces the effect of stress on chlamydia genital infection. After euthanizing the mice, necropsy was performed as previously described (Shah *et al.* 2005). As shown in Fig. 3d, IFU counts were significantly higher in the genital tract regions of stressed mice compared to that of non-stressed mice. With respect to the regions, the oviduct showed the highest IFU count, then cervix and uterus. Even though chlamydia deposition was through the cervix, it had a significantly lower IFU count than did the uterus.

## Cold water induced-stress enhances leukocyte infiltration into regions of the genital tract of mice during *Chlamydia muridarum* genital infection

We hypothesized that prolonged cold-induced stress enhances production of immunopathogenic cytokines that cause histopathological changes in the genital tract of mice during chlamydia genital infection. To test the relationship between stress-induced inflammation and chlamydial infection, the cervix, uterine and oviduct tissues were assessed independently for histologically alterations at two, 14 and 42 days during primary infection and at day 15 of secondary infection. As represented in Fig. 4, cold water-induced stress treatment resulted in a marked increase of leukocyte infiltration, especially in the cervix of stressed mice compared to non-stressed mice at day 2 of primary infection. At days 14 and 42 of primary infection, insignificant counts of leukocytes were observed (data not shown). At day 15 of secondary infection, leukocyte infiltration in the cervix was comparable to the primary infection, but the leukocyte counts in the uterine and oviduct was lowered to half the primary infection (Table 1).

Representatives of the complete genital tract of stressed and non-stressed mice after 15 days of *C. muridarum* secondary infection are shown in Fig. 5. Evaluation with naked eyes showed three to four hydrosalpinx in the oviducts and uterine horns of stressed mice compared to a few hydrosalpinx in non-stressed mice. Fluid in the uterine horns and abscesses on the oviducts and inflammatory response (redness) of genital tract were higher in stressed mice than that of non-stressed mice.

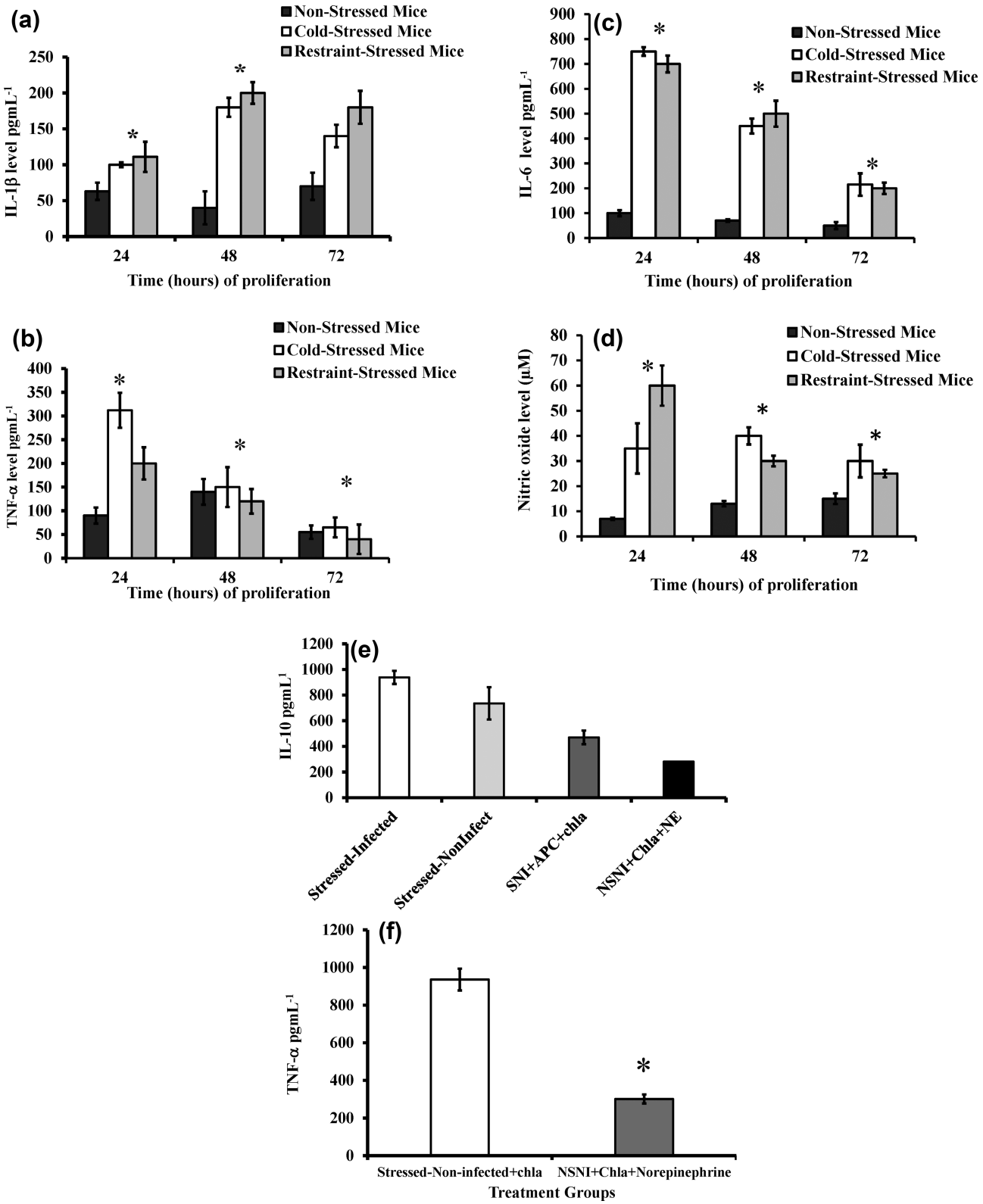


**Figure 2.** Relative production of proinflammatory cytokines in peritoneal cells of mice stressed with cold water. The levels of cytokines IL-1$\beta$ **(a)**, TNF-$\alpha$ **(b)** and IL-6 **(c)** were measured by using ELISA. Nitric oxide concentrations determined using the Greiss reagent **(d)**, IL-10 **(e)**, TNF-$\alpha$ **(f)**, IL-2 **(g)** and INF-$\gamma$ **(h)**. SI, stressed/infected; NSI, non-stressed/infected; NSNI, non-stressed/non-infected mice; APC, antigen-presenting cell; CHL, *Chlamydia trachomatis* antigen (irradiated); NE, norepinephrine. Each point value represents the means ± standard deviation of 2 or 3 wells (n = 5 or 6 mice for each group of experiments). Statistical differences between treatments were evaluated by Student's *t* test. Asterisk denotes significant statistical differences between the experimental groups at the level of ($P \leq 0.05$).

