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# Immunity and Sex Concerns on Behaviour

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***Authors' contributions***

*Author SCI designed the study, performed experiments, the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors CJ, GG and JO collaborated performing the experiments. Author JCC contributed to the design of the study, author JCM purified C tala pollen glycoprotein and collaborated in the design and statistical analysis. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** To find evidence that the immune response modifies behavior by regulating sex hormones.
**Study Design:** Experimental transversal case control study and longitudinal experimental case control study.
**Place and Duration of Study:** Biochemistry and Molecular Biology Chair, School of Medicine, Medical Sciences Faculty, National University of Córdoba. 2009-2015.
**Methodology:** Albino Swiss mice Rockefeller strain (110) weighing 30g were assigned to two experimental designs. Transversal case control physiologic solution vs or Celtis tala pollen glycoprotein T evaluated in forced swimming test along the course of antibodies production. Similar

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study but cases were treated with spironolactone(S) and immunized as previous mice. Longitudinal case control study with cases and controls the same categories as the second study but followed and evaluated in FST. Climbing, swimming floating summing a total of 8 events and delated time to contact another mouse CT (seconds) were recorded. Plasma IgE and testosterone (Tt) were also measured.

**Results:** Immunization with T increases the proportion of climbing in both sexes at day 7(c.male 0.42, T.male 0.72, c.female 0.28, T.female 0.68) and reverted at day 15 (c.male 0.8, T.male 0.35, c.female 0.47, T.female 0.43). Since climbing is more frequent in male we treated mice with S to determine if immunization effects were mediated by testosterone and reverted the changes triggered by *C. tala* (day 7, ST. males 0.22, STfemales 0.35, day 15 ST.male 0.23, ST.female 0.32). T caused a shortening of CT in males from day 7 to 15, S produced the opposite and ST was partly similar to T (T.male12.5 to 5, Smale 8,2 to 14, ST.male 11 to 4.2 in sec, T.female 2.9 to 6.2, S.female 7.8 to 17, ST.female 13 to 9.6 sec). Plasma concentration ranges of Tt (ng/mL) were: CM 0.75-6.72, TM 31.1-58.5; STM 26-29.5; all females remained between 0.3-039.

**Conclusion:** The results presented in this paper support our hypothesis that immune response could modify mice FST performance by regulation of testosterone levels.

