ASPECTOS NEUROETOLÓGICOS DEL RECONOCIMIENTO DE PARENTESCO EN EL ROEDOR CAVIOMORFO *OCTODON DEGUS*

Tesis
Entregada a la
Universidad de Chile
en cumplimiento parcial de los requisitos
para optar al grado de

Doctor en Ciencias con Mención en Ecología y Biología Evolutiva

Facultad de Ciencias

Por
Natalia Márquez Aguayo

Enero, 2012

Directores de Tesis:

Dr. Rodrigo A. Vásquez
&
Dr. Jorge Mpodozis

FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

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TESIS DE DOCTORADO

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata

NATALIA MÁRQUEZ AGUAYO

Ha sido aprobada por la comisión de Evaluación de la tesis como requisito para optar al grado de Doctora en Ciencias con mención en Ecología y Biología Evolutiva, en el examen de Defensa Privada de Tesis rendido el día 16 de Enero de 2012.

Director de Tesis:
Dr. Rodrigo A. Vásquez .....

Co-Director de Tesis:
Dr.Jorge Mpodozis .....

Comisión de Evaluación de la Tesis:

Dr. Mauricio Canals .....

Dr. Francisco Aboitiz .....

Dr. Mario Penna .....

A Eru y Luna

AGRADECIMIENTOS

Quiero agradecer a Jaime, por ser mi compañero todos estos años, y a nuestros dos hijos por ser unas personitas tan lindas. A mis padres, por darme libertad y apoyo en mis decisiones. A Juani, por ayudarme a cuidar a mis hijos en momentos cruciales. Y, muy especialmente, a mi amiga Macarena por toda la buena onda que compartió conmigo mientras trabajamos en el Rayo y vivíamos juntas.

Quiero también dar las gracias a Rodrigo Vásquez por aceptarme en su laboratorio en los inicios de mi carrera e incentivarme a realizar mi doctorado una vez que terminé la licenciatura. No puedo dejar de agradecer a Jompoma, por haber aceptado ser mi co-tutor y sobre todo por ser tan Jompoma.

Agradezco a mis compañeros del laboratorio de Ecología: a Camila, por ayudarme con los experimentos y también por ayudarme en los terrenos, junto a Claudia, Daniela y René; a Ronny, por su buena disposición y ayuda en montar los experimentos. A la gente del Rayo que ayudó a levantarlo después del incendio, en especial a Pato, Maca y Solano. A Marín, por las interesantes conversaciones; a Lete, por sus buenos consejos; a Ximena Rojas, por enseñarme a hacer inmunohistoquímica, y a Elisa, por su ayuda en el lab.

A mis compañeras inseparables del pregrado: Camila, Carolina, Daniela y Mariela.

Agradezco también a los amigos chilenos en Alemania: Loreto, JP, Álvaro, Mariela, Camila y René, que nos alegran la vida con cada visita, y a los otros amigos en Berlín: Rodrigo, Karina, Pieps, que son nuestra familia en la distancia.

Finalmente, agradezco formalmente a la beca MECESUP UCO 0214 por financiar mi doctorado y a la beca CONICYT AT-24050185 de apoyo de realización de tesis doctoral. Además, esta tesis fue financiada por el Instituto de Ecología y Biodiversidad - proyecto P05-002 de la Iniciativa Científica Milenio del Ministerio de Economía, Fomento y Turismo, y los proyectos PBF-23-CONICYT y FONDECYT 1090794 de Rodrigo Vásquez.

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LISTA DE ABREVIACIONES

ANOVA: analysis of variance
AOB: accessory olfactory bulb
BO: bulbo olfatorio
cin: cineole
DA: dorsoanterior
DL: dorsolateral
DM: dorsomedial
DP: dorsoposterior
EPL: external plexiform layer
EUC: Eucalyptol
GL: glomerular layer
Gr: granular
HS.RA: half siblings reared apart
HSD: honestly significant difference
IPL: internal plexiform layer
KPS: kind of stimuli pair
LC: locus coeruleus
M/T: mitral/tufted
MET: mean exploration time
MHC: major histocompatibility complex
MUP: major urinary proteins
NE: norepinephrine
NS.RA: non-siblings reared apart
NS.RT: non-siblings reared together
OB: olfactory bulb
OSNs: olfactory sensory neurons
PBS: phosphate-buffered saline
PBST: Triton X-100 in phosphate-buffered saline
PG: periglomerular
PN: postnatal day
r: kinship coefficient
S.RA: siblings reared apart
S.RT: siblings reared together
SP: stimuli pair
Ss: subjects
VA: ventroanterior
VL: ventrolateral
VM: ventromedial
VP: ventroposterior
Vs.: versus

RESUMEN

El reconocimiento de parentesco consiste en la discriminación conductual de individuos emparentados genéticamente. En mamíferos, las señales que participan en el reconocimiento de parentesco son principalmente olfatorias. A la fecha, no existen estudios neuroetológicos que permitan responder cuáles son los mecanismos de este fenómeno, aun cuando se considera que el aprendizaje olfatorio temprano es necesario. En esta tesis se estudió el reconocimiento de parentesco en el roedor social *Octodon degus*, en 3 niveles de análisis: (i) se evaluó la importancia de la familiaridad y del parentesco genético en la discriminación hermano-hermana, (ii) el papel del aprendizaje olfatorio temprano en el reconocimiento de parentesco, y (iii) se estudió la actividad del bulbo olfatorio asociada a señales olfativas compartidas por individuos genéticamente emparentados.

Mediante el cruzamiento recíproco de crías se evaluó la participación de la familiaridad y del parentesco genético en preferencias olfatorias hacia conespecíficos del sexo opuesto, así como en interacciones diádicas macho-hembra. Encontramos que la familiaridad cumple un rol importante tanto en conductas exploratorias en el laberinto de *Y* como durante encuentros macho-hembra. Por otra parte, encontramos una influencia del parentesco genético en otras interacciones sociales (Capítulo I).

