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# Age and isolation influence steroids release and chemical signaling in male mice

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ABSTRACT

Social interactions in mice involve olfactory signals, which convey information about the emitter. In turn, the mouse social and physiological status may modify the release of chemical cues. In this study, the influences of age and social isolation on the endocrine response and the release of chemical signals were investigated in male CD1 mice, allocated into four groups: Young Isolated (from weaning till 60 days; $N = 6$), Adult Isolated (till 180 days; $N = 6$), Young Grouped (6 mice/cage; till 60 days; $N = 18$), Adult Grouped (6 mice/cage; till 180 days; $N = 18$). Mice were transferred in a clean cage to observe the micturition pattern and then sacrificed. Body and organs weights, serum testosterone, dehydroepiandrosterone, corticosterone and the ratio Major Urinary Protein/creatinine were measured. Urinary volatile molecules potentially involved in pheromonal communication were identified. Androgen secretion was greater in isolated mice ($P < 0.05$), suggesting a greater reactivity of the Hypothalamic–Pituitary–Gonadal axis. Grouped mice presented a higher degree of adrenal activity, and young mice showed a higher serum corticosterone ($P < 0.05$) suggesting a greater stimulation of the Hypothalamic–Pituitary–Adrenal axis. The micturition pattern typical of dominant male, consisting in voiding numerous droplets, was observed in Young Isolated mice only, which showed a higher protein/creatinine ratio ($P < 0.05$). Urinary 2-s-butyl-thiazoline was higher in both Young and Adult Isolated mice ($P < 0.005$). Young Isolated mice showed the most prominent difference in both micturition pattern and potentially active substance emission, while long term isolation resulted in a less extreme phenotype; therefore social isolation had a higher impact on young mice hormone and pheromone release.



## 1. Introduction

In mammals, hormonal setting and social experience are mutually linked. In male mice, age and social conditions may influence androgen levels, that in turn affect several physiological and behavioral responses, including the production/excretion of substances used as signals to communicate the male status to conspecifics, and hence to modulate their behavior and hormonal status.

Plasma testosterone (T) concentration is affected by age: it increases at puberty, peaks in early adulthood and slowly decreases thereafter, with a strain-dependent time-course [1–3]. Mice typically exhibit episodic T secretion, where high pulsatile levels are periodically superimposed upon basal low levels of the hormone. Two types of pulsatile T release occur: spontaneous T release occurs every 3–4 h, while reflexive T secretion is triggered by encountering a novel female (anticipatory release) or by ejaculation (ejaculatory release) [4].

Testicular function and androgen concentrations can be affected by social experiences, such as different housing density [5]. Stress or other subtle factors, such as an unfamiliar environment, can depress the T increase related to the exposure to a female [4].

*Abbreviations:* BSA, Bovine Serum Albumin; C, corticosterone; CYP3A, cytochrome P450; family 3, subfamily A; EC, European Commission; DHEA, dehydroepiandrosterone; DL, Decreto Legislativo (Italian for Law Decree); GC/FID, Gas Chromatography/Flame Ionization Detector; GC/MS, Gas Chromatography/Mass Spectrometry; HPA, Hypothalamic–Pituitary–Adrenal; HPG, Hypothalamic–Pituitary–Gonadal; MUP, Major Urinary Protein; PBS, Phosphate Buffered Saline; RIA, radioimmunoassay; T, testosterone.

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There are data, thoroughly reviewed in [6], suggesting that T levels in blood can be increased in the initial stage of acute stress, mainly in animals that have winning experiences and/or dominant status in a population hierarchy [6]. In particular, social isolation can result in a significant increase in testosterone concentrations and testis weight [7,8].

Isolated male mice exhibit also smaller adrenals and lower plasma corticosterone in association with increased gonadal function [8], suggesting that social isolation may affect the adrenal function and reduce the stress response. Interestingly, glucocorticoids can modify serum testosterone both by inhibiting testosterone synthesis [9,10] and enhancing hepatic testosterone clearance [11].

Dehydroepiandrosterone (DHEA) is a multifunctional steroid with a broad range of biological effects in humans and animals. In particular, DHEA can be synthesized and metabolized in the brain, where it is claimed to exert neuroprotective and anti-glucocorticoids effects, and reduce aggressiveness [12]. In rodents, no age-related blood DHEA decrease has been documented [12], and DHEA is primarily secreted by the gonads since, in contrast to primates, rodent adrenals are not capable of DHEA synthesis [13].

Testosterone can influence different aspects of the male mouse sex behavior, such as copulatory behavior [14], ultrasonic vocalizations and territory marking [15,16], and promotes aggressive behavior [17,18].

Therefore, social interactions represent a powerful signal that shape both behavior and the responses of Hypothalamic–Pituitary–Gonadal (HPG) and Hypothalamic–Pituitary–Adrenal (HPA) axes. However, most previous data were obtained from orchydectomised and/or androgen-supplemented mice [4–20]. In this study, we modified the social setting at two different ages to unravel the relationships among the physiological release of androgens and glucocorticoid release, and the urinary excretion of chemical signals.

