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# The adaptive immune and stress responses of adult female CD1 mice following exposure to a viral mimetic

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ABSTRACT

Exposure to a bacterial endotoxin during puberty induces long-term changes to reproductive and non-reproductive behaviours. While the underlying mechanisms remain unknown, we have recently shown that there are age and sex differences in acute immune and stress responses following immune challenge. Given that it is unclear whether viral infections result in similar age and sex differences, the objective of this study was to examine the acute immune and stress responses following exposure to polyinosinic:polycytidylic acid (poly(I:C)), a viral mimetic, in CD1 mice and to investigate the role of gonadal hormones in these responses. CD1 male and female mice underwent sham-surgery or gonadectomy at 5 or 9 weeks of age. Following one week of recovery, at 6 (pubertal group) or 10 (adult group) weeks of age, mice were treated with either saline or poly(I:C). Poly(I:C) treatment induced greater sickness behaviour in males compared to females and increased peripheral corticosterone in adult mice relative to their pubertal counterparts. Changes in body temperature and central c-Fos expression were more prominent in adult females. Gonadectomy worsened poly(I:C)-induced sickness behaviour and altered body temperature in both sexes. The results demonstrate that adult females display the most pronounced acute changes in body temperature, corticosterone release, and c-Fos expression but show the fastest recovery in sickness behavior, indicating that, compared to males, females display an adaptive physiological response following immune stress due to higher circulating estradiol and progesterone.

## 1. Introduction

Puberty is a critical period of development that is sensitive to exposure to stressors. Stress exposure during this period can cause enduring changes in brain functioning and behavior [1,2]. More specifically, in mice, exposure to an immune stressor, like the bacterial endotoxin lipopolysaccharide (LPS), during puberty (at 6 weeks of age), induces enduring changes in reproductive [3,4] and non-reproductive behaviors, such as depression-, anxiety-, and Parkinson-like behaviours, and in cognitive function [5–9]. The behavioural responsiveness to treatment with female sex steroids, estradiol and progesterone, also changes in an enduring manner following LPS exposure [3]. These enduring effects are limited to treatment at 6 weeks of age, a stress vulnerable pubertal period since mice treated with LPS younger or older than 6 weeks of age do not show these behavioral changes [3,4]. However, the mechanisms underlying these enduring behavioral changes are still not known. LPS is derived from the cell wall of gram-negative bacteria (e.g., Escherichia coli). Following systemic administration, LPS binds to toll-like receptor-4 (TLR-4) resulting in activation of an intracellular signaling pathway leading to the activation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway [10]. Through this pathway, LPS stimulates the transcription and translation of cytokines [11,12] and the production of stress and immune responses [13,14].

Recent findings from our laboratory show that there are important age and sex differences in the acute immune response to LPS treatment. Adult mice mount a stronger immune response relative to pubertal mice. They display greater hypothermia and sickness symptoms and more pro-inflammatory cytokines in the blood relative to their pubertal counterparts following LPS treatment [14]. Additionally, exposure to LPS in adulthood induces an increase in c-Fos expression in many brain regions and an increase in corticosterone levels – changes that are not shown in pubertal mice [13]. These findings suggest that pubertal mice may be hypo-responsive to immune challenges compared to their adult counterparts. There are also notable sex differences in the acute response to LPS treatment. Compared to females, adult males display greater hypothermia and sickness behavior [14]. Adult females, on the other hand, display a greater increase in serum corticosterone levels at 2 h following LPS treatment [13] and show more pro-inflammatory cytokines at 8 h following LPS treatment compared to males [15].

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Together, these findings suggest that there are important age and sex differences in acute stress and immune responses that may provide mechanistic insight into the enduring behavioural alterations following pubertal immune challenge. While these age and sex differences are specific to LPS treatment (i.e., a bacterial endotoxin), similar age- and sex differences have been identified for other bacterial infections with older age and male biological sex being persistent factors that are each associated with negative outcomes like hospitalization [16,17]. However, it remains unknown whether exposure to other immune challenges, such as viral mimetics, would result in similar age and sex differences in immune and stress responses.

A commonly used viral mimetic in mice and rats is polyinosinic:polycytidylic acid (poly(I:C)), which is a synthetic analog of double-stranded RNA (dsRNA). Poly(I:C) binds to toll-like receptor 3 (TLR-3) and activates the innate immune system. This activation leads to the production of peripheral and central pro-inflammatory cytokines and increases in corticosterone in the blood. It also induces sickness symptoms including a febrile response, and a loss of appetite and body weight [18–23]. Similar to LPS, exposure to poly(I:C) during critical periods of development can lead to enduring changes in brain functioning and behavior. For example, prenatal exposure of poly(I:C) results in abnormalities in sensorimotor gating, reduced social interactions, and impaired learning and memory in juvenile spiny mice [24]. Moreover, prenatal poly(I:C) treatment increases anxiety-like behaviour, impairs working memory, and accelerates puberty via an increase in body mass [25–27]. Neonatal poly(I:C) treatment administered intravenously also results in neuroanatomical abnormalities such as reduced total volume in the hippocampus, striatum, and prefrontal cortex, and larger ventricular volume [25,28]. These regional and ventricular abnormalities as well as the behavioral alterations are delayed in females, compared to males, following neonatal poly(I:C) treatment [28]. While poly(I:C) has been examined in prenatal period, its effects during puberty remain unknown.

The current study aims to examine the age and sex differences in the acute immune response to a single intraperitoneal injection of poly(I:C) and whether these effects are modulated by circulating levels of gonadal steroid hormones in pubertal CD1 mice. Sickness symptoms, body temperature, plasma corticosterone, and c-Fos expression in the paraventricular nucleus (PVN) and medial prefrontal cortex (mPFC) were examined. The PVN and mPFC were selected due to their role in the neural circuitry of stress reactivity. As the immune and stress systems are intricately connected, examining age and sex differences in the functioning of the aforementioned systems following poly(I:C) treatment will contribute to a better understanding of how the acute response from a viral infection differs from a bacterial one. Understanding the role of gonadal steroid hormones in the acute immune and stress responses will contribute to our understanding of the sex differences following a viral infection especially with regards to pubertal infection.