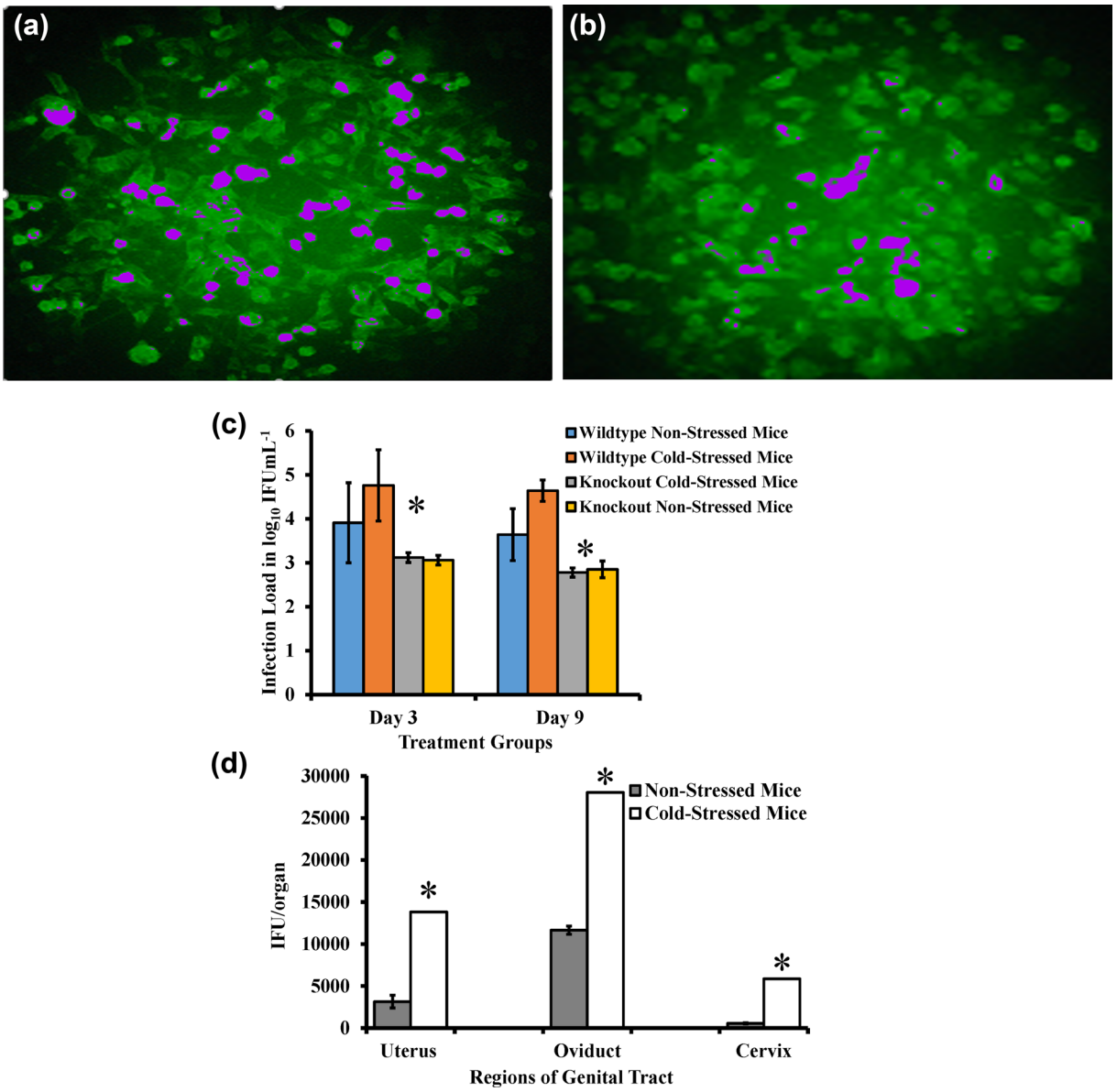


**Figure 3.** Determination of *C. muridarum* shedding in the genital tract of wild type and knockout stressed mice. Stressed (**a**) and non-stressed (**b**) mice were infected with *C. muridarum*. Cervicovaginal swabs were collected at 3-day intervals (**c**). Alternately, cervix, uterus and oviduct sections were harvested after 48 h of infection (**d**). McCoy mouse fibroblasts were seeded into 96-well plates and incubated at 37°C with 5% carbon dioxide supply until monolayers were 90%–95% confluent. After 32 h of infection, *C. muridarum* isolation was performed by staining with fluorescence-tagged anti-chlamydia antibodies and inclusion bodies were counted. Each data point is mean ± standard deviation of log10 inclusion forming unit/milliliter representing combined results of two separate experiments (n = 5 to 6 mice per group). False color has been added to (**a**) and (**b**) to aid visualization of the inclusion bodies.

This study indicates that stress can induce acute inflammation in the cervical region of mice, suggesting that this inflammation might predispose mice to increased susceptibility to chlamydial genital infection and histopathological changes. Although the shedding of *C. muridarum* and hydrosalpinx development was significant in the oviduct of stressed mice, leukocyte infiltration was modest in uterine and oviducts.