Además, camadas de *O. degus* fueron impregnadas diariamente con un odorante

artificial (*eucalyptol*) durante su primer mes de vida para evaluar el efecto del ambiente olfativo temprano en preferencias olfativas y conductas de reconocimiento de parentesco en degus adultos hacia conespecíficos no familiarizados impregnados con el odorante artificial (Capítulo II). Degus sin previa experiencia con *eucalytol* mostraron neofobia en el laberinto de *Y* hacia el brazo donde el estímulo olfatorio era un conespecífico impregnado con *eucalyptol* (brazo *Euc*). Si bien animales impregnados con *eucalyptol* durante su desarrollo temprano no mostraron preferencia por el brazo *Euc*, tampoco se observó neofobia. Por otra parte, en encuentros de parejas en arenas experimentales se observó que la ocurrencia de conductas evasivas y de escape durante encuentros agonistas dependía de la experiencia olfativa temprana y del sexo.

Adicionalmente, se estudió la actividad neuronal del bulbo olfatorio en sujetos expuestos durante tres días consecutivos a un conespecífico (sesiones de habituación), seguido por la exposición al cuarto día (sesión de deshabituación) de otro conespecífico que podía ser hermano o no-hermano del individuo utilizado como estímulo olfatorio durante las sesiones de habituación (Capítulo III). La actividad neuronal del bulbo olfatorio, medida como expresión del gen de expresión temprana *c-fos*, difirió entre sexos y tratamientos. No hubo diferencias en la expresión de *c-fos* entre machos de ambos grupos, sin embargo el grupo de hembras estimuladas con dos conespecíficos no emparentados expresó mayores niveles de *c-fos*. Esto resultados sugieren que las señales olfatorias aprendidas tempranamente en un contexto social determinan preferencias y sesgos conductuales en degus adultos y por otra parte sugieren que estas señales podrían ser familiarmente distintivas, al menos en el caso de hembras.

ABSTRACT

Kin recognition is the behavioural discrimination of genetically related individuals. In mammals, the signals used in kin recognition are mainly olfactory. Until now there are no neuro-ethological studies addressing the mechanisms underlying this phenomenon, even though early learning is necessary. In this thesis kin recognition in the social rodent *Octodon degus* was studied at three levels of analysis: (i) the role of familiarity and genetic relatedness was assessed in the context of male-female sibling discrimination, (ii) the role of olfactory learning during early ontogeny in kin recognition, and (iii) the activity of the olfactory bulb associated to olfactory similarities between subjects genetically related.

Cross-fostering allowed us to evaluate the importance of familiarity and genetic relatedness on olfactory preferences and male-female dyadic interactions. We found that familiarity mediated olfactory exploration in the *y-maze* and also some social interactions. However, genetic relatedness also influenced other social behaviours (Chapter I).

In order to evaluate the effects of early olfactory environment, *O. degus* litters were daily impregnated with an artificial odour (*eucalyptol*) during their first month of life. Olfactory preferences and kin discrimination behaviours toward non-familiarized conspecifics impregnated with *eucalyptol* were assessed in adult degus (Chapter II). Degus without previous experience with *eucalyptol* showed neophobia

toward the *y-maze* arm where the olfactory stimulus was a conspecific impregnated with *eucalyptol* (*Euc* arm). Animals impregnates with *eucalyptol* during early ontogeny did not showed a preference for the *Euc* arm, but they did not show neophobia either. In the other hand, evasive behaviour within aggressive encounters in pair encounters in experimental arenas was affected by the interaction of early experience and sex.

Additionally, the neuronal activity of the olfactory bulb was studied in subjects exposed three consecutive days to a conspecific (habituation trials), followed by the exposure to either the sibling or the non-sibling of the stimulus animal used in the habituation trials (Chapter III). The neuronal activity of the olfactory bulb, measured as the expression of the immediate early gene *c-fos*, differed between sexes and treatments. No difference in *fos* expression was found between males, however the female group stimulated with to non-genetically related conspecifics expressed higher levels of *c-fos*. These results suggest that olfactory signals learned within the early social environment determine behavioural bias and behavioural preference in adult degus, and that this signals could be shared by family members, at least in females.

INTRODUCCION GENERAL

El reconocimiento de parentesco consiste en la discriminación conductual de individuos emparentados genéticamente. Este proceso requiere que al menos se cumplan dos requisitos, primero que un individuo sea capaz de reconocer a sus parientes mediante algún mecanismo sensorial y segundo que presente un sesgo conductual frente a estos (Hepper, 1991, Mateo, 2002, Pfennig, 2002, Mateo, 2004). Estudios sobre las señales relevantes en la discriminación de parentesco en mamíferos han demostrado que éstas varían inter-específicamente. Se ha observado en muchas especies la utilización de señales olfativas, mientras que en otras se utilizan predominantemente señales visuales y/o auditivas (Klopfer and Gamble, 1966, Lindsay and Fletcher, 1968, Gubernick, 1981, Hepper, 1991). En el caso particular de roedores, el reconocimiento de parentesco estaría mediado por señales olfativas (Halpin, 1991). El papel fundamental del olfato en la discriminación de hermanos se demostró en la ardilla *Spermophilus tridecemlineatus*, donde los individuos que son capaces de discriminar entre hermanos y no-hermanos pierden esta capacidad al ser sometidos a anosmia con sulfato de zinc (Holmes, 1984). Estos resultados sugieren que las mismas vías neuronales del sistema olfatorio principal que están involucradas en la percepción de odorantes del ambiente participan en la percepción de señales

olfativas involucradas en el reconocimiento de parentesco (Tang-Martinez, 2001).

,Por otra parte, estudios conductuales sobre el reconocimiento de parentesco han llevado a proponer cuatro mecanismos mediante los cuales individuos podrían llegar a distinguir específicamente señales sensoriales provenientes de otros individuos con los cuales están emparentados genéticamente (véase Dawkins, 1976, Lacy and Sherman, 1983, Holmes, 1984, Halpin, 1991, Hepper, 1991, Tang-Martinez, 2001). Brevemente, un primer mecanismo corresponde al de distribución espacial común, en el cual los individuos reconocen lugares utilizando señales ambientales características, y presentan sesgos conductuales positivos hacia los individuos que utilizan o han utilizado el mismo lugar. Un segundo mecanismo corresponde a la asociación o familiaridad, por el cual aquellos individuos con los cuales existe asociación espacio-temporal y/o con los cuales existe una alta familiaridad (i.e., han vivido juntos por largo tiempo) son discriminados favorablemente. Los individuos aprenderían a discriminar, durante un período del desarrollo, señales características de aquellos conespecíficos que están en contacto directo con ellos. Un tercer mecanismo de reconocimiento se conoce como comparación de fenotipos (phenotype matching), en el cual un individuo aprende a discriminar algunos aspectos de su propio fenotipo o del fenotipo de parientes familiarizados, y posteriormente puede comparar este fenotipo aprendido ("molde") con el fenotipo de otro conespecífico con el cual no ha tenido contacto previo. Este mecanismo requiere una correlación entre similitudes genotípicas y fenotípicas. También requiere