*Keywords: Immunization; behavior; sex; testosterone; synergy.*

## 1. INTRODUCTION

Functional integration of nervous and immune systems is well documented from a phenomenological point of view in medicine since Hans Seyle described thymus variations in shape in response to stress. Immune components can modulate the endocrine function and components of the endocrine system participate of immune functions i.e. the impact of sterile inflammation on fertility is well accepted [1], acute exercise alters IL10 in salivary glands [2], lacrimal glands secret IL 22 [3], IL17 action is sex dependent [4]. Another type of interactions involves nervous and endocrine systems like the anti-depressive effects of sexual hormones [5,6] and the suppressive action of stress on the plasmatic levels of sexual steroids [7]. A fourth branch covers the interaction of nervous and immune systems like the immunosuppression due to stress or the influence of cytokines on the course of adaptation to stress [8,9]. But the information relating the impact of immune system activation on hormonal responses involved in mood or behavior is scarce. The immune competent regulation of behavior by recruiting sex hormones was partially covered in an interesting experimental design to test the evolution of mood in mice with sciatica ligation as a model of neuropathic pain [10]. An outstanding point is that pain was increased by injection of BCG or it was attenuated by immune suppression through systemic injection of Cyclosporin A. While ligation caused no effect initially, it resulted in “antidepressant” at 30 days, measured by forced swimming test (FST). On the contrary, BCG was depressogenic at first and then turned to an antidepressant. BCG also increased testosterone and decreased estradiol. On the contrary, Cyclosporine decreased testosterone and augmented estradiol. One case of widespread immune modulation of behavior that is important for human health is allergy, particularly rhinitis because of comorbid depression with outcomes that are sex dependent [11,12,13]. *Celtis tala* (Gill. Ex Planchon) is an autochthonous tree from South America widely distributed in Argentina. The average pollen count is 15-30 grains/m3, with a maximum airborne pollen concentration of 275 grains/$m^3$. Its significance is determined by a 50 Kd glycoprotein that may be considered as the specific allergen causing allergic rhinitis [14]. We are interested on the impact of immune response on the interplay of nervous and endocrine systems. To gain knowledge on the mechanisms involved in this type of interaction we immunized mice with a common allergen of our country, *C. tala* glycoprotein. Alternatively, mice were testosterone suppressed by spironolactone treatment and immunized. Forced swimming test (FST) was performed at specific time points during IgE synthesis. FST behavior presents three main categories that represent corresponding pharmacological characteristics: climbing, adrenergic; swimming, the midpoint between adrenergic and serotoninergic; floating, antiadrenergic or depressed. In this work, we report the results of these experiments with a focus on sex differences along the immune response and the modification of behavioral patterns when spironolactone is introduced.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Albino Swiss mice Rockefeller strain weighing 30 g were assigned to different experimental designs. In one set Albino Swiss mice were separated into four groups, control just subcutaneous physiological solution, males (CM, 7), females (CF,9) and immunized with *C. tala* glycoprotein (10 µg prot. /mice) male (IM,8), and females (IF,12). Mice were weighted before immunization and subjected to forced swimming test (FST) for 2 minutes and swimming (s), climbing (c) and floating (f) events were registered every 15 seconds among 8 possible events. In another experiment mice without immunization or treatment underwent FST and then when returned to their cage, the lag time to make physical contact with another mouse of the same cage was registered. We called this variable Contact Time (CT).

In another set of experiments mice were separated into four treatment groups, control, (C) injected with physiologic solution, (T) mice injected with *C. tala* pollen glycoprotein once at day one subcutaneously, and (ST) mice primed with *C. tala* pollen glycoprotein at day one and spironolactone 25 mg/Kg every 15 days. All groups were evaluated as the first set with FST at 7, 30 and 90 days.

### 2.2 Antibody Production

*C. tala* glycoprotein was obtained according to Baronia et al. [14]. Quantitation of specific IgE to *C. tala* was assessed in sera of rats of two groups, immunized and control. Briefly plates were coated with *C. tala* pollen glycoprotein (1 ug/100ml) in carbonate – bicarbonate buffer (pH 9.6) overnight at 4°C. The unbound proteins were removed by washing five time with PBS-0.1 % Tween 20. Wells were the blocked for 1 hr with 4% BSA/ PBS at 37°C and incubated for 30 min with a 1:100 dilution in 1% BSA/ PBS in both groups. Plates was washed as described previously and incubated with a 1:1000 dilution of anti-rat IgG or IgE peroxidase (Sigma) for 30 min. Again, the plates were washed as described previously and bound secondary antibody was allowed to react with substrate, σ-phenylenediamine (Sigma) for 30 min. The reaction was stopped by adding 4 N $SO_4H_2$ and the optical density was determined in a microplate reader at 492 nm. A sample was considered positive if the optical density was two or more standard deviation above the mean of normal control group [15,16].

### 2.3 Forced Swimming Test (FST)

Each mouse was overlaid on the top of the water in a 5 liters tank and allowed to swim for two minutes. Mice behavior was registered by visual observation and by every 15 seconds. The number of events registered in 2 minutes for each behavior category swim, climb or float, for each treatment and sex, were loaded to a sheet.

### 2.4 Contact Time (CT)

Each mouse that completed the FST, was taken from the tank, dried gently with a towel, and then placed in a cage containing another randomly selected mouse of the same sex and group in the opposite corner. From that moment time was recorded until the recently introduced mouse touched with the nose the previously introduced one. Times were averaged by sex and group.

### 2.5 Statistics

Statistical analysis was performed with SPSS and GraphPad software. Differences among different groups and categories were analyzed by Independent Samples t Test, by non-parametric Kruskal -Wallis Test, and one way ANOVA, we considered significantly p= 0.05.