## Cold water-induced stress enhances *Chlamydia muridarum* genital infection-induced infertility in mice

This study was undertaken to examine the effect of stress on induction of infertility in a mouse model. After recovery from secondary infection, mice were mated with proven breeder male mice and monitored for fertility for 19–21 days. As shown in Table 2, non-stressed and non-infected mice had 100% fertility rates. Non-stressed but infected mice had a lower fertility rate (84%) but had significantly higher fertility rates than stressed and infected mice (56%) ($P < 0.05$). All stressed mice had two or three times fewer embryos than control animals, and non-stressed, non-infected mice had significantly more embryos than stressed and infected mice ($P < 0.05$). This significant impact of cold water-induced stress in combination with chlamydia genital infection on fertility indicates that stress increases infertility complications associated with *C. trachomatis* genital infection. This study is in agreement with the finding that stressed and *C. muridarum* infected mice had the least fertility outcome compared to other experimental groups.

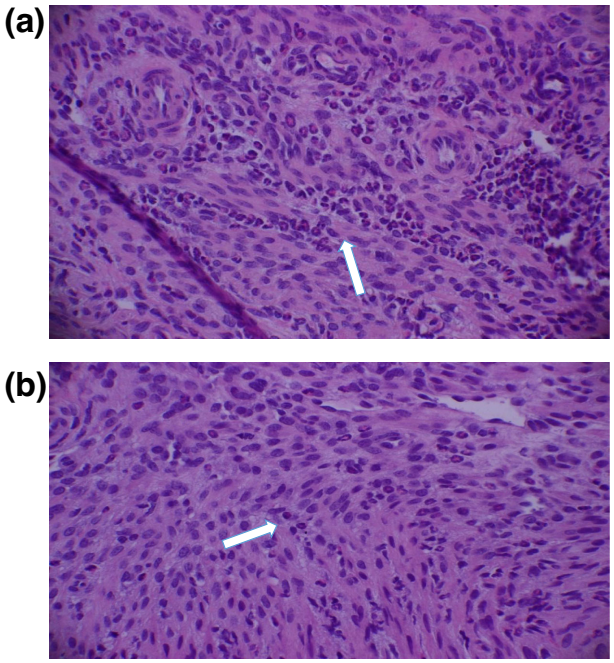


**Figure 4.** Infiltration of leukocytes to the regions of genital tract of mice during *C. muridarum* genital infection. Arrows show neutrophils the cervix of stressed mice (**a**) and non-stressed mice (**b**) during *C. muridarum* infection. Mice were stressed, infected as above. After 48 h of infection, mice were euthanized and the tract was aseptically harvested and excised into cervix, uterus and oviduct sections and placed in buffered formalin. The sections were stained with hematoxylin and eosin (H&E) at Marshall University pathology laboratory services.

**Table 1.** Effect of cold-induced stress on infiltration of leukocytes to the regions of genital tract of mice during *C. muridarum* genital infection.

| | | Treatment groups | | |
|---|---|---|---|---|
| Infection Phase[a] | Genital Tract region | Non-stressed Non-infected (n = 5) | Non-stressed Infected (n = 10) | Stressed Infected (n = 10) |
| Primary[b] | Cervix | 130 ± 42 | 198.5 ± 20 | 154 ± 32 |
| | Uterus | 88.5 ± 57 | 65 ± 22 | 133.5 ± 7 |
| | Oviduct | 1 ± 0 | 1 ± 0 | 124 ± 17 |
| Secondary[c] | Cervix | 20 ± 0 | 130 ± 92 | 149.5 ± 21 |
| | Uterus | 2 ± 0 | 53 ± 17 | 65 ± 42 |
| | Oviduct | 2 ± 0 | 14 ± 11 | 71.3± 67 |

[a]Genital tract samples were harvested 48 h after primary infection.
[b]Genital tract samples were harvested 48 h after secondary infection.
[c]Results are the average of two separate experiments.

Overall, mice mated 40 days following secondary infection had a significant decrease in fertility rate compared with the control groups, as shown by a reduction in pregnancy and a decrease in the number of embryos.

In summary, we have demonstrated that prolonged exposure of mice to cold water-induced stress results in (i) elevated levels of NE and EP in the spleen and genital tract of stressed mice; (ii) elevated IL-1$\beta$, TNF-$\alpha$, IL-6 and NO production in macrophage-rich peritoneal cells of mice; (iii) supplement of NE *in vitro* exerts an immunosuppressive effect on splenic T-cell production of cytokines; (iv) increased intensity of *C. muridarum* genital infection; decreased *C. muridarum* shedding in the genital tract of $\beta$1Adr/$\beta$2Adr receptor KO mice; and (v) a higher rate of infertility in infected mice.

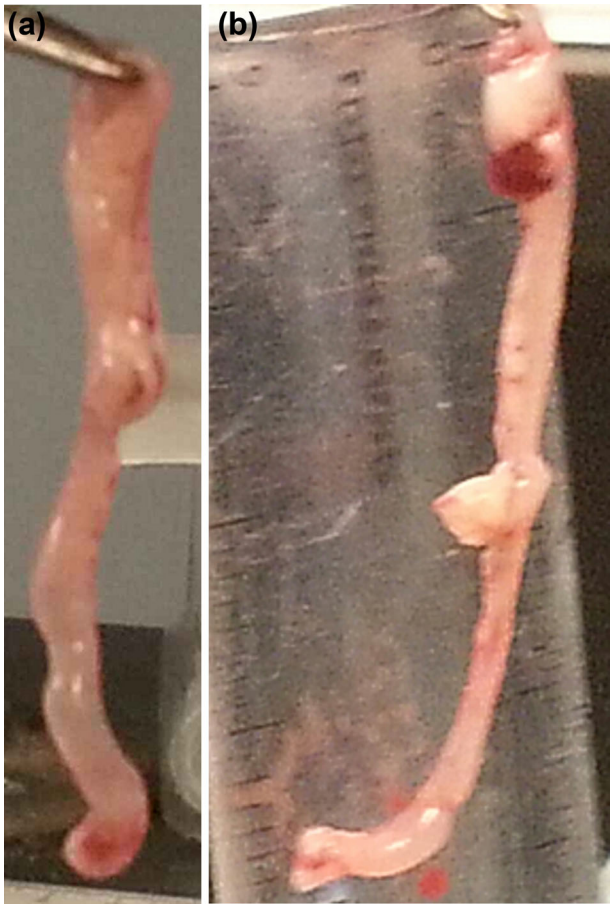


**Figure 5.** Gross genital tract pathology in stressed and non-stressed *C. muridarum* infected mice. At day 15 after secondary infection, tissues were removed from mouse for evidence of the number of oviduct hydrosalpinx scored by observing clearly visible with naked eyes. Uterine horn filled with clear fluid and any other related abnormalities of the entire genital tract carried out by gross examination.

