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# *Toxoplasma gondii* infection and testosterone congruently increase tolerance of male rats for risk of reward forfeiture

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ABSTRACT

Decision making under risk involves balancing the potential of gaining rewards with the possibility of loss and/or punishment. Tolerance to risk varies between individuals. Understanding the biological basis of risk tolerance is pertinent because excessive tolerance contributes to adverse health and safety outcomes. Yet, not much is known about biological factors mediating inter-individual variability in this regard. We investigate if latent *Toxoplasma gondii* infection can cause risk tolerance. Using a rodent model of the balloon analogous risk task, we show that latent *T. gondii* infection leads to a greater tolerance of reward forfeiture. Furthermore, effects of the infection on risk can be recapitulated with testosterone supplementation alone, demonstrating that greater testosterone synthesis by the host post-infection is sufficient to change risk tolerance. *T. gondii* is a frequent parasite of humans and animals. Thus, the infection status can potentially explain some of the inter-individual variability in the risky decision making.



## Introduction

Animals and humans typically make decisions in ambivalent situations and under risk of forfeiture. Biological factors play an important role in such decision making. Two such biological factors have attracted greater scientific interest: first, mesolimbic dopaminergic system which pivots around nucleus accumbens; and second, steroid hormones secreted by peripheral glands. Testosterone secreted by male gonads enhances risk-taking behavior in human subjects (Coates and Herbert, 2008; Cooper et al., 2014; Peper et al., 2013; Stanton et al., 2011). Extraneous testosterone can be used as a positive reinforcement in rodents (Wood, 2004; Wood et al., 2004), suggesting its ability to intersect with dopaminergic reward system in the brain. Consistent with this, placement of testosterone or its metabolites in nucleus accumbens facilitates conditioned place preference (Frye et al., 2002), again suggesting that testosterone can activate mesolimbic dopaminergic pathways involved in decision making under risk.

Interestingly a widely prevalent protozoan parasite (Jones et al., 2014), *Toxoplasma gondii*, alters both testosterone synthesis and nucleus accumbal dopamine content in laboratory rats (Lim et al., 2013; Tan et al., 2015). *T. gondii* invades testes in this animal model (Hari Dass et al., 2011; Vyas, 2013), resulting in a long-term increase of testosterone synthesis (Lim et al., 2013). In addition, the infection results in greater synthesis of arginine vasopressin in brain regions afferent to nucleus accumbens (Hari Dass and Vyas, 2014), structural diminution of nucleus accumbens neurons and decrease in total dopamine concentration (Tan et al., 2015). Retrospective studies suggest that chronic *T. gondii* infection enhances behaviors reminiscent of risk-taking in human subjects like being involved in traffic accidents (Flegr et al., 2002, 2009; Yereli et al., 2006).

These observations suggest that *T. gondii* increases tolerance to reward forfeiture through associated increase in testosterone availability. In this report, we experimentally test this hypothesis.

## Materials and methods

### *Animals*

Male Wistar rats were used. Rats were 8 weeks of age at the start of experiments, housed 2 per cage with 12 h light–dark cycle (lights on at 7 AM). Rats were provided with *ad libitum* access to food and water, except during operant experiments when rats were maintained on a restricted diet to 85% of their free-feeding weight and allowance of 3–5 g per week body weight gain. Animals were obtained from the vivarium of National University of Singapore. All animal procedures were approved by Nanyang Technological University's institutional animal care and use committee.

### *Parasites*

*T. gondii* tachyzoites of type 2 Prugniaud strain were maintained in human skin fibroblast cultures. Infected fibroblasts were syringe-lysed to release tachyzoites. Animals were either infected with tachyzoites

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($5 \times 10^6$, intraperitoneal) or mock-infected with sterile phosphate buffered saline. Eight weeks elapsed between infection and the start of the behavioral experiment; an incubation period consistent with the presence of chronic infection and absence of acute parasitic proliferation (Vyas et al., 2007a).

*Castration and testosterone treatment*

Surgery was performed using aseptic techniques under isoflurane anesthesia (2.5% gaseous isoflurane with pure $O_2$). After placing animals in dorsal recumbency, testes were approached through a mid-scrotal incision. Testes, vas deferens and testicular fat pad were bilaterally removed followed by suturing of spermatic blood vessels. Scrotum was subsequently sutured. One micro-infusion pump was placed subcutaneously supplying either vehicle (grape seed oil) or testosterone cypionate. Microinfusion pumps (iPRECIO SMP-200; Durect) delivered their cargo for several months requiring only monthly refills through the septum of the pumps accessed through subcutaneous route. Pumps were programmed to deliver 0.8 μL/day of vehicle or testosterone cypionate (200 mg/mL dissolved in grape seed oil; Pfizer) at a constant rate. This dose of the testosterone is in slight excess to physiological norms of circulating testosterone (Aubele et al., 2008).

Animals were given pre-operative prophylaxis antibiotic (Baytril 10 mg/kg, sc; Bayer) and pain relief (Carprofen 5 mg/kg, sc; Pfizer). After surgery, animals were housed singly for >3 days with supplemental pain relief daily (Carprofen 5 mg/kg, sc). Animals were re-housed with prior cage-mates once wound healing was visually confirmed. At least one week elapsed between surgery and start of food restriction for operant testing. Pumps were programmed to start infusion only after the recovery period.