## 3. RESULTS AND DISCUSSION

### 3.1 Antibody Production of Immunized Mice

The subcutaneous injection of *C. tala* glycoprotein induced the production of IgE as shown in Fig. 1. Plasma was obtained at day 23 and immediately subjected to ELISA protocol. All immunized animals were positive to the ELISA reaction.

### 3.2 Allergenic Immunization Influence on FST Performance

Results of these groups performance are resumed in (Fig. 2). Mice of both sexes showed a different pattern of performance in the FST. At 7 days mice of both sexes immunized with *C. tala* glycoprotein at the beginning of the experiment, diminished swimming activity in favor of climbing (Fig. 2, a & b). Immunized males at 15 days presented an increment in swimming with respect to control males and decreased climbing.

The opposite occurred in control males which increased climbing. In control and immunized males appeared floating as a third alternative. By day 23 both groups presented no statistically significant differences although a predominance of swimming in control mice and of climbing in immunized mice may be appreciated (Fig. 2 a).

Results of female performance are depicted in Fig. 2b. At day 7 climbing was favored, at day 15 swimming and climbing activities were similar in both groups. Although there are no significant differences, is important to note that the control group did not present float behavior. At day 23 both groups presented similar trends as day 15 but we highlight that immunized female mice increased flotation although the difference with day 15 is not statistically significant.

This kind of experiment let us hypothesize that the fate of an immune response was determined at least in part by sexual hormones.

### 3.3 Testosterone Suppression on Immune Biased FST Performance

We explored the influence of sex on the FST performance of immunized mice. Results are presented in (Fig. 3). At day 7, male mice (Fig. 3a) treated with spironolactone presented swimming as the dominant activity, a behavior characteristic of control females at day 7. Mice treated with spironolactone and immunized with *C. tala* glycoprotein differed, presenting a pattern with less climbing activity and increased floating while swimming had no significant difference with control and spironolactone treated mice. This profile was like that of immunized females at day 23. Both groups with spironolactone decreased climbing activity compared to swimming. This pattern was the opposite to that of immunized mice without spironolactone at day 7 (Fig. 2a). At day 15 control male mice conserved a climber profile while both spironolactone treated groups presented a clear dominance of swimming activity (Fig. 3b). This swimmer profile of spironolactone treated immunized male mice was like that of immunized males without spironolactone (Fig. 2a), suggesting that the prevalence of swimming was due to immunization effects.

Female mice of the same experiment presented similar behavior profiles among the three groups at day 7 with clear predominance of swimming (Fig. 3c). This profile was in contrast with that of immunized females without spironolactone which behaved as mainly climbers (Fig. 2b). At day 15 spironolactone treated females presented a significative increment on climbing activity compared to control ones (Fig. 3d). Immunized spironolactone treated female mice at the same time presented increased floating behavior, that could be interpreted as a deleterious consequence of immunization.

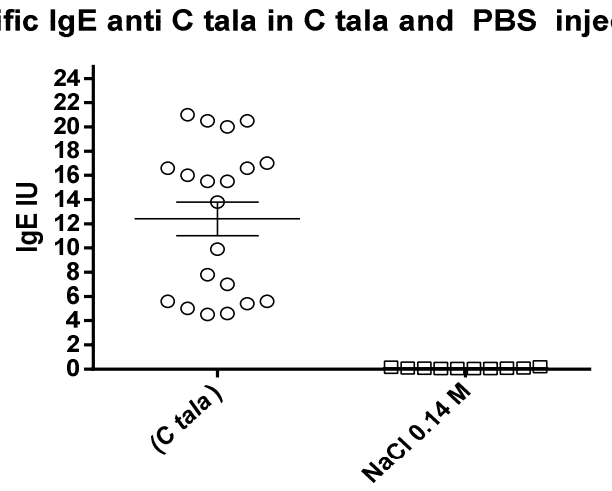



**Fig. 1. Antibody plasma antibodies of mice immunized with *C. tala* pollen glycoprotein.**
*Thirty mice plasma samples were analyzed by ELISA to determine IgE. Ten mice were injected with 145 mM saline (control) and twenty with C. tala pollen glycoprotein subcutaneously. Mann Whitney test: p <0.0001, IU: IgE 2.3ng of IgE*