## DISCUSSION

We have investigated the possible role of cold water-induced stress in the outcomes of chlamydia genital infection and subsequent complications in a mouse model to help understand the differential incidence and disease severity of chlamydia genital infection in humans. The significance of this study intends to expand a mounting evidence that stress increases susceptibility to infections by modulating the immune system in animal models, which may be applicable in humans.

Evidence in the literature shows that, depending on the duration and timing, cold stress has either immunosuppressive effects leading to decreased resistance to infection or enhancement in immune response (Yin *et al.* 1996). In establishing our study, preliminary experiments indicated that application of cold water-induced stress at least for 8 days increased productions of interferon gamma by splenic T cells, suggesting enhancement of the immune response. These observations led us to adopt extending the stressing frequency of the mouse model for 5 min every day for 24 days in order to establish chronic stress. This was a crucial step because many published studies reported that long-term stress suppresses the ability of the im-

**Table 2.** Effect of cold water-induced stress on the rate of pregnancies and litter sizes in BALB/c mouse strain after *C. muridarum* genital infection[a].

| Animal group | Fertility at day 19 | Number of embryos/mouse | Gross appearance of genital tract |
|---|---|---|---|
| Non-stress | 19/19 (100%) | 7 (11[b]), 8(2 [b]), 9(6 [b]) | Normal appearance |
| Non-infected | | Average no. of embryos [c] = 7.74 ± 0.9 | |
| Non-stressed | 21/25 (84%) | 6 (16 [b]) to 7 (6 [b]) | Inflammation and necrosis |
| Infected | | Average no. of embryos [c] = 6.6 ± 0.6 | |
| Stressed | 14/25 (56%) | 3 (11 [b]), 3 (6 [b]) | Inflammation and necrosis |
| Infected | | Average no. of embryos [c] = 3.6 ± 0.8 | |

[a]Pregnancy testing was performed after the resolution secondary *C. muridarum* genital infection. Mating occurred 120 days after primary infection or 40 days after secondary infection

[b]The number in parenthesis denotes the number of mice that had the specified number of embryos.

[c]The average number of embryos of each animal group was obtaining by dividing the total number of embryos over the total number of the mice of the group.

mune system to fight viral, bacterial, fungal and parasitic infections (Yin *et al.* 1996; Kubera *et al.* 1998). All data presented in this study were from mice subjected to chronic stress compared to non-stressed mice.

Similarly, preliminary experiments included two stressing methods: cold and restraint. Initial results were that the restraint method of stressing produces comparable results to cold stressing. In an effort to simplify the experiment and reduce the number of experimental groups, and thus the number of mice, required for testing, we needed to select one stressing method. We chose the cold-stress method because it was observationally clear that even after repeated exposure, the mice were experiencing discomfort, whereas with the restraint stress it seemed that repeated stressing induced habituation, as the mice would no longer struggle in the restraint syringe. Unless indicated to be restraint stress, data presented in this study were from mice subjected to cold stress.

Previous studies in a mouse model showed a correlation between susceptibility to chlamydia genital infection and increased stress hormone production in response to cold water-induced stress application (Belay and Woart 2013). Our previous results also revealed decreased production of stimulatory cytokines, such as IFN-$\gamma$, and chemokines after cold-stress treatment, which are consistent with previous reports that exposure of mice to cold water leads to a decrease in the secretion of cytokines (Aviles and Monroy 2001). These findings prompted us to investigate further the impact of cold water-induced stress on immune response, pathological changes and fertility following chlamydia genital infection in the mouse model we developed.

Our current data indicate that cold water-induced stress modulates production of proinflammatory cytokines and nitric oxide by peritoneal macrophages. These results are consistent with previous reports that exposure of mice to cold water leads to an increase in the secretion of proinflammatory cytokines, suggesting that cold-water treatment of mice stimulates the release of peptide substance that may alter the function of peritoneal macrophages (Zhu *et al.* 1996). Whether the increased production of proinflammatory cytokines by peritoneal cells favors protection or disease remains to be determined. The increased production of proinflammatory cytokines by peritoneal macrophages and failure to clear *Chlamydia muridarum* suggests that these cytokines probably are involved in the development of immunopathology, rather than protection. There is a possibility that catecholamines may actually enhance IL-1$\beta$, TNF-$\alpha$ and primarily IL-6 productions that are detrimental effects of proinflammatory cytokines. Previous studies showed that altered macrophages and dysregulated macrophage function may well contribute to the generalized suppression of the immune response in the host as described (Cheng *et al.* 1990). Although produced by $CD8^+$ T cells, it has been demonstrated that TNF-$\alpha$ contributes significantly to upper genital tract pathology in mice (Murthy *et al.* 2011).

The present data also show that exposure of mice to cold water-induced stress leads to elevated shedding of chlamydia, indicating suppression of the immune system and subsequently leading to infection that is more progressive. Our results are consistent with other reports that stress suppresses the ability of the immune system to fight viral, bacterial, fungal and parasitic infections (Bonneau 1996; Borghetti *et al.* 2009;; Zieziulewicz *et al.* 2013). However, the mechanism(s) involved in the increased intensity of the infection process are not well defined.