## 2. Materials and methods

### 2.1. Animals

Eighty CD-1 mice were used in each experiment (Experiments 1 and 2). The mice were shipped at three weeks of age from Charles River Laboratories (St-Constant, Quebec) and were housed in groups of 3–4 per cage (Lexan cage dimensions of 17 × 28 × 12 cm) with ad libitum access to food and water. Cages were bedded with Teklad Corn Cob (Harlan Laboratories, Inc., Madison, WI, USA). Enrichment was provided in the cages and consisted of one square piece of Nestlet (Ancare Corp., Bellmore, NY, USA) and a refuge hut made of cardboard (Ketchum Manufacturing, Inc., Brockville, ON, CA, USA). For both experiments, mice were segregated based on sex, with males housed in all-male and females in all-female colony rooms. The rooms were kept at 24 ± 2 °C, 45% humidity and 14L:10D light cycle (lights out at 10:00 AM). Dawn and dusk were gradually induced over a one-hour period. During all the procedures, mice received equal handling from male and female researchers. All experimental procedures were approved by the Animal Care Committee at the University of Ottawa.

#### 2.1.1. Polyinosinic:polycytidylic acid (poly(I:C)) treatment

Poly(I:C) (Sigma Aldrich Co., Oakville, ON) was purchased in the form of a lyphophilized sodium salt powder and was dissolved at a concentration of 2 mg/ml in sterile water prior to injection. Mice were injected intraperitoneally either with sterile 0.9% saline solution or with poly(I:C) at a dose of 12 mg poly(I:C)/kg of body weight at 6 weeks old (pubertal group) or at 10 weeks old (adult group). This dosage of poly(I:C) was chosen based on previous literature demonstrating that this dose causes significant hypothermia, like LPS [19].

#### 2.1.2. Sickness monitoring

Sickness monitoring consisted of assessing mice for the presence of four sickness behavioural symptoms (huddling, piloerection, ptosis, and lethargy) at 30 min and 4, 8, and 10 h after exposure to saline or LPS. At each time-point, mice were examined independently by two experienced observers who were blind to treatment conditions. Each rater assigned a sickness score ranging from 0 (no symptoms) to 4 (all four sickness symptoms observed), and the final sickness score was an average score of the two raters [29].

#### 2.1.3. Bilateral gonadectomy, data-logger implantation, and body temperature recording

At 5 or 9 weeks of age, male and female mice were anesthetized and kept sedated using Isoflurane. While all mice underwent data-logger implantation, only half the mice underwent bilateral gonadectomy. The other half underwent sham-surgery. For all mice, the ventral abdominal surface was shaved and cleaned with an Endure 400 soap and chlorhexidine gluconate solution. A small incision was made on the ventral skin and underlying muscle layer. Previously programmed and sterilized mini data-logger (Subcue Data-loggers, Canadian Analytical Technologies, Calgary, Alberta) was gently placed in the abdominal cavity. For the females undergoing ovariectomies, the uterine horns were tied with absorbable suture, and the ovaries were removed. Likewise, incisions were made to the scrotum of males undergoing castrations. Testicles were excised after tying the spermatic cord with absorbable suture. The muscle layer was sutured, and the skin incision was closed with wound clips. The incision at the scrotum was sutured as well. Transdermal Bupivicaine (0.3 mg) was applied on the incision site and mice were given a subcutaneous injection of Carprofen analgesic (5 mg/ml). The mice were returned to their cages and given water bottles with 3% Children's Tylenol. Cages were placed on top of Gaymar T/Pump classic heating pads set to 38 °C ( ± 1 °C) for two days post-surgery. Male and female mice were allowed 7 days to recover.

#### 2.1.4. Euthanasia

Following poly (I:C) treatment, mice were euthanized with Euthanyl (pentobarbital) injection at 10 h (Experiment 1) or 2 h (Experiment 2). In Experiment 1, mice were euthanized with Euthanyl injection, and the mini data-loggers were removed from the mice. Transmitters were gently washed with 1% alkaline liquid detergent, rinsed with distilled water, and allowed to dry. The silicone layer covering the connector was removed with a razor and the data was downloaded using Subcue Analyzer software. In Experiment 2, mice were euthanized with an injection of Euthanyl. Blood samples were collected from the ascending aorta using 27 gauge 1 cc syringes and stored in microvette CB 300 Z tubes with a coagulant. The samples were refrigerated at 4 °C for 24 h, after which samples were centrifuged at 10,000 *g* at 20 °C for 5 min in order to separate the serum. 50 µl serum aliquots were distributed into microcentrifuge tubes, stored at −80 °C, and used for an enzyme-linked immunosorbent assay to quantify corticosterone concentration.

*2.1.5. Tissue collection*

Immediately after euthanasia, intracardial perfusion was performed only in Experiment 2 with 10 ml of 0.9% saline to wash out blood, followed by 10 ml of 4% paraformaldehyde to fix the tissue. After perfusion, the brains were quickly extracted and placed in vials containing 4% paraformaldehyde to further fix the tissue. The brains were later switched to vials containing a 30% sucrose solution for cryoprotection. The brains were sliced at 40 µm into two equal brain series using a Leica VT1200 S automated vibrating blade microtome. Sliced brains were stored in a -20 °C freezer in a cryoprotectant solution until the ICC was carried out.