In mice, social interactions are mediated by chemical communication [21,22]. Volatile molecules and proteins released in body fluids (urine, tears, saliva) are detected by conspecifics, which decode the signal and consequently modify both their neurohormonal status and their behavior [23]. In turn, hormonal changes are redirected downstream to the production of chemical signals, emitted from the receiver mouse, which modulate its pheromonal blend according to the incoming information.

Androgens play an important role in regulating the inter-individual chemical communication. Testosterone stimulates the release of aggression-promoting pheromones in urine [24,25], possibly acting on both volatiles and the Major Urinary Proteins (MUP). However, the precise relationship between androgens and MUP release is not yet documented. On the other hand, the perception of chemosignals is partly mediated by the accessory olfactory system [26], whose sensitivity is modulated by centrifugal noradrenergic fibers that differentially respond to sex steroids [27], so that testosterone facilitates responsiveness to male and female odors [28]. Both the release of urinary molecules and the micturition pattern typical of adult males develop at puberty, when testicular testosterone prompts male secondary characters [29,30]. Typical male behavior, including aggressiveness and territory marking, depends on the effect of testosterone on the medial preoptic area of the hypothalamus [15].

The rationale for the present study stems from the need to provide data on the link between controlled social condition and hormonal output. This link is still obscure, yet it may subsequently affect the excretion of molecules involved in intraspecific communication, thus providing a feedforward loop aimed at influencing intraspecific relationships.

In the present experiment, the effects of isolation on the HPG and HPA axes activities were investigated in young and adult intact male mice in relation to the excretion of molecules putatively involved in pheromonal communication. In this way we intend to explore the physiological responses of the whole organism to isolation and ageing.

## 2. Experimental

### 2.1. Animals and treatments

Male CD1 mice ($N = 48$) from different litters were raised in our animal facility and were allocated into four experimental groups, which varied for age (Young or Adult) and social conditions (Grouped or Isolated). Isolated mice (Young, YI, $N = 6$; or Adult, AI, $N = 6$) were individually housed in $42 \times 27 \times 15$ cm cages from weaning (at 21 days) and sacrificed at 60 or 180 days of age; they showed no sign of distress related to isolation. Grouped mice (Young YG, $N = 18$; or Adult, AG, $N = 18$) were maintained 6 mice/cage from weaning to 60 or 180 days of age. Groups were made so that no sibling was present in any cage. The age at group formation was chosen to maximize the differences with non-grouped mice, according to [31]. All animals had free access to water and were fed *ad libitum*.

Before being sacrificed, mice were placed on a clean cage with a wire mesh grid ($4 \times 4$ mm), in order to observe the micturition pattern in a new environment. After the first micturition act, urine drops were counted and collected. Mice were immediately sacrificed with a lethal injection of Tanax (20 mg/kg, I.P.). Blood was collected between 10:00 and 13:00 by heart puncture, clotted at room temperature and centrifuged to obtain serum (5 min at 2500*g*). The urine left within the bladder was also collected and its volume determined. The bodies were weighted and dissected, and the weight of liver, heart, right and left kidney, bladder, right and left testis, seminal vesicles, penis, preputial glands, left and right adrenals, spleen and thymus was recorded.

The experiment was approved by the competent authorities and conducted according to the European Union law on animal experiments and welfare (EEC directive 86/609/EEC) and the Italian legislation on animal care (DL n.116/92).

### 2.2. Hormone analysis

The serum concentrations of steroids under investigation were determined by solid phase radioimmunoassay (RIA) methods using 96-well microtitre plates (Optiplate, Perkin-Elmer Life Science). Plasma testosterone (T) and corticosterone (C) were measured following diethyl ether extraction. For both hormones plasma (0.1 ml) were extracted with 8 ml diethyl ether (J.T. Baker, NL), and the ether fractions were transferred into fresh glass tubes and dried under nitrogen. The dry extracts were carefully dissolved in RIA buffer (PBS, 0.1% BSA, pH 7.4) and used for the analysis at appropriate dilutions.

Plates were coated with anti-rabbit γ-globulin serum raised in a goat by incubating overnight the antiserum in 0.15 mM sodium acetate buffer, pH 9, at 4 °C. The plates were then carefully washed with RIA buffer and incubated overnight at 4 °C with 200 μl of a solution of the specific antisera raised in rabbits.

Testosterone was measured according to [32]. The antiserum was raised in the rabbit against testosterone-3 carboxymethyloxime-BSA and showed the following cross reactions: testosterone 100%, 5α-dihydrotestosterone 38%, 5α-androstan-3α, 17β–diol 13.7%, 5α-androstan-3β, 17β–diol 13.7%, 19-nortestosterone 8.6%, androstenedione 1.6%, 5-androstene-3β, 17β-diol 1.2%, DHEA 0.01%, cortisol <0.001%. The detection limit was 3.125 pg/well. The intra- and inter-assay coefficients of variation were 8.8% and 11.8% respectively.

Serum DHEA was measured as previously described [33], using a commercial anti-DHEA-7-carboxymethyloxime–BSA (Biogenesis,