We hypothesized that NE through binding to androgenic receptors expressed on immune cells leads to decreased resistance to chlamydia genital infection. Our results revealed that the catecholamines, EP and NE, were significantly more expressed in the lymphoid and genital tract tissues of stressed, compared to non-stressed mice. Because increased intensity of chlamydia genital infection was associated with high levels of stress in stressed mice (Belay and Woart 2013), we questioned whether mice deficient in adrenergic receptors would develop reduced infection. We infected stressed $\beta$1Adr/$\beta$2Adr receptor KO mice with *C. muridarum* and found that the NE receptor-deficient mice developed low burden of *C. muridarum* compared to the stressed wild type, indicating that the receptors are associated with suppressing the immune response against *C. muridarum* infection. In addition, stress-mediated suppression of host immune response against *Chlamydia* and the increased susceptibility of the animals may be due to a lack of recruitment and activation of inflammatory cells by chemotactic factors (Mormède *et al.* 2000; Simhan *et al.* 2003). Our results appear to be substantiated by documented studies that sympathetic nerves in the lymphoid organs release the stress hormone, NE, which stimulates the $\beta$2Adr receptor expressed on $CD4^+$ and B cells, leading to modulation of the immune system (Yin *et al.* 1996; Swanson, Lee and Sanders 2001; Shimizu *et al.* 2005). Interestingly, our results also showed that NE *in vitro* significantly alters the production of different cytokines possibly exerting an immunosuppressive effect on proliferation of T cells. We believe that the increased release of cytokines from T cells of stressed mice compared to naïve T cells is a result of combined influences such as other stress hormones compared to action of NE.

Because T cells express the $\beta$2Adr, which is the receptor of NE, we decided to examine the influence of synthetic NE on cytokine production. To determine whether synthetic NE has an inhibitory effect on T cells, we evaluated the proliferation of T cells supplemented with synthetic NE *in vitro*. Different cytokine

production profiles *in vitro* and *in vivo* are obtained in this study. Although the findings *in vivo* and *in vivo* are difficult to compare, it is important to note that the effects observed *in vivo* could be a result of a combined influence from other hormones such as corticosteroids (Ziemessen *et al*. 2007). Compared to the in *vitro* action of synthetic NE, a number of cellular and molecular events and mechanisms, such as coordination with different signaling pathways and/or transcription factors that occur *in vivo*, may influence the level of cytokine production. As previously stated, the actual effects of NE on lymphocytes can be blocked by other types of immune cells (Slota *et al*. 2015). NE appears to play an important role in modulating T-cell function, and thus further studies are underway utilizing T lymphocyte subsets to have a better understand the impact of NE on these cells.

Inoculation of *C. muridarum* intravaginally showed persistent infiltration of polymorphonuclear leukocytes into the cervix and a rapid spread to the upper regions of the genital tract as shown in IFU counts. Our results demonstrate that stress worsens the pathological outcomes, as well as increased infertility outcomes following chlamydia genital infection.

These results confirm that the rate and extent of the acute inflammatory infiltrate corresponds with severe pathological outcomes (Patton and Kuo 1989; Shah *et al*. 2005; Danville 2010). Increased infiltration of neutrophils shows active inflammation, whereas challenged mice displayed significant decreases in tissue infiltration of inflammatory leukocytes, with marked reduction in the frequency of neutrophils. Moreover, previous studies have reported that large infiltration of neutrophils in the genital tract is directly related to increased burden of *C. muridarum* in the genital tract (Maxion *et al*. 2004). Greater infiltration of polymorphonuclear cells in the cervical tissue, a large number of viable chlamydia IFU, more hydrosalpinx in the oviduct and dilatation of the uterus with clear fluid were observed in stressed mice compared to controls. This shows that the immune response is insufficient to clear *C. muridarum* in the genital tract of stressed mice.

One objective of the study was to test the hypothesis that cold-induced stress enhances infertility in infected mice. We showed that chlamydia genital-infected mice were more infertile with the cold-induced stress treatment, whereas unstressed controls were fertile. This is in agreement with previous studies (Tuffrey, Alexander and Taylor-Robinson 1990). Our results are also consistent with previous results in decreased fertility and premature delivery in mice (de la Maza *et al*. 1994). Previous studies have shown that the fertility of BALB/c strains following chlamydia genital infection was not different from that of controls (Tuffrey *et al*. 1992). Our results, however, showed that stress enhances chlamydia-induced infertility, which is consistent with previous observations that cold water-induced stress modulates cytokine production that subsequently leads to inhibition of host defense against *C. muridarum*.

Although several studies have demonstrated that *C. muridarum* infection results in pathological outcomes and infertility, the current study is the first report of its kind that stress likely worsens pathological disease and increases chlamydia-induced infertility in mice. The results of this study showed a greater number of hydrosalpnix linked with low rate of fertility in stressed group compared to non-stressed *C. muridarum*-infected mice. The poor fertility outcome exhibited in *C. muridarum*-infected stressed mice could be linked to the negative impact of hydrosalpnix in the host. Our results are in agreement with previous reports that hydrosalpnix fluid inhibits *in vitro* fertilization and embryonic development in a mouse model (Beyler *et al*. 1997). We speculate that stress is a problem and may play a major role in enhancing acute inflammation during chlamydia genital infection that leads to worsened pathology and infertility in our mouse model.