que los rasgos fenotípicos aprendidos bajo un conjunto de circunstancias sean recordados y subsecuentemente utilizados en otras circunstancias. Por último, un cuarto mecanismo denominado alelos de reconocimiento, postula que los parientes genéticos son identificados como resultado de la posesión de alelos que tendrían tres efectos en conjunto: (i) expresión de una señal fenotípica característica, (ii) percepción de dicha señal por otro individuos, y (iii) generación de conductas preferenciales hacia los poseedores de señales similares. Este mecanismo ha sido cuestionado, ya que si bien muchos estudios han mostrado evidencia para el punto (i) (Yamazaki et al., 2000, Hurst et al., 2001, Carrol et al., 2002, Beauchamp and Yamazaki, 2003), no existen evidencias para los puntos (ii) y (iii) (véase Hepper, 1991, Tang-Martinez, 2001)

La mayoría de los estudios de las últimas dos décadas han tratado de determinar, en distintas situaciones naturales, cual de los dos mecanismos más estudiados y aceptados, reconocimiento por familiaridad y comparación de fenotipos, es el más relevante (Hepper, 1991; Tang-Martinez, 2001), aun cuando ambos mecanismos podrían no ser mutuamente excluyentes, e incluso posiblemente operen de manera conjunta (Hepper, 1991). Basada en evidencias experimentales acumulada en más de 20 años, Tang-Martínez (2001) argumenta que esta dicotomización del problema es inadecuada, ya que obstaculiza el entendimiento de los mecanismos neuroetológicos básicos por los cuales los animales reconocen y discriminan a sus parientes. Como lo destaca dicha autora, en todos los mecanismos, con excepción del de alelos de reconocimiento, ocurre aprendizaje de señales las que luego son comparadas con las señales del

individuo con el cual se interactúa. En el caso del reconocimiento por familiaridad, los animales aprenderían señales distintivas individualmente, mientras que en la comparación de fenotipos, las claves aprendidas estarían asociadas al individuo focal o a individuos genéticamente emparentados, y serían compartidas entre parientes (Tang-Martinez, 2001).

Adicionalmente, la discriminación de parentesco ha sido propuesta como uno de los mecanismos conductuales involucrado en la evitación de la endogamia (Hoogland, 1982, Blouin and Blouin, 1988). Interesantemente, estudios en mamíferos han determinado que es la familiaridad, y no el parentesco genético, la que determina las preferencias de apareamiento (Hill, 1974, Gavish et al., 1984, Beauchamp et al., 1988, Yamazaki et al., 1988). Por ejemplo, individuos criados por padres adoptivos eligen aparearse con conespecíficos que poseen haplotipos del complejo de histocompatibilidad mayor (MHC, por sus siglas en inglés) distintos a los que poseen sus padres adoptivos (Beauchamp et al., 1988, Yamazaki et al., 1988). Muchos de los estudios sobre preferencia olfatoria han sido realizados en laberintos de Y, sin embargo, dichas preferencias no han sido estudiadas manipulando la familiaridad entre hermanos.

Por otra parte, el aprendizaje olfatorio temprano dentro de un contexto social juega un papel importante en la preferencia hacia olores con los cuales se tuvo experiencia durante la ontogenia temprana. Al respecto, se ha reportado que el apego a la madre en ratas neonatas es aprendido, que ocurre en un período crítico, y que requiere de estímulos pareados olfatorios y táctiles, los cuales

normalmente ocurren durante el período de amamantamiento (Sullivan et al., 1989). Si bien estudios en mamíferos han evidenciado que el aprendizaje olfatorio temprano determina preferencias olfativas en adultos (Marr and Lilliston, 1969, Carter and Marr, 1970, Holmes, 1984, Sullivan et al., 1989; véase además Apfelbach (1986) para efectos de presencia de odorantes artificiales durante la ontogenia temprana en preferencia por alimentos), tales preferencias no han sido estudiadas en el contexto del reconocimiento de parentesco.

Como ya se ha dicho, el reconocimiento de parentesco en muchos mamíferos es mediado por olfato. La naturaleza de las señales olfativas ha sido campo de intensa investigación en las últimas décadas (Boyse et al., 1991, Halpin, 1991, Yamazaki et al., 2000, Hurst et al., 2001, Carrol et al., 2002, Beauchamp and Yamazaki, 2003). Se ha determinado que señales individualmente distintivas presentes en la orina son influenciadas por diferencias genéticas (Boyse et al., 1987, Schaefer et al., 2002). Mediante habituación/ deshabituación se ha mostrado que roedores son capaces de discriminar a individuos que difieren en alelos del MHC o de proteínas urinarias mayores (MUPs) (Hurst et al., 2001, Carrol et al., 2002). A partir de estos experimentos se ha sugerido que este tipo de moléculas actuarían como señales familiarmente distintivas y que al menos en mamíferos serían percibidas olfativamente.

El sustrato neuronal del olfato ha sido estudiado intensamente en las últimas décadas. Resultan relevantes en este contexto los estudios que se han realizado sobre patrones de activación glomerular evocados por diferentes odorantes en el

bulbo olfatorio principal, así como los estudios que muestran que estos patrones de actividad están determinados por la novedad del estímulo. Se ha sugerido que la especificidad y disposición de las neuronas receptoras en el epitelio olfatorio junto a su conectividad particular con los glomérulos determinaría estos patrones espaciales característicos para cada odorante (Rubin and Katz, 1999, Uchida et al., 2000, Belluscio and Katz, 2001; véase además Shepherd and Greer, 1998 para detalles de la conectividad neuronal a nivel del bulbo olfatorio). Si bien, utilizando genes de expresión temprana (c-*fos*), se han reportado mapas de actividad característicos en el bulbo olfatorio evocados incluso por odorantes de conespecíficos que difieren en alelos del MHC (Schaefer et al., 2002), se ha descrito que la novedad del estímulo también es crucial para observar patrones característicos de actividad (Montag-Sallaz et al., 1999; véase además Anokhin et al., 1991, Schettino and Otto, 2001, Rojas et al., 2009 para el papel de la novedad del estímulo en la expresión de *c-fos* en otros sistemas). Sin embargo, estudios de este tipo no se han realizado en el contexto de señales olfativas características de individuos emparentados (i.e., hermanos), ni en especies silvestres que presenten conductas sociales complejas.