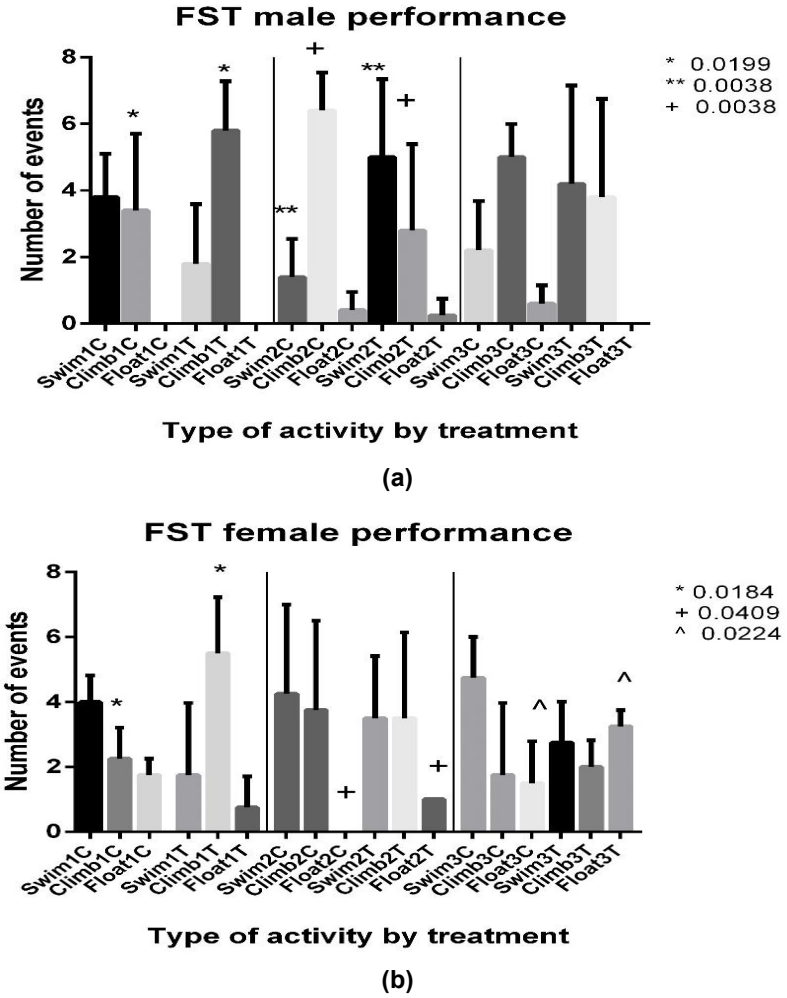


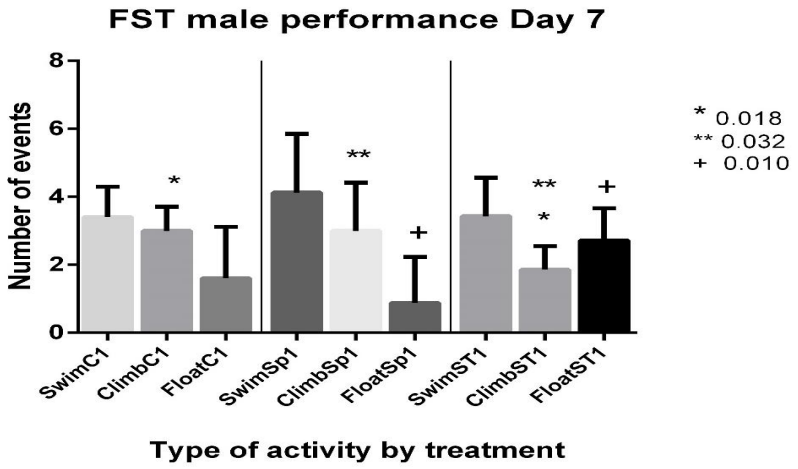

**Fig. 2. Forced swimming test performance of mice immunized with *Celtis tala* pollen glycoprotein (T)**

*a) Male b) Female. Mice were separated into control (C) and (T) injected once with C. tala pollen glycoprotein subcutaneously. Vertical lines separate 7(1) 15(2) and 23(3) days post injection. Values with significative difference are indicated with symbols on top of the bar and p values are depicted on the right.*