Despite the fact that *C. trachomatis* is the causative agent of humans, we used the mouse agent, *C. muridarum,* in this study for the following reasons. Studies show that inoculation of *C. trachomatis* in mice does not cause productive infection, nor does it cause reproductive pathologies as observed following *C. muridarum* infections in mice (Darville *et al*. 1997; Lyons *et al*. 2005). Furthermore, *C. trachomatis* in mice may not directly mimic *C. trachomatis* infections in human because *C. trachomatis* and *C. muridarum* have evolved host-specific mechanisms for IFN-gamma evasion (tryptophan synthetize and GTPase, respectively (Nelson *et al*. 2005). It is true that human and mouse immunology differs with respect to downstream IFN-gamma signaling that controls chlamydial replication. Such features are only produced by the naturally adapted pathogen in mice, *C. muridarum*. Moreover, studies have shown that primary genital infection of mice with *C. trachomatis* induces immunity against challenge with E and L human biovars (Ramsey *et al*. 1999; Igietseme *et al*. 2009). Thus, the mouse strain, *C. muridarum* has been used as a model organism for the study of human *C. trachomatis* urogenital and respiratory tract infections (Shao *et al*. 2012; De Clercq, Kalmar and Vanrompay 2013). Several studies of the mouse immune system have led to significant advances in our knowledge of the human immune system. While we agree that no model is perfect, extensive literature presents little evidence to suggest that immunological findings made using *C. muridarum* infection in mice is vastly different from those that occur with *C. trachomatis* infections in human. The closeness of the mouse and human serovars has been described, and thus the results of our studies in mice might extend to what happens in humans that are stressed and exposed to *C. trachomatis*, which could explain the increased incidence of chlamydial genital infection in some populations.

In conclusion, the initial observations in this study could serve as the basis for designing studies to assess in detail how stressful conditions may promote susceptibility to chlamydia genital infection and disease onset, the associated immunological alterations and immunomodulatory mechanisms. We feel that this study is important because psychological or physical stressors may lead to increased stress hormone production and have profound effects on health, including prevalence of chlamydia genital infection. Evidence shows that these stressors generally are greater in populations of lower socioeconomic status, in which there are increased health concerns. However, this study is preliminary and does not fully define the underlying similarities between our animal model and humans. The present finding, taken together within previous observations (Belay and Woart 2013) indicates that cold-water induced stress increases the intensity of chlamydia genital infection in mice. Furthermore, the present results show that stress increases disease progression, contributing to pathology and increased chlamydia-induced infertility. The long term of our study will employ the cold water-induced stress in a $\beta$2Adr receptor KO mouse model, and the hypothesis to be tested is that cold water-induced stress leads to NE modulation of the immune response against *C. muridarum* genital infection by enhancing the production of immunopathogenic cytokines that result in disease sequelae. We believe that this study may offer important direction for further investigation of the mechanisms of stress on chlamydia genital infection and immunity in humans.

## ACKNOWLEDGEMENT

The authors are grateful to Dr Joseph Igiesteme; at the Centers for Disease Control and Prevention, for his expertise and consultation in the *Chlamydia* project and for providing us *Chlamydia* stock cultures throughout the study. The authors are also thankful to Dr Guy Sims's Title III Office at Bluefield State College for the financial assistance to prepare the manuscript. The authors are also very thankful to Dr Julie Kalk, Associate Professor of Physics and MS. Beth Hash at Bluefield State College, for editing our complete manuscript.

## FUNDING

This work was supported by National Institution of Health (NIH) grant P20GM103434 to the West Virginia IDeA Network Biomedical Research Excellence (WV-INBRE), awarded to Bluefield State College, and National Institute of Allergy and Infectious Diseases at NIH grant R15AI124156 awarded to Bluefield State College.

***Conflict of interest.*** None declared.

## REFERENCES

Aviles H, Belay T, Vance M *et al.* Active hexose correlated compound enhances the immune function of mice in the hindlimb-unloading model of spaceflight conditions. *J Appl Physiol* 2004;**97**:1437–44.

Aviles H, Johnson MT, Monroy FP. Effects of cold stress on spleen cell proliferation and cytokine production during chronic *Toxoplasma gondii* infection. *Neuroimmunomodulation* 2004;**11**:93–102.

Aviles H, Monroy FP. Immunomodulatory effects of cold stress on mice infected intraperitoneally with a 50% lethal dose of *Toxoplasma gondii*. *Neuroimmunomodulation* 2001;**9**:6–12.

Beatty WL, Morrison RP, Byne GI. Persistent *Chlamydia*: form cell culture to a paradigm for *Chlamydial* pathogenesis. *Microbiol Rev* 1994;**58**:686–99.

Belay T, Eko FO, Ananaba GA *et al.* Chemokine and chemokine receptor dynamics during genital chlamydia infection. *Infect Immun* 2002;**70**:844–50.

Belay T, Woart A. Cold-induced stress increases the intensity of Chlamydia genital infection in mice. *J Microbiol Immunol* 2013 **45**:330–7.

Beyler SA, James KP, Fritz MA *et al.* Hydrosalpingeal fluid inhibits *in-vitro* embryonic development in a murine model. *Hum Reprod* 1997;**12**:2724–28.

Bonneau RH. Stress-induced effects on integral immune components involved in herpes simplex virus (HSV)-nonspecific memory cytotoxic T lymphocyte activation. *Brain Behav Immun* 1996;**10**:139–63.

Borghetti P, Saleri R, Mocchegiani E *et al.* P. Infection, immunity and the neuroendocrine response. *Vet Immunol Immunop* 2009;**130**:141–62.

Bylund DB, Eikenberg DC, Hieble JP *et al.* International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol Rev* 1994;**46**:121–36.

Cain TK, Rank RG. Local Th1-like responses are induced by intravaginal infection of mice with the mouse pneumonitis biovar of *Chlamydia trachomatis*. *Infect Immun* 1995;**63**: 1784–9.

Center of Disease Control: National surveillance Data for Chlamydia, Gonorrhea, and Syphills. *Published in CDC's report, Sexually Transmitted Disease Surveillance*, 2008, www.cdc.gov/std/stats (18 April 2017, date last accessed).

Cheng GJ, Morrow-Tesch JL, Beller ID *et al.* Immunosuppression in mice induced by cold water stress. *Brain Behav Immun* 1990;**4**:278–91.

Cohen CR, Brunham RC. Pathogenesis of Chlamydia induced pelvic inflammatory disease. *Sex Transm Infect* 1999;**75**:21–4.