*Quantification of serum testosterone levels*

The method was modified from French (2013). 98 μL of serum and 2 μL of internal standard (10 ng/mL Testosterone-2,3,4-$^{13}C_3$ in acetonitrile) were suspended in 1.1 mL of hexane:ethyl acetate (90:10 v/v). The mixture was vortex-mixed and centrifuged at 3000 rpm for 10 min at 4 °C. The aqueous layer was frozen on dry ice and the supernatant was pipetted into in a clean tube. The solvent was then evaporated to dryness. Extracted testosterone was reconstituted in 100 μL of 20% acetonitrile. After reconstitution, the extracted sample was centrifuged at 13,200 rpm for 10 min at 4 °C and 85 μL of the supernatant was transferred to HPLC vials for liquid chromatography electrospray tandem mass spectrometry (LCMS/MS) analysis for the detection of testosterone. Detection of testosterone using LCMS/MS spectrometry is described in Takyi-Williams et al. (2015). The quantitation limit of the method was 0.06 ng/mL and the method was linear within a range of 0.06 ng/mL to 1.95 ng/mL.

*Balloon analogous risk task*

Operant performance under risk of reward forfeiture was measured using a balloon analogous risk task, adapted from Jentsch et al. (2010) (Fig. 1). Operant chambers used for training and testing were provisioned with a house light and internal stimulus lights (30 × 24 × 30 cm, Med-Associates; programmed using K-Limbic, Conclusive Solution). Chambers were enclosed in a sound-attenuating and ventilated outer cabinet. Ventilating exhaust fan mounted on the outer cabinet provided a masking white noise (88 dB, linear scale). Operation of the pellet dispenser delivered 45 mg food pellets (formula 5TUM; TestDiets) into the food receptacle within the operant chamber. In addition, two retractable stainless steel response levers were mounted on either side of the food delivery receptacle (8.5 cm above the floor, 7 cm lateral to the outer edge of food tray).

During initial training, rats were individually placed in the operant chambers and one of the levers was extended for 30 min (phase 1). Each operant response of one lever press was reinforced with the delivery of one food pellet. The process was repeated for the other lever and animals were subsequently returned to their home cage. This phase of training was repeated daily until all rats committed ≥60 responses for each lever during a 30-min session.

Subsequently, one of the levers was randomly designated as the 'add' lever (phase 2). The left or right lever was designated as the 'add' lever in a counterbalanced manner across animals; and kept consistent for each animal across training and testing. Rats were trained to increase lever presses on the 'add' lever by successively increasing requisite responses from 1 to 3 and then 10 before delivery of one food pellet ensued (session duration = 20 min; 1 trial/day). This phase of the training continued till individual animals accumulated ≥30 lever presses per session.

Next, subjects were trained in sessions comprising 54 trials (phase 3; 1 session/day). The 'add' lever was presented. Animals were required to accumulate a pre-determined number of lever presses (varied randomly between 2 and 15) before the 'add' lever was retracted and an alternative lever designated as the 'cash-out' was presented. Pressing the 'cash-out' lever resulted in delivery of food pellets equal in number to presses of the 'add' lever required for that trial.

After completion of training in the phase 3, rats began daily testing on the actual task (Fig. 1; 54 trials/session, 1 session/day). Initially animals did not encounter any risk of reward forfeiture (baseline). During each trial, 'add' and 'cash-out' levers were presented simultaneously. Animals were required to execute >1 lever presses on the 'add' lever and follow it up by pressing 'cash-out' lever. This resulted in delivery of delivery of food pellets equal in number to the total number of 'add' lever presses. Pressing the 'cash-out' lever before the 'add' lever resulted in an aborted trial without delivery of food. Failure to respond within 3 s resulted in a mistrial. Both mistrial and aborted trials resulted in zero yields. Only gainful trials were included in the analysis. The process was repeated daily till they reached a stable baseline ($p > 0.05$ for mean lever presses on 'add' lever when analyzed for three consecutive days). Stable baseline was observed after 12–18 successive sessions had elapsed.

Once a stable baseline had been achieved, animals were tested under a risk of forfeiture. Each successive press of the 'add' lever added one pellet to the accrued reward, but also linearly increased the probability to total forfeiture. Three forfeiture probabilities were used (Δ increase in forfeiture probability per 'add' press: 0, 0.111 and 0.167; assigned pseudo-randomly and non-alternating; one session per day). For experiments involving castrated animals with/without testosterone supplementation, only 0 and 0.167 risk schedule was used. Trials comprising of zero risk of reward forfeiture were signaled by the illumination of a house light during sessions. Response of animals in zero forfeiture trials before introduction of risk (baseline) was compared with zero risk trials after introducing risk of forfeiture (probe). Trials comprising of risk of reward forfeiture were signaled by the illumination of a distinct stimulus light within the operant chamber. Mixed-risk sessions continued till stable responding was achieved after 12 to 18 successive sessions. Probe trials with zero risk of reward forfeiture were interspersed with forfeiture risk trials in a pseudorandom manner. Mean lever presses during gainful trials was used as the endpoint during both baseline and mixed-risk sessions.

Animals were assigned in the groups in a random manner. Training and subsequent testing for control and infected animals was conducted >7 weeks post-infection. All animals in control and infected groups were tested using continuous reinforcement schedule (FR1). For experiment involving testosterone supplementation, gonad-intact animals were first trained to a stable baseline before surgery. After at least one week of post-surgery recovery, animals were again trained till stable baseline and then testing commenced. Castrated animals with or without testosterone supplementation were tested at continuous reinforcement schedule (FR1), although these animals had been initially trained on intermittent schedule (FR3) and then shifted to FR1 till stable baseline had been achieved.