*2.1.6. Immuncytochemistry (ICC)*

Free-floating tissue sections from one of the series for each mouse were rinsed in tris-buffered saline (TBS; pH 7.6) for 3 × 5 min. The sections were incubated in a solution of 2.205 g sodium citrate in 150 ml of TBS for 30 min. For antigen retrieval, the sections were washed again with TBS for 3 × 5 min and then incubated for 30 min in 0.9375 g of glycine in 125 ml of TBS. Following additional 3 × 5 min TBS rinses, the sections were incubated in a concentrated blocking solution containing TBS, 20% normal goat serum, 0.3% Triton-X, and 1% hydrogen peroxide for 30 min. The sections were then incubated overnight at room temperature in a solution containing the primary antiserum specific to c-Fos (Ab-5 rabbit anti c-Fos; PC38 Millipore; 1:10,000). Then, the tissue sections were washed in a diluted blocking solution of TBS for 3 × 5 min to remove any unbound antibody and incubated for 60 min in the secondary antibody (goat anti-rabbit biotinylated secondary antibody solution in TBS (biotinylated goat Anti-rabbit IgG (H + C); BA-1000 Vector Labs; 1:500). The sections were inse d 3 × 5 min in a 0.2% Triton-X solution, which was followed by a 60-minute incubation in the Vectastain ABC system (Vector Vectastain ABC kit Elite Pk-6100 standard; Vector Laboratories, Burlingame, CA, USA). The tissues sections were then rinsed in a TBS solution for 3 × 10 min to remove unbound antibodies. The sections were incubated in freshly prepared diaminobenzidine (DAB) solution (DAB Peroxidase Substrate kit, SK-4100 Vector Laboratories, Burlingame, CA, USA), followed by rinses in TBS. Finally, the tissue sections were mounted on microscope slides and coverslipped using Permount (Fisher Scientific, Permount Adhesive, SP15-500). Brain tissues from the mice were run in two separate immunocytochemistry runs.

*2.1.7. Image analysis and cell counting*

The medial prefrontal cortex (mPFC) and paraventricular nucleus (PVN) were examined. The number of c-Fos expressing cells were quantifed based on the best-matched section for the mPFC and PVN for each mouse. The identification of these regions was based on The Mouse Brain Atlas in Stereotaxic Coordinates [30]: medial prefrontal cortex (plate #16) and paraventricular nucleus (plate #36). An Olympus BX51 light microscope connected to a Jenoptik ProRes MF scan camera was used along with the Image J software (version 1.48), created by the National Institutes of Health. Adjusted selections for the general shape of the mPFC and PVN were then built with Image J and the same selections were used to analyze this region across all mice. Two raters performed manual cell counts on each image to identify the number of c-Fos positive cells. The average of the two raters was used for the final counts.

*2.1.8. Enzyme-linked immunosorbent assay (ELISA)*

ELISA kits were used in accordance with the supplier's instructions (Enzo Life Sciences, Cat. No. ADI-901-097) and samples were plated in duplicate for all assays. Plasma was used to identify corticosterone concentrations with the inter- and intra-assay precision for the kit used being less than 10%. All plates were read with Biotek Powerwave XS2 and analyzed with the Gen 5 V2.0 software.

*2.1.9. Data analysis*

A repeated measures ANOVA (sex x treatment x age x time x surgery) was used to assess the sickness behavior and body temperature. A three-way ANOVA (sex x treatment x age) was used to assess the statistical significance of plasma corticosterone and c-Fos expression. Significant interactions were followed by pairwise comparisons using the Bonferroni correction factor. Data that were 1.96 SD away from the mean were considered as outliers and removed from the analyses. Statistical analyses were performed using the SPSS V22 software. The criterion for statistical significance was set at $p \leq 0.05$.

*2.2. Procedures*

*2.2.1. Experiment 1: age and sex differences in poly(I:C)-induced sickness behavior and changes in body temperature*

Eighty male and female mice were gonadectomized or sham-operated and implanted with a mini data-logger at either 5 or 9 weeks of age. One week later, at 6 or 10 weeks of age, all mice were treated with either saline or poly(I:C) and changes in body temperature were recorded and sickness behavior was monitored at various time points. The mice were euthanized and the mini data-loggers were excised 10 h following saline or poly(I:C) treatment. The telemetry data was then extracted from the mini data-loggers and analyzed.

*2.2.2. Experiment 2: age and sex differences in poly(I:C)-induced increases in serum corticosterone and brain c-Fos expression in the mPFC and PVN*

Eighty male and female mice were treated with saline or poly(I:C) at 6 or 10 weeks of age. At 2 h, mice were euthanized and aortic blood was collected. Brain tissue was perfused and underwent immunocytochemistry for examination of c-Fos expression. Serum aliquots were analyzed with a commercially available corticosterone ELISA kit.

## 3. Results

*3.1. Experiment 1: age and sex differences in poly(I:C)-induced sickness behavior and changes in body temperature*

*3.1.1. Sickness behaviour following poly(I:C) treatment*

Poly(I:C) treatment increased sickness behaviour in all mice relative to their saline counterparts and induced sex- and age-specific differences at various time points (Fig. 1). Four-way ANOVA revealed significant main effects of time ($F_{(3, 165)} = 6.42$, $p < 0.05$, $\eta_p^2 = 0.105$), sex ($F_{(1, 55)} = 7.26$, $p < 0.05$, $\eta_p^2 = 0.117$), and treatment ($F_{(1, 55)} = 152.92$, $p < 0.05$, $\eta_p^2 = 0.735$). There was also a significant time x sex x treatment x age x surgery ($F_{(3, 165)} = 4.96$, $p < 0.05$, $\eta_p^2 = 0.083$) interaction.