Cotter TW, Ramsey KH, Miranpuri GS *et al.* Dissemination of *Chlamydia trachomatis* chronic genital tract infection in gamma interferon gene knockout mice. *Infect Immun* 1997;**65**:2145–52.

Danville T. Pathogenesis of genital tract disease due to *Chlamydia trachomatis*. *J Infect Dis* 2010;Suppl **2**:S114–25.

Darville T, Andrews CW, Laffoon K *et al.* Mouse strain-dependent variation in the course and outcome of chlamydial genital tract infection is associated with differences in host response. *Infect Immun* 1997;**65**:3065–73.

De Clercq E, Kalmar I, Vanrompay D. Animal models for studying female genital tract infection with *Chlamydia trachomatis*. *Infect Immun* 2013;**81**:3060–7.

de la Maza LM, Pal S, Khamesipour A *et al.* Intravaginal inoculation of mice with the Chlamydia trachomatis mouse pneumonitis biovar results in infertility. *Infect Immun* 1994;**62**:2094–7.

Ellen JM, Kohn RP, Bolan GA *et al.* Socioeconomic differences in sexually transmitted disease rates among black and white adolescents, San Francisco, 1990 to 1992. *Am J Public Health* 1995;**85**:1546–8.

Elenkov IJ, Papanicolaou DA, Wilder RL *et al.* Modulatory effects of glucocoricoids and catecholamines on human interlukin-12 and interleukin-10 production: clinical implication. *P Assoc Am Physician* 1996;**108**:474–81.

Goyarts E, Matsui M, Mammone T *et al.* Norepinephrine modulates human dendritic cell activation by altering cytokine release. *Exp Dermatol* 2008;**17**:188–96.

Haggerty C, Gottlieb S, Taylor B *et al.* Risk of sequelae after *Chlamydia trachomatis* genital infection in women. *J Infect Dis* 2010;**201**(Suppl 2):S134–5.

Igietseme JU, Eko FO, Black CM. Chlamydia vaccines: recent developments and the role of adjuvants in future formulations. *Expert Rev Vaccines* 2011;**10**:1585–96.

Igietseme JU, He Q, Joseph K *et al.* Role of T lymphocytes in the pathogenesis of Chlamydia Disease. *J Infect Dis* 2009;**200**:926–34.

Igietseme JU, Portis JL, Perry LL. Inflammation and Clearance of *Chlamydia trachomatis* in enteric and non-enteric mucosae. *Infect Immun* 2001;**69**:1832–40.

Igietseme JU, Rank RG. Susceptibility to reinfection after a primary chlamydial genital infection is associated with a decrease of antigen specific T cells in the genital tract. *Infect Immun* 1991;**59**:1346–51.

Johansson M, Schon K, Ward M *et al.* Genital tract infection with *Chlamydia trachomatis* fails to induce protective immunity in gamma interferon receptor-deficient mice despite a strong local immunoglobulin A response. *Infect Immun* 1997;**65**:1032–44.

Kelly AK, Walker JC, Jameel SH *et al.* Differential regulation of CD4 lymphocyte recruitment between the upper and lower regions of the genital tract during *Chlamydia trachomatis*. *Infection Infect Immun* 2000;**68**:1519–28.

Kohm PA, Tang Y, Sanders VM *et al.* Activation of antigen-specific CD4+$Th_2$ cells and B cell *in vivo* increases norepinephrine release in the spleen and bone marrow. *J Immunol* 2000;**165**:725–33.

Kubera M, Basta-Kaim A, Holan V *et al.* Effect of mild chronic stress, as a model of depression, on the immunoreactivity of C57BL/6 mice. *Int J Immunopharmaco* 1998;**20**:781–9.

Lorton D, Bellinger DL. Molecular mechanisms underlying β-adrenergic receptor-mediated cross-talk between sympathetic neurons and immune cells. *Int J Mol Sci* 2015;**16**: 5635–65.

Lyons JM, Ito JI, Peña AS *et al.* Differences in growth characteristics and elementary body associated cytotoxicity between *Chlamydia trachomatis* oculogenital serovars D and H and *Chlamydia muridarum*. *J Clin Pathol* 2005;**58**:397–401.

Madden KS, Sanders VM, Felten DL. Catecholamine influences and sympathetic neuralmodulation of immune responsiveness. *Annu Rev Pharmacol* 1995;**35**:417–48.

Maxion HK, Liu W, Chang MH *et al.* The infecting dose of Chlamydia muridarum modulates the innate immune response and ascending infection. *Infect Immun* 2004;**72**:6330–40.

Mormède C, Castanon N, Médina C *et al.* Chronic mild stress in mice decreases peripheral cytokine and increases central cytokine expression independently of IL-10 regulation of the cytokine network. *Neuroimmunomodulation* 2000;**10**:359–66.

Morrison RP, Feilzer K, Tumas DB. Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in *Chlamydia trachomatis* genital tract infection. *Infect Immun* 1995;**63**:4661–8.

Murthy AK, Li W, Chaganty BK *et al.* Tumor necrosis factor alpha production form CD8+ T cell mediates oviduct pathological sequelae following primary genital *Chlamydia muridarum* infection. *Infect Immun* 2011;**79**:2928–35.

Nakai A, Hayano Y, Furuta F *et al.* Control of lymphocyte egress form lymph nodes through β2-adrenergic receptors. *J Exp Med* 2014;**211**:2583–98.

Nelson DE, Virok DP, Wood H *et al.* Chlamydial IFN-gamma immune evasion is linked to host infection tropism. *P Natl Acad Sci USA* 2005;**102**:10658–1063.

Owusu-Edusei K, Chesson WH, Leichliter JS *et al.* The association between racial disparity in income and reported sexually transmitted infections. *Am J Public Health* 2013;**103**:910–6.

Pal S, Hui W, Peterson EM *et al.* Factors influencing the induction of infertility in a mouse model of Chlamydia trachomatis ascending genital tract infection. *J Med Microbiol* 1998;**47**:599–605.