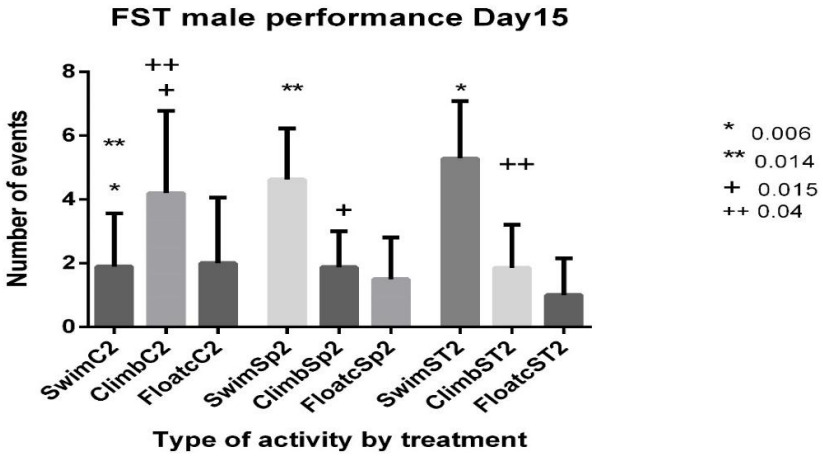
### 3.4 Contact Time

Through the course of the experiments we observed what seemed to be another sex dependent variable: the time for each mouse to make physical contact with another mouse of the same group and sex. So, we decided to measure that time lag and called the variable Contact Time. We measured that period for mice of both sexes that underwent each treatment and FST at day 7 and 15 and found some interesting differences. The effect of immunization with *C. tala* is presented in Fig. 4a. Control mice of both sexes differ, with female presenting shorter CT and male increasing from day 7 to 15 indicating discomfort. Immunized mice of both sexes presented more prolonged CT after immunization at day 7 and reduced CT at day 15. This could be interpreted as less discomfort in male mice and the contrary for female.

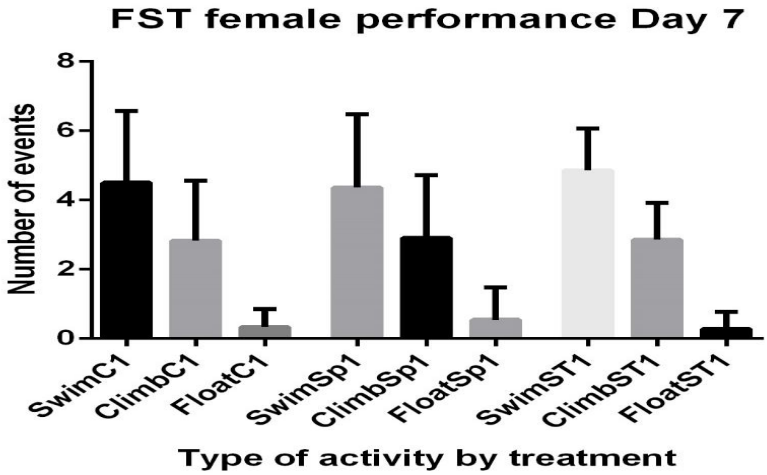
As shown in Fig. 4 b immunized male mice trend was to decrease CT from day 7 to 15 while spironolactone treated males behaved exactly the opposite. The immunization concomitantly with spironolactone reverted the effect of spironolactone alone. Immunized females and spironolactone treated ones presented similar pattern between day 7 and 15. Spironolactone immunized CT for day 7 and 15 differences are not significative (Fig. 4c).