Pairwise comparisons showed that all sham-operated and gonadectomized adult males and females treated with poly(I:C) displayed significantly more sickness behaviour than their saline-treated counterparts at 30 min following treatment (MD = 1.1, SE = 0.36, $p < 0.05$; MD = 1.1, SE = 0.36, $p < 0.05$; MD = 0.9, SE = 0.36, $p < 0.05$; MD = 1.1, SE = 0.36, $p < 0.05$, respectively). Pubertal females treated with poly(I:C) showed a delayed onset of sickness behaviour occurring at 8 h after treatment (MD = 2.3, SE = 0.33, $p < 0.05$). However, by 8 h, all adult females had recovered, as there was no difference between this poly(I:C)-treated group and saline-treated controls ($p > 0.05$). All females displayed full recovery at 10 h while both sham-operated and gonadectomized poly(I:C)-treated pubertal and adult males continued to display sickness behaviour (MD = 0.8, SE = 0.26, $p < 0.05$; MD = 1.3, SE = 0.26, $p < 0.05$; MD = 0.6, SE = 0.22, $p < 0.05$; MD = 1.0, SE = 0.22, $p < 0.05$, respectively). Moreover, adult males treated with poly(I:C) displayed significantly more sickness behavior relative to their pubertal male counterparts at 4 and 8 h (MD = 1.0, SE = 0.29, $p < 0.05$; MD = 0.9, SE = 0.25, $p < 0.05$, respectively). However, gonadectomy worsened sickness

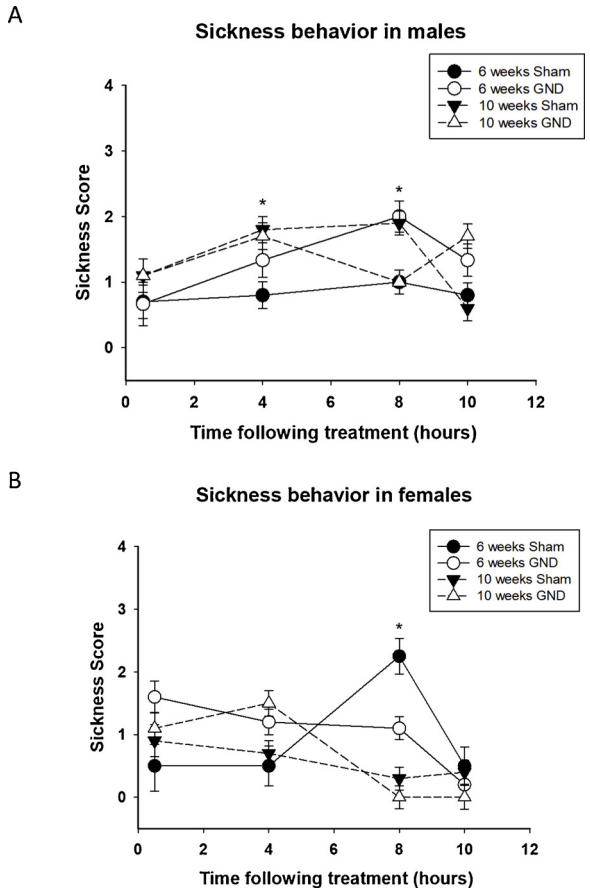


**Fig. 1.** Mean ( ± SEM) sickness score following poly(I:C) treatment in sham-operated and gonadectomized pubertal and adult (A) male and (B) female mice. The asterisk (*) denotes significant differences ($p < 0.05$) in sickness behavior between sham-operated and gonadectomized poly(I:C)-treated pubertal and adult mice at specified time points.

behaviour among poly(I:C)-treated mice. Gonadectomized and poly (I:C)-treated pubertal and adult females displayed significantly more sickness behavior at 30 min and 4 h, compared to their sham-operated counterparts (MD = 1.1, SE = 0.48, $p < 0.05$; MD = 0.8, SE = 0.29, $p < 0.05$, respectively). Similarly, at 8 h, gonadectomized pubertal males treated with poly(I:C) displayed significantly more sickness behaviour than their sham-operated counterparts (MD = 1.0, SE = 0.28, $p < 0.05$). Taken together, these findings show that poly(I:C) treatment induced sickness behaviour in all mice, but gonadectomy worsened poly(I:C)-induced sickness behaviour, and intact adult females displayed the fastest recovery after poly(I:C) treatment (Fig. 1).

#### 3.1.2. Age, sex, and surgery differences in body temperature following poly (I:C) treatment

Poly(I:C) treatment induced changes in body temperature and gonadectomy appeared to limit or reverse the temperature fluctuations in sex- and age-specific manners (Figs. 2 and 3). Three-way ANOVA revealed significant main effects of time ($F_{(20, 1280)} = 87.60$, $p < 0.05$, $\eta_p^2 = 0.578$) and treatment ($F_{(1, 64)} = 4.92$, $p < 0.05$, $\eta_p^2 = 0.071$). Significant time x age ($F_{(20, 1280)} = 5.58$, $p < 0.05$, $\eta_p^2 = 0.080$), time x sex ($F_{(20, 1280)} = 19.90$, $p < 0.05$, $\eta_p^2 = 0.237$), and time x treatment ($F_{(20, 1280)} = 18.55$, $p < 0.05$, $\eta_p^2 = 0.225$) interactions were also observed.

Pairwise comparisons showed that poly(I:C)-treated sham-operated pubertal females displayed a delayed hyperthermic response from 450 to 480 minutes and from 540 to 570 minutes compared to their saline counterparts (Fig. 2A). Sham-operated adult females displayed an initial hypothermic response from 30 to 60 minutes following poly(I:C) treatment compared to saline controls (Fig. 2B). No significant enduring body temperature changes (i.e., body temperature changes lasting for two time-points or more) were identified among the other groups. However, pairwise comparisons revealed sex differences such that poly (I:C)-treated sham-operated pubertal females showed a mild increase in body temperature from 480 to 540 minutes and 600 min following treatment compared to their male counterparts (Figs. 2A and 3 A). Sham-operated adult females showed a mild increase in body temperature in comparison to their sham-operated adult male counterparts from 510 to 600 minutes following poly(I:C) treatment (Figs. 2B and 3 B). Gonadectomy altered body temperature in pubertal mice following poly(I:C) treatment but blocked poly(I:C)-induced body temperature changes in adults. Gonadectomized pubertal females treated with poly (I:C) displayed lower body temperature relative to their sham-operated counterparts from 240 to 270 minutes following treatment (Fig. 2C). Gonadectomized poly(I:C)-treated adult females, on the other hand, showed no significant difference compared to their saline controls (Fig. 2D). Gonadectomy changed the body temperature of gonadectomized pubertal males, as they displayed increased body temperature from 510 to 540 minutes following poly(I:C) treatment compared to saline controls (Fig. 3C). To summarize, the body temperature fluctuations following poly(I:C) treatment were most prominent in gonadally intact females with adult females displaying greater body temperature changes immediately after poly(I:C) treatment, whereas the pubertal females displayed significant changes at later timepoints (Figs. 2 and 3).