Modelo de estudio

La mayoría de los estudios sobre el papel de las señales olfativas en las conductas sociales de mamíferos se han realizado con roedores de los subórdenes Sciuromorpha y Myomorpha, donde se distinguen los grupos que comprende a las ardillas y las ratas, respectivamente. Otro grupo completo de roedores, el suborden Hystricognathi, con una gran cantidad de familias casi no

se ha estudiado con dicha perspectiva (véase Ciszek, 2000 para una excepción), a pesar de poseer muchas especies altamente sociales (Ebensperger, 1998). Dentro de Hystricognathi, *Octodon degus* (Rodentia, Hystricognathi) es un roedor endémico de Chile central, diurno y altamente social (Fulk, 1976, Yánez and Jaksic, 1978), en el cual se ha descrito amamantamiento comunal, en donde hembras aceptan crías de otras hembras (Ebensperger et al., 2002, Ebensperger et al., 2004). Sin embargo, las madres parecen asignar más cuidado (e.g., leche) a las crías propias (Jesseau, 2004, Jesseau et al., 2008). Los degus presentan otras conductas sociales como vigilancia anti-depredatoria grupal (Vasquez, 1997), y llamadas de alarma situacionalmente específicas (C. Cecchi, datos no publicados), conductas que podrían ser mediada por reconocimiento de parentesco (Sherman et al., 1997, Mateo and Johnston, 2000). Antecedentes recientes indican que este roedor presenta conductas nepóticas, siendo menos agresivos con sus hermanos así como con individuos no-emparentados con los que han estado familiarizados (R. Vásquez, datos no publicados). Además, los degus asignan más tiempo a vigilancia anti-depredatoria al encontrarse en grupos de hermanos en relación a grupos de no-emparentados (R. Vásquez, datos no publicados). La información disponible revela que la familiaridad determina en gran medida los sesgos conductuales observados en encuentros diádicos en degus (Davis, 1975, Villavicencio et al., 2009). Sin embargo, los degus también presentan sesgos conductuales hacia hermanos con los cuales no han co-existido, sugiriendo la presencia de señales familiares distintivas (Villavicencio et al., 2009). Por lo tanto, esta especie constituye un sujeto de

estudio apropiado para analizar mecanismos conductuales, cognitivos y neuroetológicos que median en el reconocimiento de parentesco.

En esta tesis propongo estudiar el reconocimiento de parentesco en el roedor social *Octodon degus* en tres niveles de análisis: (i) evaluar la importancia de la familiaridad y del parentesco genético en la discriminación hermano-hermana (ii) analizar el papel del aprendizaje olfatorio temprano en el reconocimiento de parentesco, y (iii) analizar la actividad del bulbo olfatorio (BO) asociada al señales familiares distintivas olfativas.

HIPÓTESIS

i) Si el reconocimiento de parentesco en *Octodon degus* es influenciado por el parentesco genético, individuos debieran presentar sesgos conductuales positivos hacia hermanos aun cuando no hayan vivido junto a ellos en su ontogenia temprana.

ii) La experiencia olfativa temprana en un contexto social es necesario para determinar el reconocimiento de parentesco. Animales cuyo ambiente olfativo temprano es alterado artificialmente, deberían mostrar sesgos conductuales asociados al reconocimiento hacia conespecíficos no familiarizados que tengan la señal artificial a la cual fueron expuestos durante la ontogenia temprana.

iii) Individuos genéticamente cercanos presentan claves olfativas similares, a diferencia de individuos no emparentados. El bulbo olfatorio de individuos focales habituados al olor de un individuo y luego expuestos a un hermano del animal previamente utilizado en la habituación, no expresarán marcadores de actividad neural gatillados por estímulos novedosos. Por el contrario, si se le presentan odorantes de un individuo no-emparentado con el animal al que fue previamente expuesto, se debiera expresar el marcador.

OBJETIVO GENERAL

Estudiar la importancia del comportamiento, aprendizaje y actividad neuronal sobre el reconocimiento de parentesco en el roedor social *Octodon degus* (Rodentia, Octodontidae)

OBJETIVOS ESPECÍFICOS

- Analizar la importancia de la familiaridad y parentesco genético en el reconocimiento de parentesco

- Analizar la importancia del aprendizaje olfatorio temprano en el reconocimiento de parentesco

- Analizar la actividad neuronal del bulbo olfatorio asociada a señales familiares distintivas.

CAPÍTULO I

MALE-FEMALE SIBLING DISCRIMINATION IN *OCTODON DEGUS*: EFFECTS OF FAMILIARITY AND GENETIC RELATEDNESS

ABSTRACT

Kin recognition refers to the discrimination and subsequent behavioural bias toward kin, in the particular case of rodents the available evidence suggests that kin recognition would be mediated by olfaction. In order to evaluate the effects of familiarity and genetic relatedness on male-female behavioural bias, *Octodon degus* were cross-fostered within the 24 hours after birth. Olfactory preferences were assessed presenting a sibling and a non-sibling with whom they had different previous experience in a *y-maze* labyrinth (experiment 1). In addition, male-females sibling discrimination was studied using an experimental arena observing male-females dyadic interactions (experiment 2). No difference was

observed between the exploration of the two conspecifics in the *y-maze* experiment. However an effect of the familiarity with the pair of stimuli presented was observed on exploration frequency of the stimuli arms. When both stimuli animals were unfamiliar, experimental subjects explored significantly more than subjects exposed to the pairs where a familiarized sibling was present. We also found an effect of the sex of the focal subjects on the exploration time, with females exploring significantly longer than males. A negative correlation was observed between exploration frequency and the time spent in each exploratory visit, animals exposed to two unfamiliar conspecifics showed significantly shorter visit time, thus degus exposed to two unfamiliar stimuli pair were more actives. Dyadic encounters in the other hand, revealed that familiarity but not genetic relatedness affected exploratory and agonistic behaviours, however social contact was significantly higher between siblings than between non-siblings even though they had no previous contact, indicating that this behaviour is influenced by genetic relatedness.