FST male performance Day 7
Number of events
0
2
4
6
8
SwimC1
ClimbC1
FloatC1
SwimSp1
ClimbSp1
FloatSp1
SwimST1
ClimbST1
FloatST1
Type of activity by treatment
* 0.018
** 0.032
+ 0.010

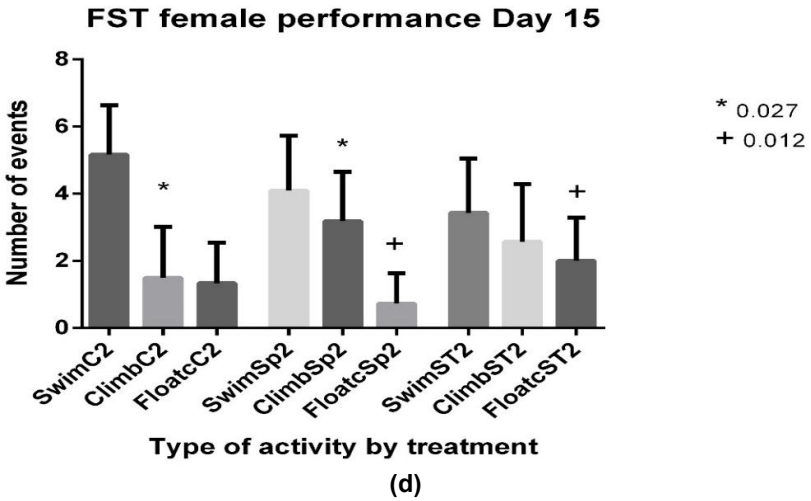
(a)


FST male performance Day15
Number of events
0
2
4
6
8
SwimC2
ClimbC2
FloatcC2
SwimSp2
ClimbSp2
FloatcSp2
SwimST2
ClimbST2
FloatcST2
Type of activity by treatment
* 0.006
** 0.014
+ 0.015
++ 0.04

(b)


FST female performance Day 7
Number of events
0
2
4
6
8
SwimC1
ClimbC1
FloatC1
SwimSp1
ClimbSp1
FloatSp1
SwimST1
ClimbST1
FloatST1
Type of activity by treatment

(c)




(d)

**Fig. 3. Forced swimming test performance of mice treated with spironolactone alone or with spironolactone and immunized with *Celtis tala* pollen glycoprotein (T) separated by sex**

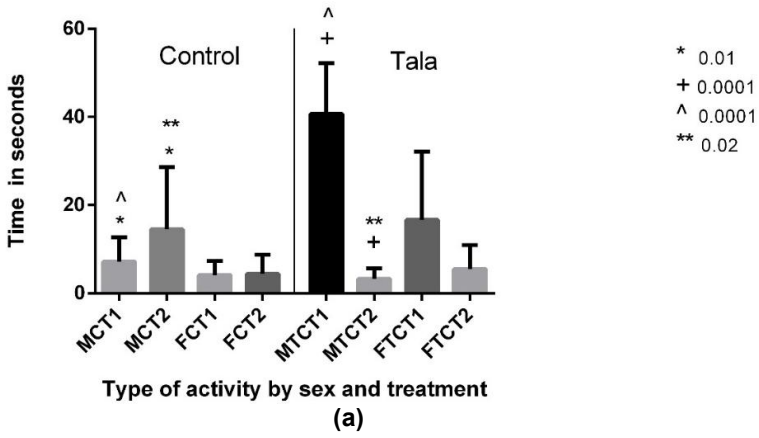
*a) males day 7, b) males day 15, c) females day 7 d) females day 15. injected with Celtis tala glycoprotein subcutaneously. p values for significative differences are on the right upper corner of each graph.*

## 3.5 Plasma Testosterone

We observed that control mice presented sex dependent profile of performance in the FST with males being more climber and females more swimmer. Immunization with *C. tala* altered the behavior profile in the FST with females becoming more climbers at day 7 and males more swimmers by day 15. Treatment with spironolactone made males behave like females in the same test at day 7 and 15, while females reinforced the swimmer profile except for a little increase in climbing at day 15. This trend was counter balanced in part by *C. tala* immunization in males. These results led us to hypothesize that *C. tala* immunization could increase male sexual hormones, so we determined the plasma levels of testosterone in control, immunized and immunized spironolactone treated mice of both genders. Results are presented in Table 1.