### 3.2. Experiment 2: age and sex differences in poly(I:C)-induced increases in serum corticosterone and c-Fos expression in the mPFC and PVN

#### 3.2.1. Serum corticosterone concentration following poly(I:C) treatment

Poly(I:C) treatment induced an increase in corticosterone concentration in the blood (Fig. 4). Three-way ANOVA revealed main effects of sex ($F_{(1, 65)} = 11.90$, $p < 0.05$, $\eta_p^2 = 0.155$), treatment ($F_{(1, 65)} = 130.80$, $p < 0.05$, $\eta_p^2 = 0.668$), age ($F_{(1, 65)} = 7.68$, $p < 0.05$, $\eta_p^2 = 0.106$) and a significant age x treatment ($F_{(1, 65)} = 8.49$, $p < 0.05$, $\eta_p^2 = 0.116$) interaction. As expected, pairwise comparisons revealed significantly greater corticosterone levels in pubertal and adult male and female poly(I:C)-treated animals relative to their saline counterparts (MD = 1061.3, SE = 255.77, $p < 0.05$; MD = 1804.8, SE = 274.33, $p < 0.05$; MD = 1176.7, SE = 248.95, $p < 0.05$; MD = 1963.5, SE = 270.49, $p < 0.05$, respectively). Moreover, both adult males and females treated with poly(I:C) displayed increased corticosterone levels relative to their pubertal counterparts (MD = 812.96, SE = 280.53, $p < 0.05$; MD = 679.85, SE = 255.77, $p < 0.05$, respectively). Pubertal females treated with poly(I:C) also showed higher corticosterone levels relative to their pubertal male counterparts (MD = 587.99, SE = 255.77, $p < 0.05$). Overall, pubertal males and females displayed a blunted corticosterone response relative to their adult counterparts following poly(I:C) treatment (Fig. 4).

#### 3.2.2. Age and sex differences in c-Fos expression in the PVN following poly (I:C) treatment

No significant differences were observed in the PVN following poly (I:C) treatment.

#### 3.2.3. Age and sex differences in c-Fos expression in the mPFC following poly(I:C) treatment

Poly(I:C) treatment did not increase acute c-Fos expression in the mPFC rather a basal sex difference was identified (Fig. 5). Three-way ANOVA revealed a main effect of sex ($F_{(1, 35)} = 7.79$, $p < 0.05$, $\eta_p^2 = 0.182$). Pairwise comparisons indicated that adult females treated with poly(I:C) displayed significantly more c-Fos expression than their adult male counterparts (MD = 19.24, SE = 7.67, $p < 0.05$). Similarly, adult females treated with saline tended to display higher c-Fos