INTRODUCTION

Kin recognition refers to the ability of individuals to discriminate between kin and non-kin. Individuals recognizing their kin should exhibit behavioural bias towards them, thus implying the existence of a sensorial mechanism capable of discriminate between kin and non-kin conspecifics (Hepper, 1991, Mateo, 2002, Pfennig, 2002, Mateo, 2004). Experimental studies on the discrimination of close related conspecifics have focused mainly on exploratory, amicable and/or aggressive behaviours. Behavioural bias in some species has been reported to be the result of prior association (i.e., recognition by familiarization) (Holmes, 1984, Paz-y-Miño and Tang-Martinez, 1999b), while in other species such behaviours have been observed even in individuals that had no previous contact with their close kin (i.e. recognition by phenotype matching) (Holmes and Sherman, 1982, Holmes, 1986, Heth et al., 1998).

In the other hand, kin recognition has been proposed to be one of the behavioural mechanisms mediating inbreeding avoidance (Hoogland, 1982, Blouin and Blouin, 1988). Observations of social mammalians have reported the occurrence of low levels of inbreeding in natural populations (i.e., prairie dogs:

Hoogland, 1982, 1992; spiral-horned antelopes: Apio et al., 2010) as well as in captive animals (i.e., meadow vole: Bollinger et al., 1991; mice: Sherborne et al., 2007; see also Pusey and Wolf, 1996 for a review about inbreeding avoidance). Interestingly, several studies in mammals have shown that is familiarity, and not genetic relatedness what determine mate preferences. In prairie voles cross-fostering experiments show that animals reared together don't breed between them, while non-familiarized subjects successfully breed whether they are siblings or not (Gavish et al., 1984). In the same sense, evaluations of prepubertal experiences in prairie deer mice reveal that siblings paired after sexual maturity present similar reproductive success than non-siblings. However, placing couples together during prepuberty produces low reproduction rate regardless of their genetic relationship (Hill, 1974). In addition, prairie vole siblings separated for more than 15 days revoke inbreeding avoidance (Gavish et al., 1984) but not other kind of behavioural biases (aggressive or amicable), which disappear only after 20 days of isolation, suggesting that caution must be taken when assuring the lost of discrimination considering only inbreeding avoidance as an indicator (Paz-y-Miño and Tang-Martinez, 1999a).

Kin recognition, weather mediated by direct familiarity or phenotype matching, has been proposed to be the result of learning of distinctive signals which can be associated with kinship or not (Tang-Martinez, 2001, Mateo and Holmes, 2004). In rodents kin discrimination would be predominantly mediated by distinctive olfactory signals (Holmes, 1984, Halpin, 1991, Sherborne et al., 2007), therefore olfactory discrimination abilities and olfactory preferences have been intensely

studied. Olfactory preferences in the *y-maze* labyrinth have shown that bank vole females prefer the odours from males carrying major histocompatibility complex (MHC) haplotypes different from their own (Radwan et al., 2008), thus, suggesting the possibility of an olfactory discrimination of kin individuals. However, caution must be taken in the interpretation of olfactory preferences toward conspecifics of the opposite sex in the *y-maze*: since no copulation is allowed in these experiments, mating preferences cannot be inferred from them, only social preferences (Clarke and Faulkes, 1999).

By the other hand, foster-parental studies in mice revealed that early social experience indeed influences mate preference. In these experiments, subjects reared by foster parents choose to mate with individuals carrying MHC haplotypes that differs from that of their foster parents, suggesting that simple familiarity can account for the natural preference of mice to mate with individuals carrying MHC haplotypes different from their own (Beauchamp et al., 1988, Yamazaki et al., 1988).

A previous study in degus showed that sibling discrimination in this specie is strongly mediated by familiarity even though the participation of genetic relatedness could not be ruled out (Villavicencio et al., 2009). Here we implement a cross-fostering strategy to 1) characterized olfactory preferences of a focal individual towards familiar or unfamiliar siblings or non-siblings of the opposite sex in a *y-maze* labyrinth and 2) to quantifying exploratory, amicable and aggressive behaviours during dyadic encounters between male and female

couples that differ in familiarity and/or degree of genetic relatedness. To our knowledge this is the first study addressing olfactory preferences toward siblings as well as sibling discrimination between individuals of the opposite sex.

MATERIAL AND METHODS

Subjects

Adult *Octodon degus* were captured and maintained as described by Villavicencio et al. (2009). To assure coordination of females' oestrus, we used wild animals captured from three populations in central Chile: la Campana (32° 55'S, 71° 05'W), Lampa (33° 17'S, 70° 53'W) and Rinconada de Maipú (33° 29'S, 70° 53'W). Within each population animals were captured using Sherman live traps from different sites separated at least 400 m in order to collect non-related females (see Ebensperger et al., 2004). Once at the University of Chile they were housed in metal cages (50 x 40 x 35 cm) with wood shavings in an air-conditioned room under natural photoperiod. Animals were feed with rabbit pellet and alfalfa and provided with water *ad libitum*. Males and females were kept separated four months prior the experiments to ensure that females were not pregnant before being captured. After this period, 16 mate groups consisting in one male and three females from the same population, but different capture sites (in order to obtain parental half siblings) were placed together during the reproductive period (May-June). Parturition was inspected daily (after 3-month of gestation period), and any female that gave birth was housed separated with her litter. Later this enabled us to manipulate litter members and create cross-

fostered groups (see below).

All procedures of capture, maintenance and experimentation were approved by the ethics committee of the Faculty of Sciences of the University of Chile, and followed Chilean regulations on wild-life management.

Cross fostering

Female's litters from different groups but from the same population that gave birth within 24 hours were cross-fostered (see Villavicencio et al., 2009). Half of the pups were exchanges when litters had even number of pups (male by male and female by female), or half plus one in the cases of odd number of newborn degus. For permanent identification each pup was marked with eartags (National Band & Tag Co., Newport, KY, U.S.A). Through this cross fostering protocol we produced 14 litters where animals grew up with full sibs (coefficient of kinship, $r = 0.5$) and genetically unrelated conspecifics ($r = 0$).