**Table 1. Immunization and spironolactone combined effect on plasma testosterone in ng/ml**

| | Male | Female |
|---|---|---|
| Control | 0.75-6.76 | 0.3-0.37 |
| *C. tala* immunized | 31.1-58.05 | 0.36- 039 |
| Spironolactone + *C. tala* | 26-29.5 | 0.3-0.3 |




(a)

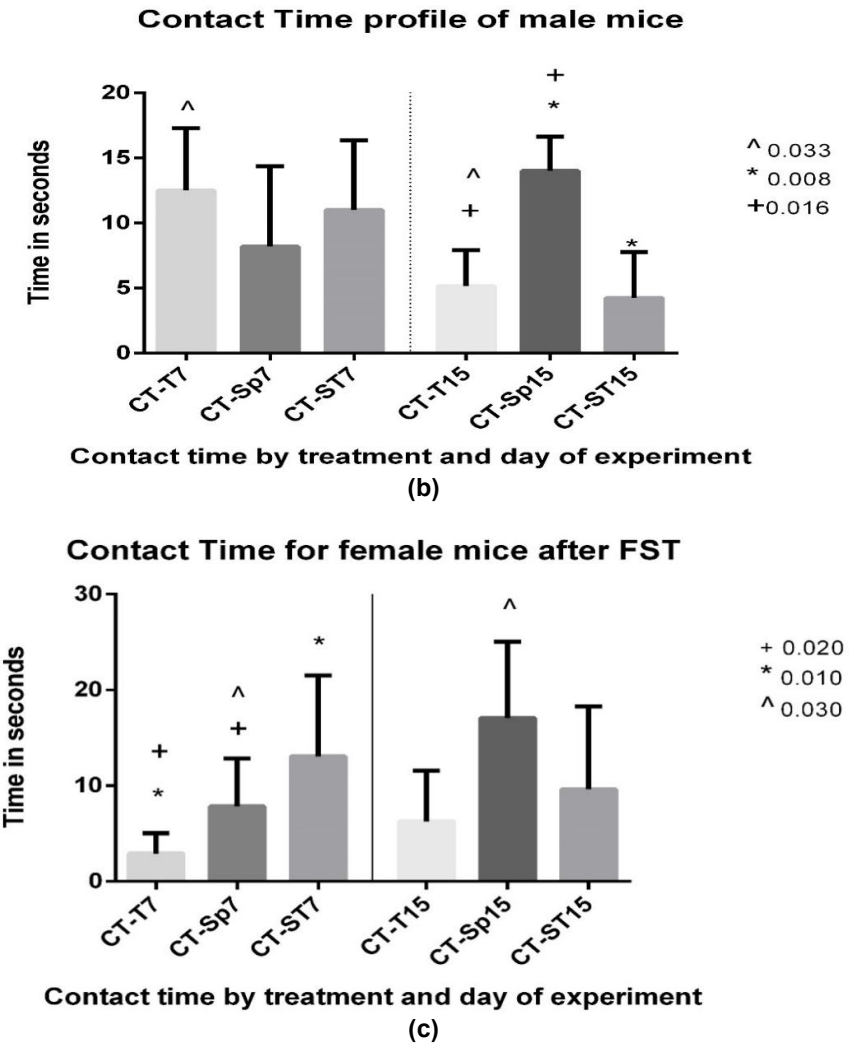



**Fig. 4. CT time lag to make physical contact with another mouse of the same group and gender after performing FST measured at 7(T1) and 15(T2) days in control (MC/FC) and *C. tala* immunized mice (a) and spironolactone (Sp) and spironolactone *C. tala* immunized (ST) mice of both sexes (b, male, c, female). p values are on the right legends**

As we suspected immunization with *C. tala* increased 20 fold plasma testosterone levels and the increment was reverted in part by spironolactone treatment. The results are presented in ranges since it is well known that mice present great circadian fluctuations on these values [17].