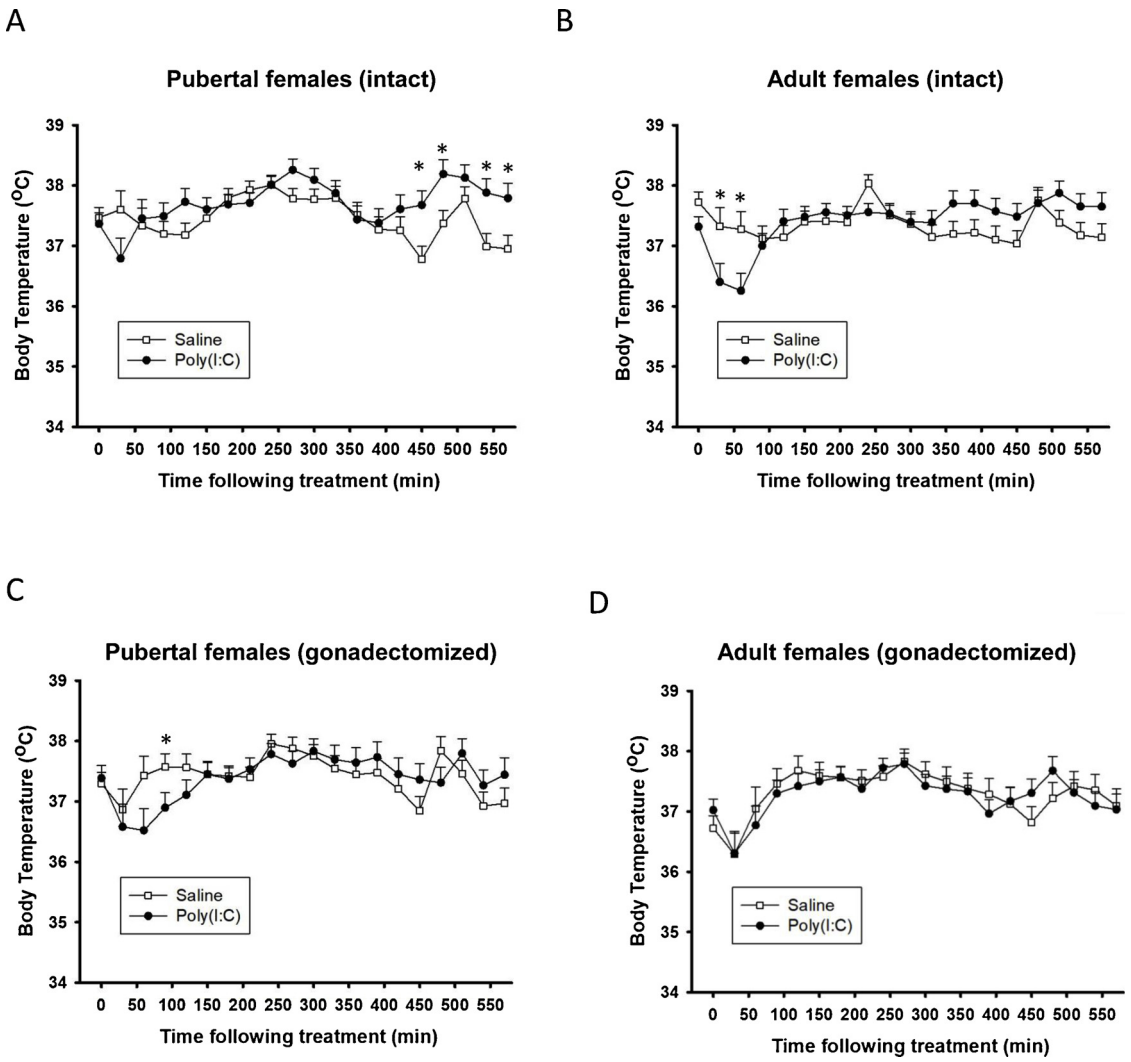


**Fig. 2.** Mean ( ± SEM) body temperature recordings following saline and poly(I:C) treatments in (A) sham-operated pubertal females, (B) sham-operated adult females, (C) gonadectomized pubertal females, and (D) gonadectomized adult females. The asterisk (*) denotes a significant difference between saline- and poly(I:C)-treated mice at the specified time points ($p < 0.05$).

expression than their adult male counterparts (MD = 14.77, SE = 8.03, $0.05 < p < 0.1$). Saline-treated adult females also displayed significantly more c-Fos expression in the mPFC than their pubertal female counterparts (MD = 16.67, SE = 7.67, $p < 0.05$). Therefore, a basal sex difference in central c-Fos expression was identified with adult females displaying greater c-Fos expression compared to adult males (Fig. 5).

## 4. Discussion

This study reveals important age and sex differences in stress and immune responses following poly(I:C) treatment. Adult males and females display greater corticosterone relative to their pubertal counterparts during the initial phase (i.e., first 2 h) of poly(I:C) treatment. However,

poly(I:C) does not significantly increase c-Fos expression in the PVN or mPFC compared to saline treatment. Adult males display the most sickness behaviour relative to their pubertal male and adult female counterparts following poly(I:C) treatment. Sham-operated adult females display the most pronounced acute change in body temperature. However, gonadectomy appears to increase sickness behaviour, especially in females, and it appears to limit or reverse the body temperature fluctuations experienced by pubertal and adult females and pubertal males.

Like LPS treatment, poly(I:C) induced sickness behavioural symptoms in all mice irrespective of sex, age, and gonadal status. However, the extent of sickness behaviour was less than that observed following LPS treatment [14,31]. This may be attributed to differences in signal transduction and localization between the TLRs. LPS binds to TLR-4 whereas poly(I:C) binds to TLR-3. Signaling differences between these receptors may result in altered cytokine profiles following LPS and poly (I:C) treatment [19,32,33]. For example, a greater dose of poly(I:C) in relation to LPS has been required to induce IL-6 mRNA and plasma IL-6 [18,32,34]. As with IL-6, LPS is also more potent than poly(I:C) at inducing an increase in IL-1β concentrations [18,35]. In contrast, poly (I:C) is more potent at inducing type 1 interferons than LPS. Type 1 interferons contribute to poly(I:C)-induced symptoms of immune activation, such as hypoactivity, anhedonia, hypothermia, and anorexia [36]. Thus, the differences in the sickness symptoms induced by the two immune mimetics may be due to the stronger ability of LPS at inducing cytokines linked to sickness behaviour observed in the current study.

Poly(I:C)-induced changes in body temperature were more prominent for pubertal and adult females relative to their male counterparts. The change in body temperature in sham-operated pubertal and adult females coincides with the onset of their sickness behaviour. However, pubertal females display a mild hyperthermic response in conjunction