Experimental groups

As result of the mating group design and the cross fostering manipulation we could create five experimental groups that differed in familiarity (i.e., animals reared together or apart) and genetic relatedness (i.e., kin or non-kin): (1) siblings reared together (S.RT), (2) siblings reared apart (S.RA), (3) non-siblings reared together (NS.RT), (4) non-siblings reared apart (NS.RA) and (5) half siblings reared apart (HS.RA). Male-female sibling discrimination was assessed by observation of (i) olfactory exploration in a *y-maze* labyrinth where two

conspecifics of the opposite sex were used as olfactory stimuli (see below) and (ii) pair encounters in an experimental arena allowing social interactions.

***Y-maze* (Experiment 1)**

Olfactory exploration and preferences toward conspecifics of opposite sex were assessed in a *y-maze* labyrinth (see Fig. 1.1). Animals were carried in individual plastic cages from the housing rooms to the experimental rooms. The experiment consisted in 1 min of acclimatization and 3 min of test time. As olfactory stimuli two degus of the same sex (that we will call a Stimuli Pair, SP) were placed in the stimulus chambers. We formed four kinds of SP (KSP): (1) S.RT versus NS.RA, (2) S.RT versus NS.RT, (3) S.RA versus NS.RT and (4) S.RA versus NS.RA. A total of 14 focal degus (7 males, 7 females) were exposed to each KSP presented. In all cases KPS members were of the same sex between them, but from the opposite sex respect to the focal subject. To avoid scent contamination between animals, we used different sets of gloves to manipulate animals in the maze, and disposable gloves to clean the *y-maze* after each experiment with 95° Ethanol to eliminate odour traces. The arm in which the sibling member of a SP was placed was randomized between presentations, to control for labyrinth arm preferences of the focal subjects. No such preferences were found (paired *t*-test $t = -0.97$, d.f. = 83, $p = 0.33$). All experiments were video recorded (colour CCTV camera connected to a Sony video recorder) from above the *y-maze* system. From the video recordings, an observer blind to the treatment and subjects' sex quantified frequency and duration of a) arm's exploration, b) division plate smelling, and c) still in each arm, using the

JWatcher 1.0 software (Dan Blumstein, University of California, Los Angeles, U.S.A)

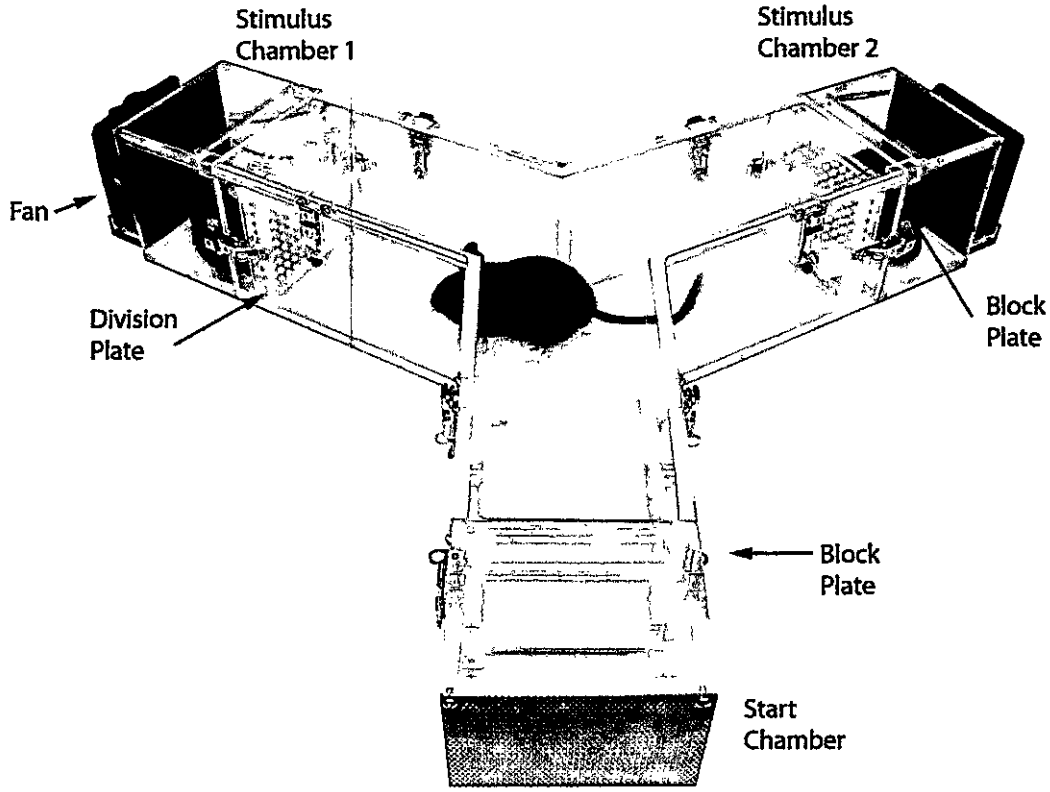

Fig.1.1 The *y-maze*. The system was designed to allowed a focal degu explore freely the three arms of the labyrinth (50 cm long x 15 cm with x 15 cm high Plexiglas arms forming angles of 120°). Stimulus degus were maintained in chambers attached to the extremes opposite the start chamber. A perforated Plexiglas plate prevent from direct contact between focal and stimulus animal, but allowed the airflow produced by a fan behind each stimulus chamber to circulate through the labyrinth. During acclimatization time a Plexiglas plate (Block plate) avoided the movement of the focal subject, and also prevented volatile odours from the stimulus chambers reach the start chamber. Once the experiment began the block plates were removed, and the fans next to the stimulus chambers switched on.

Statistics

To evaluate differences in exploration of SP presented in the *y-maze* the measured variables described above for each experimental stimuli pair were

compared using paired *t*-test (Sokal and Rohlf, 1995). Further analysis were carried out to examine the effects of sex and experimental pairs used as olfactory stimuli on the exploration of the labyrinth using two-way ANOVA or Scheirer-Ray-Hare test extension of the Kruskal-Wallis test for non-parametric data (Sokal and Rohlf, 1995). For comparison between pairs of groups we used Tukey's honestly significant difference (HSD) (Sokal and Rohlf, 1995) or multiple comparisons for non-parametric data using the Behrens–Fisher's approach as described by Munzel and Hothorn, (2001). For all the analysis we used the software R (R Foundation for Statistical Computing, Vienna, Austria 2009).