In this work, we show that FST performance of Albino Swiss mice is different for both sexes. While male become more climbers through day 15 and 23, female remained swimmers. When mice were immunized previously with the allergen *C. tala* glycoprotein, both sexes modified their performance. The changes in behavior occurred during the course from innate immune response to adapted immune response with antibody production. Male switched from climber to swimmer profile by day 15 while female begun as climbers (opposite to their control) returning to swimmer profile by day 15. So it seems that the allergen immunization drove male to female behavior by day 15 while female reacted like control male at day 7 and then returned to their native profile.

To test the influence of sex hormones and separate allergen immunization effects, both sexes were treated with spironolactone which suppresses testosterone synthesis. Another

group of mice were treated with spironolactone and immunized at the onset of the experiment. In male at day 7, S enhanced swimming and floating appeared, while immunization did not change that pattern but increased floating. These behavioral patterns were similar to that of control female. At day 15 treatment with spironolactone and spironolactone with *C. tala* immunization produced female like swimmer profiles.

In female at day 7 spironolactone induced a swimmer pattern and *C. tala* immunization failed to induce climbing as it was the case in the first experiment with immunized female. At day 15 female of both treatments presented the same pattern of behavior.

Taken together, these results let us hypothesize that immunization, in our case with an allergen, could modify the behavior of mice under FST through the interaction with sex hormones.

Each type of behavior of rodents undergoing FST has a specific interpretation that constitutes a code that has been widely employed in pharmacological sciences [18,19,20,21]. Climbing is a response to stress triggered mainly by adrenergic stimulation as was proven by the fact that norepinephrine uptake inhibitors increase climbing events in FST. Following the same type of evidence it can be assumed that swimming involves a dominance of serotonergic innervation since swimming increments after serotonin reuptake inhibitors fluoxetine, desimipramine [22] and floating is related with simultaneous increase of norepinephrine and dopamine in the prefrontal cortex [23]. Using this scheme to interpret our results we could propose that allergenic immunization triggers a climber, norepinephrine prevailing profile and that this is accomplished by augmenting plasma testosterone.

This behavior is expected to be more evident in males although it was observed in females on day 7. But female plasma testosterone was not modified. This suggests that the increase of testosterone may not be the cause of the change in performance in the FST but it must be considered that the determination of testosterone was not performed on day 7 but between day 7 and day 15.

We also found that time to contact another mouse of the same sex (CT) after performing FST was sex dependent. Male mice at day 7 presented larger CT when immunized, while at day 15 CT was smaller than control. Control male increased CT from day 7 to 15 while immunized mice diminished CT. Female control mice presented short CT on both days and immunized female presented the same behavior since differences were not significative. Immunization affected only males. Spironolactone increased CT at day 15 and spironolactone plus immunization restored short CT like mice with *C. tala* alone.

Females presented significative differences only on day 7. Spironolactone increased CT and the addition of immunization to spironolactone increased it further.

CT needs more data to be considered as a standard behavioral variable but it seemed suitable to show sex behavioral differences and that immunization with an allergen may influence the interaction among sex hormones and nervous system. In fact, immunization with *C. tala* increased plasma testosterone in male mice without any effect on female ones.

## 4. CONCLUSION

Forced Swimming Test is a well documented method for measuring mice behavior under defined conditions with clear interpretation. Hormone plasma levels and IgE titers were also determined making a simple but straightforward methodology. We provide evidence that immune response involvement in FST performance is accomplished by regulation of testosterone levels. This interaction has not been described to our knowledge and further studies are needed to characterize molecular mediators. Our results introduce another scope to think on immunization as a regular procedure of preventive medicine particularly during early years. Successive preventive immunizations or those produced by contact with allergens might introduce fluctuations on sexual hormone levels with consequences that should be discovered and understood.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-

23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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