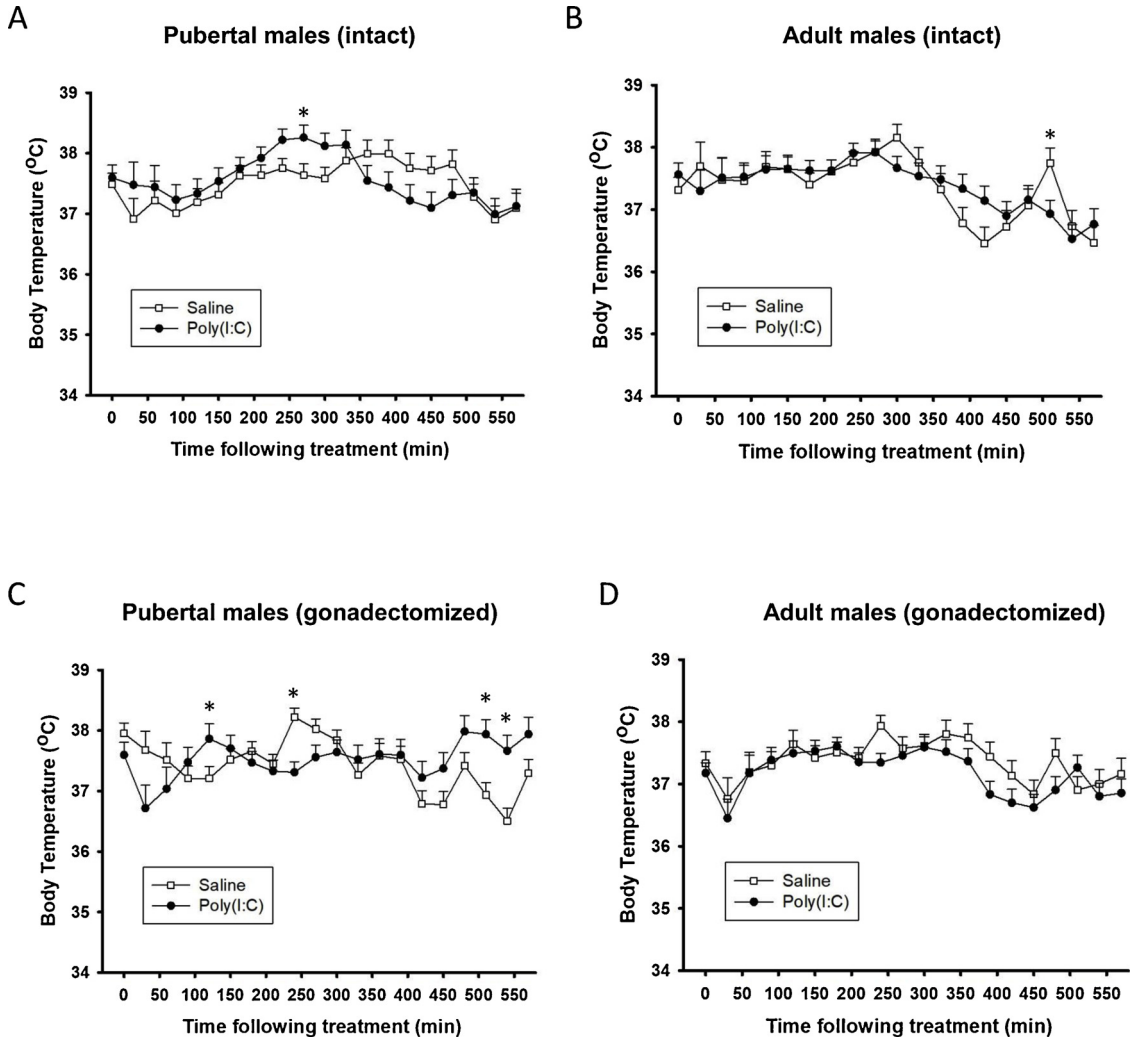


**Fig. 3.** Mean ( ± SEM) body temperature recordings following saline and poly(I:C) treatments in (A) sham-operated pubertal males, (B) sham-operated adult males, (C) gonadectomized pubertal males, and (D) gonadectomized adult males. The asterisk (*) denotes a significant difference ($p < 0.05$) between saline- and poly(I:C)-treated mice at the specified time points.

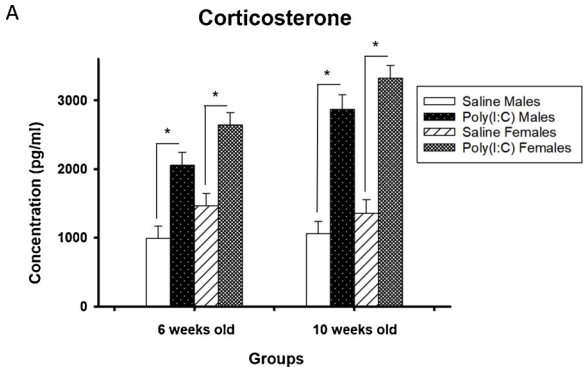


**Fig. 4.** Mean ( ± SEM) concentration of corticosterone in all mice following saline and poly(I:C) treatments 2 h later. The asterisk (*) denotes a significant difference ($p < 0.05$) between saline- and poly(I:C)-treated mice.

with their sickness behaviour, while the adult females display a mild hypothermic response when their sickness behavioural symptoms are present. The co-occurrence of sickness behaviour and body temperature changes may be mediated by differential changes in pyrogenic and anti-pyretic cytokines, such as IL-1β and TNFα, respectively, following poly (I:C) treatment [19,37]. The degree of hypothermia observed among sham-operated adult females in the current study is not consistent with that observed following LPS treatment, suggesting that hypothermia displayed by adult males and females depends on the nature of the immune challenge (i.e., bacterial vs. viral). Cai et al. (2016) found that CD1 adult males show greater hypothermia compared to adult females following LPS challenge. However, adult female mice show a greater hypothermic response following the influenza virus [38]. Hypothermia has been shown to be more advantageous than fever following LPS treatment in rats [39]. Similarly, a hypothermic response following a viral infection may be a positive response. The hypothermic response of females may be mediated by circulating and central cytokines. Adult female mice display a higher induction of pro-inflammatory cytokines in their lungs including TNFα, IL-6, and IFNγ following treatment with the influenza virus [38]. Increased levels of TNFα and IFNγ have especially been identified in the lungs and periphery following poly(I:C) exposure and are associated with a hypothermic response [19,40,41].

Our findings also show that sickness behavior in females recovers faster from the poly(I:C) treatment compared to males. Testosterone and estradiol are known to have opposite roles in the modulation of the immune response [42,43]. Estradiol suppresses inflammation and has immunoprotective effects, while testosterone increases inflammatory responses and has immunosuppressive effects by targeting the NF-κB pathway [44,45]. Therefore, in the current study, the faster recovery of pubertal and adult females may be due to enhanced activation of anti-