Pair encounters (Experiment 2)

Behavioural discrimination was assessed in encounters of male-female pairs that differed in familiarity and/or genetic relatedness (see experimental groups above). Animals were carried in individual plastic cages from the housing rooms to the experimental room. Tests were carried out in two 80x80x50 cm metal arenas that could be divided by placing a division plate (see Villavicencio et al. 2009). The floor of the arena consisted in a removable white-painted metal plate that was cleaned with detergent before each test to remove any trace of scent. For each group 7 pairs were tested. If the subjects did not interact and stayed still for more than 5 min the test was repeated on a different day (8 of 35 pairs). 5 pairs tested the second time did not interact and therefore were not considered in the analysis. In each experiment one of the animals was painted with non-toxic painting, to allow us to distinguish the animals in the video

recordings. Previous studies have shown no changes in locomotion, vigilance (Vasquez et al., 2002) or social exploratory behaviour (Villavicencio et al., 2009) due to marking. In agreement with those previous studies, we found no effects of marking on exploratory ($H = 0.59$, 1 d.f. = 1, $p = 0.44$), agonistic ($H = 0.046$, 1 d.f. =1, $p = 0.83$) or any other measured behaviour (data not shown). After a 10 min acclimatization period, the division plate was removed; when any of the two subjects started to explore the arena, we began the quantification of a 20 min trial. All experiments were video recorded (colour CCTV camera connected to a Sony video recorder) from above the arena. An observer blind to the treatment and animals sex analyzed from the recordings the different behaviours considered in our study (see below) using the JWatcher 1.0 software (Dan Blumstein, University of California, Los Angeles, U.S.A).

Four behavioural categories were defined based on behavioural descriptions of intraspecific interactions in degus (see Wilson and Kleiman, 1974, Kleiman, 1975, Fulk, 1976) and mice (Baudoin et al., 1991): (1) olfactory exploratory behaviour consistent of exploratory approaches to the mouth, head, flanks and/or anogenital area of its partner; (2) social contact, was considered when degus were in contact either side by side, on right angle to each other, grooming, or huddling one over the other, (3) agonistic encounters of two kinds, (i) evasive, whenever a focal subject by turning aside or running away avoiding the partner in hostile contexts, and (ii) aggressive, if the animal performed tail wagging, hindleg kick, foreleg push, defensive burying, chasing or fighting against its conspecific, and (4) sexual behaviour considered when the male

mounted or attempted to mount the female and when the female remained passive under the male.

Statistics

To assess the effects of familiarity and genetic relatedness, the measured variables described above were analyzed performing a two-way ANOVA after square-root transformation or Scheirer-Ray-Hare test extension of the Kruskal-Wallis test for data that even after being transformed did not accomplish parametric assumptions (Sokal and Rohlf, 1995). Between groups, comparisons were analysed using nonparametric multiple comparison using the Behrens–Fisher's approach. For all the analysis we used the software R (R Foundation for Statistical Computing, Vienna, Austria 2009).

RESULTS

Y-maze (Experiment 1)

As a first significant result, we found that SPs formed by non-siblings and siblings of the focal subjects did not elicit in them any difference in exploration frequency or exploration time, irrespectively of the genetic relatedness or familiarity that the members of the SPs hold with the focal subjects (see Table 1).

Table 1. Arms' exploration frequency and duration differences in *O. degus* exposed to a sibling ($r = 0.5$) and a non-sibling ($r = 0$) with different familiarity in a *y-maze* labyrinth.

Pairs of stimuli	Exploration frequency				Exploration time (s)			
	$r = 0.5$ M (SE)	$r = 0$ M (SE)	t	p	$r = 0.5$ M (SE)	$r = 0$ M (SE)	t	p
S.RT vs. NS.RT	13.9 (1.1)	13.6 (1.2)	-.135	.89	44.2 (4.2)	51.2 (3.6)	1.25	. 24
S.RT vs. NS.RA	12.3 (1.4)	14.9 (1.4)	1.26	.23	57 (5.4)	41 (4)	-1.90	.08
S.RA vs. NS.RT	19.1 (1.6)	20.2 (1.8)	.299	.77	63 (5.9)	62.2 (5.3)	.055	.96
S.RA vs. NS.RA	26.8 (2.1)	26.4 (2.2)	.225	.83	67.8 (5.2)	61.9 (4)	-.894	.39

Data expressed as frequency and time mean (standard error), M (SE). t indicates the paired t-test statistic (d.f. =13). Stimuli pairs: siblings reared together (S.RT) versus non-siblings rear apart (NS.RA), S.RT versus non-siblings reared together (NS.RT), siblings reared apart (S.RA) versus NS.RA.

However, we found that exploration frequency of the *y-maze* arms was influenced by the KSPs presented ($F_{[3.48]} = 4.77$, $p = 0.0055$). Subjects exposed to a SPs formed by a sibling and a non-sibling, both unfamiliar, explored

significantly more the two arms than subjects exposed to a S.RT/NS.RA SPs (post hoc Tukey's HSD test, $p = 0.011$) and than subjects exposed to sibling-non-sibling, both familiar SPs (post hoc Tukey's HSD test, $p = 0.012$; Fig.1.2 A).