Pampel FC, Krueger PM, Denney JT. Socioeconomic disparities in health behaviors. *Annu Rev Sociol* 2010;**36**:349–70.

Patton DL, Kuo CC. Histopathology of *Chlamydia trachomatis* salpingitis after primary and repeated reinfections in the monkey subcutaneous pocket model. *J Reprod Fertil* 1989;**85**:647–56.

Patton DL, Kuo CC, Wang SP *et al.* Chlamydial infection of subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys. *J Infect Dis* 1987;**155**:229–35.

Perry LL, Feilzer K, Caldwell HD. Immunity to *Chlamydia trachomatis* is mediated by T helper cells through INF-gamma-dependent and independent pathways. *J Immunol* 1997;**158**:3344–352.

Ramer-Quinn DS, Baker RA, Sanders VM. Activated $Th_1$ and $Th_2$ cells differentially express the b2-adrenergic receptor: a mechanism for selective modulation of Th1 cell cytokine production. *J Immunol* 1997;**15**:4857–456.

Ramsey KH, Cotter TW, Salyer RD *et al.* Prior genital tract infection with a murine or human biovar of *Chlamydia trachomatis* protects mice against heterotypic challenge infection. *Infect Immun* 1999;**67**:3019–302.

Rank RG. Animal models of urogenital infections. *Methods Enzymol* 1994;**235**:83–93.

Rank RG. Models of immunity. In: Stephens RS (ed). *Chlamydia: Intracellular Biology, Pathogenesis And Immunity*. Washington, DC: ASM Press, 1999, 239–95.

Rank GR, Bowlin AK, Kelly KA. Characterization of lymphocyte response in the female genital tract during ascending chlamydial genital infection in the guinea pig model. *Infect Immun* 2000;**68**:5293–8.

Rank RG, Sanders MM, Patton DL. Increased incidence of oviduct pathology in the guinea pig after repeat vaginal inoculation with the chlamydial agent of guinea pig inclusion conjunctivitis. *J Sex Transm Dis* 1995;**22**:48–54.

Rober SO, Obermeier LM, Straub HR *et al.* Chronic intermittent psychosocial stress (social defeat/overcrowding) in mice increases the severity of an acute DSS-induced colitis and impairs regeneration. *Endocrinology* 2006;**147**: 4968–76.

Sanders M, Kasprowicz DJ, Swanson-Mungerson MA *et al.* Adaptive immunity in mice lacking the beta (2)-adrenergic receptors. *Brain Behav Immun* 2003;**17**:55–67.

Sanders M, Virginia M. The beta2-adrenergic receptor on T and B lymphocytes: do we understand it yet? *Brain Behav Immun* 2012;**26**:195–200.

Shah AA, Schripsema JH, Imtiaz MT *et al.* Histopathologic changes related to fibrotic oviduct occlusion after genital tract infection of mice with *Chlamydia muridarum*. *Sex Transm Dis* 2005;**32**:49–56.

Shao R, Wang X, Wang W *et al.* From mice to women and back again: causalities and clues for Chlamydia-induced tubal ectopic pregnancy. *Fertil Steril* 2012;**98**:1175–85.

Sheridan JF, Dobbs C, Brown D *et al.* Psychoneuroimmunology: stress effects on pathogenesis and immunity during infection. *Clin Microbiol Rev* 1994;**7**:200–12.

Shimizu N, Kaizuka Y, Hori T *et al.* Immobilization increases norepinephrine release and reduces NK cytotoxicity in spleen of conscious rat. *Am J Physiol-Reg I* 2005;**271**: R537–44.

Simhan N, Caritis N, Krohn A *et al.* Decreased cervical proinflammatory cytokines permit subsequent upper genital tract infection during pregnancy. *Am J Obstet Gynecol* 2003;**189**: 560–57.

Slota C, Shi A, Chen G *et al.* Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation. *Brain Behav Immun* 2015;**46**:168–79.

Solomon GF. Psychoneurology: interaction between central nervous system and immune system. *J Neurosci Res* 1987;**18**: 1–9.

Stamm WE. Chlamydia trachomatis infections of the adult. in: Holmes KK *et al.* (eds). *Sexually Transmitted Diseases*. New York: McGraw Hill, 1999, 407–22.

Swanson MA, Lee WT, Sanders VM. INF-gamma production by Th1 cells generated form naïve CD4+ T cells exposed to Norepinephrine. *J Immunol* 2001;**166**:232–40.

Tuffrey M, Alexander F, Taylor-Robinson D. Severity of salpingitis in mice after primary and repeated inoculation with a human strain of *Chlamydia trachomatis*. *J Exp Pathol* 1990;**71**: 403–10.

Tuffrey M, Alexander F, Woods C *et al.* Genetic susceptibility to chlamydial salpingitis and subsequent infertility in mice. *J Reprod Fertil* 1992;**95**:31–8.

Westrom L, Joesoef R, Reynolds G *et al.* Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with

laparoscopically verified disease and 657 control women with normal laparocacople results. *Sex Trans Dis* 1992;**19**: 185–92.

Yin D, Tuthill D, Mufson RA *et al*. Chronic restraint stress promotes lymphocyte apoptosis by modulating Cd95 expression. *J Exp Med* 1996;**191**:1423–8.

Zhu FG, Chancellor-Freeland C, Berman AS *et al*. Endogenous substance P mediates cold water stress-induced increase in interleukin-6, peritoneal macrophages. *J Neurosci* 1996;**16**:3745–52.

Ziemessen T, Mondal TK, Gao D *et al*. Psychoneuroimmunology-cross-talk between the immune and nervous system. *J Neurol* 2007;**254**(Suppl 2): II8–11.

Zieziulewicz T J, Mondal TK, Gao D *et al*. Stress-induced effects, which inhibit host defenses, alter leukocyte trafficking. *Cell Stress Chaperones* 2013;**18**:279–91.