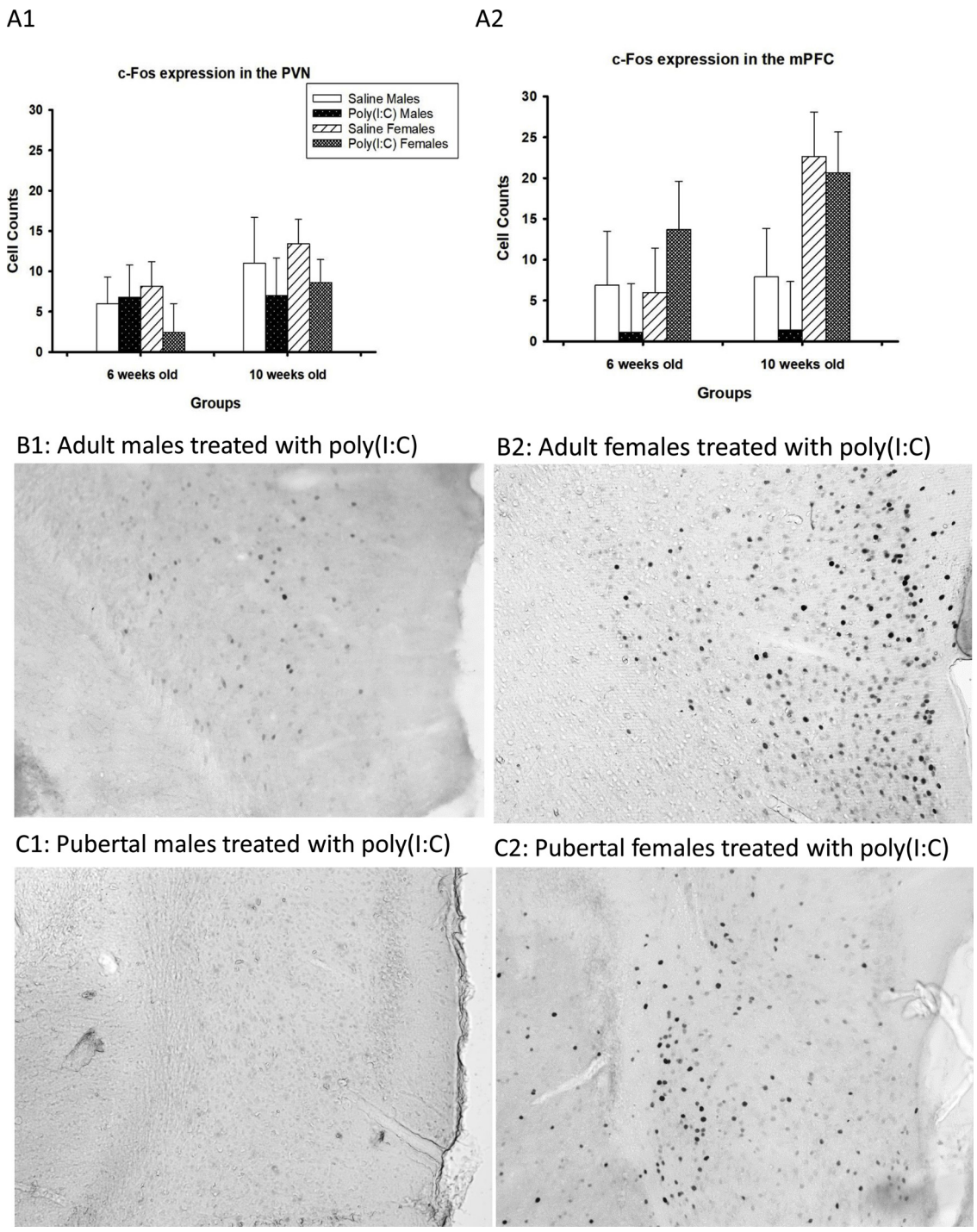


**Fig. 5.** Mean ( ± SEM) number of c-Fos + cells in all mice following saline and poly(I:C) treatment in (A) the paraventricular nucleus and (B) medial prefrontal cortex2 h following treatment. The asterisk (*) denotes a significant difference ($p < 0.05$) between saline- and poly(I:C)-treated mice. (C) Representative photomicrographs of c-Fos expression in the medial prefrontal cortex in pubertal and adult males and females treated with poly(I:C).

inflammatory immune pathways that confer increased immune protection. This faster recovery in females following poly(I:C) treatment compared to males is also consistent with previous work using LPS [14,46] and can be regarded as an adaptive psychophysiological mechanism [47]. Clinical studies on other types of viral infections also support these findings. For example, men infected with the influenza virus mount a faster IL-1β mRNA expression, and display blunted corticosterone secretion compared to women [48]. Similarly, female patients have a higher survival rate to the Ebola virus and spend a shorter amount of time hospitalized [49]. Interestingly, poly(I:C)-treated gonadectomized pubertal and adult females recovered quicker than their gonadectomized male counterparts. Therefore, it appears that additional factors beyond the circulating levels and activational effects of gonadal sex hormones contribute to the faster recovery in females following an immune challenge.

Following poly(I:C) treatment, adult mice display increased corticosterone levels relative to their pubertal counterparts. Similar age effects on corticosterone release have been found with other types of stressors, like LPS treatment and restraint stress [13,50,51]. We postulate that the hypo-responsive stress reactivity of pubertal mice is

maladaptive, as it may set the stage for altered brain functioning and behaviour later in life.

Poly(I:C) treatment did not increase c-Fos expression in the PVN and mPFC. However, consistent with other findings, this study points to adult females showing a greater stress response when exposed to certain environmental factors (i.e., handling during injections) than their male counterparts [52–54]. The higher stress reactivity among females, in general, may be due to circulating gonadal hormones. Estrogens stimulate the HPA axis, and there is also a greater number of estrogen receptors in stress-responsive brain regions. Thus, adult females may experience greater hormonal stimulation of this neuroendocrine pathway, in general, due to circulating estradiol levels. In the present study, the increased stress response following immune stress may confer physiological benefits to females.

## 5. Conclusions

These findings highlight important age and sex differences in CD1 mice following poly(I:C) treatment. This study depicts many similarities and differences between the effects of poly(I:C) and LPS treatments in the CD1 strain of mice with respect to the age groups examined. Overall, the findings point to adult females mounting the most adaptive physiological response to fight a viral infection. Adult females display a hypothermic response and increased peripheral and central stress reactivity following poly(I:C) treatment, yet a faster recovery, in comparison to age-matched males and pubertal females. Gonadectomy worsens poly(I:C)-induced sickness behaviour and alters body temperature profiles. These results advance our understanding of the age and sex differences following exposure to a viral infection and of the impact of circulating gonadal steroid hormones on immune and stress responses following an immune challenge.

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