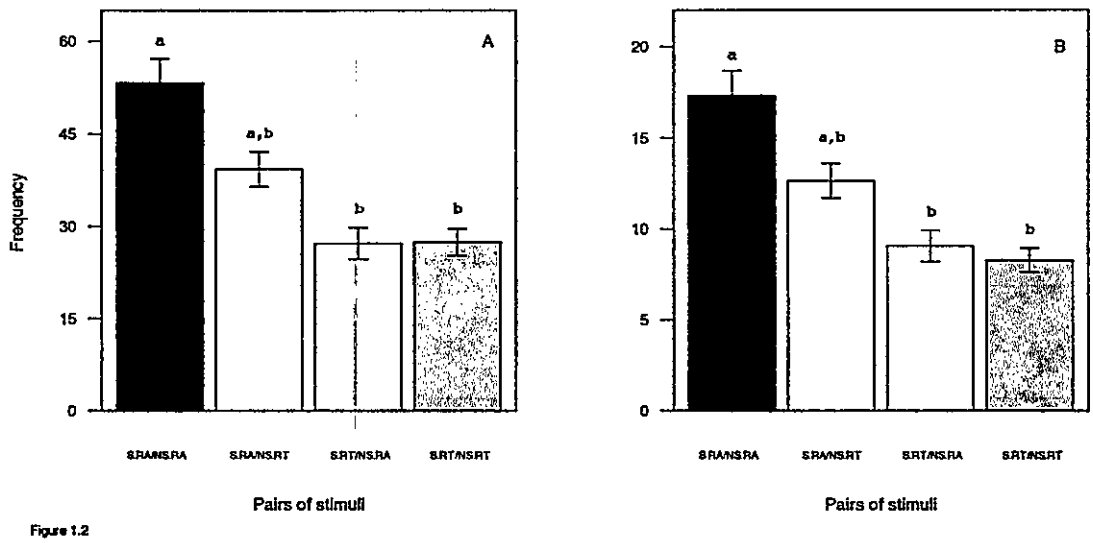

Figure 1.2. Effects of pair of stimuli on the frequency (mean ± SE) of exploration of both stimuli arms (A) and frequency of direct investigation of both division plates (B). Focal subjects were exposed to one of the four kinds of stimuli pairs (KSP): (1) siblings reared together (S.RT) versus non-sibling reared apart (NS.RA), (2) S.TR versus non-sibling reared together (NS.RT), (3) sibling reared apart (S.RA) versus NS.RT and (4) S.RA versus NS.RA. Different letters represent statistical significant differences between groups (Tukey's HSD post hoc test, $p < 0.05$, see text for exact p-values).

In close agreement, we found that the frequency of smelling of the of the division plates of the *y-maze* was affected by the KSPs ($F_{[3,48[} = 4.72$, $p = 0.0058$) in the same way that the total exploration frequency. Focal subjects exposed to S.RA/NS.RA SPs explore significantly more the division plates than focal subjects exposed to S.RT/NS.RA SPs (post hoc Tukey's HSD test, $p = 0.018$), and

than focal subjects exposed to S.RT/NS.RT SPs (post hoc Tukey's HSD test, p = 0.008) (Fig. 1.2 B).

In contrast to what was found in frequency analysis, we found no effect of the KSPs on total exploration time ($F_{[3,48]} = 1.71$, $p = 0.18$). An effect of the sex of the focal subject was found instead: females explored significantly more than males ($F_{[1,48]} = 5.43$, $p = 0.024$; Fig. 1.3 A). There was no effect of the interaction between KSPs and sex of the focal subjects ($F_{[3,48]} = 2.70$, $p = 0.06$). To further understand this result we calculated the mean time the focal subjects used exploring both the stimuli arms and the division plates (mean exploration time, MET), dividing the exploration time by the number of exploring events. A correlation was found between the MET and the frequency of exploring events (Pearson's product-moment correlation: $r = -0.49$, d.f. = 54, $p = 0.0001$), indicating that focal subjects that explored less frequently the labyrinth arms spend more time in each exploratory event. If the MET is analyzed performing a non-parametric two-way ANOVA, an effect of the interaction between sex and the KSPs (Scheirer-Ray-Hare test: $H = 5.17$, d.f. = 3, $p = 0.02$; Fig 1.3 B) and an effect of KSPs alone ($H = 10.3$, d.f. = 3, $p = 0.001$) was observed. Multiple comparisons for non-parametric data using the Behrens–Fisher's approach revealed that focal subjects exposed to SPs in which both members are unfamiliar (S.RA/NS.RA) perform exploring events significantly shorter than focal subjects exposed to SPs containing at least one familiar sibling (S.RT/NS.RT ($p = 0.017$), and S.RT/NS.RA ($p = 0.016$)).

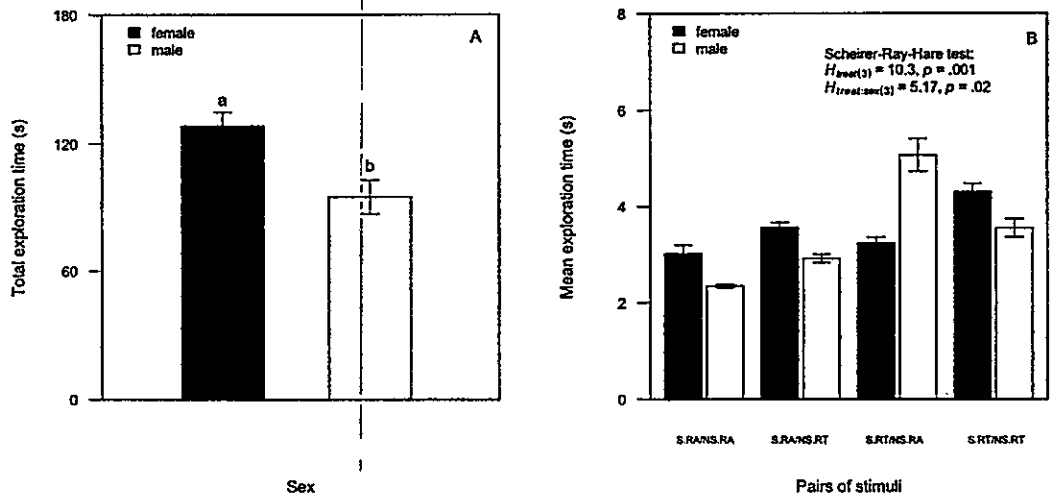

Figure 1.3

Figure 1.3. Exploration time in the *y-maze* labyrinth. In (A) an effect of sex was observed on total exploration time of both stimuli arms. Different letters represent statistical significant differences, two-way ANOVA ($p < 0.05$, see text for exact F and p values). In (B) Exploration time (mean ±SE) per event of visit was affected by the pair of conspesific presented and the interaction of stimuli and sex (Scheirer-Ray-Hare test).

Dyadic encounters (Experiment 2)

We found that olfactory exploration with direct nose-to-nose contact was significantly higher between members of unfamiliar pairs of opposite sex degus ($F_{[1,55]} = 11.50$, $p = 0.0013$; Fig. 1.4 A). No effects of genetic relatedness ($F_{[2,55]} = 0.16$, $p = 0.85$) or of interaction between familiarity and genetic relatedness ($F_{[1,55]} = 0.23$, $p = 0.63$) were observed for this behaviour.

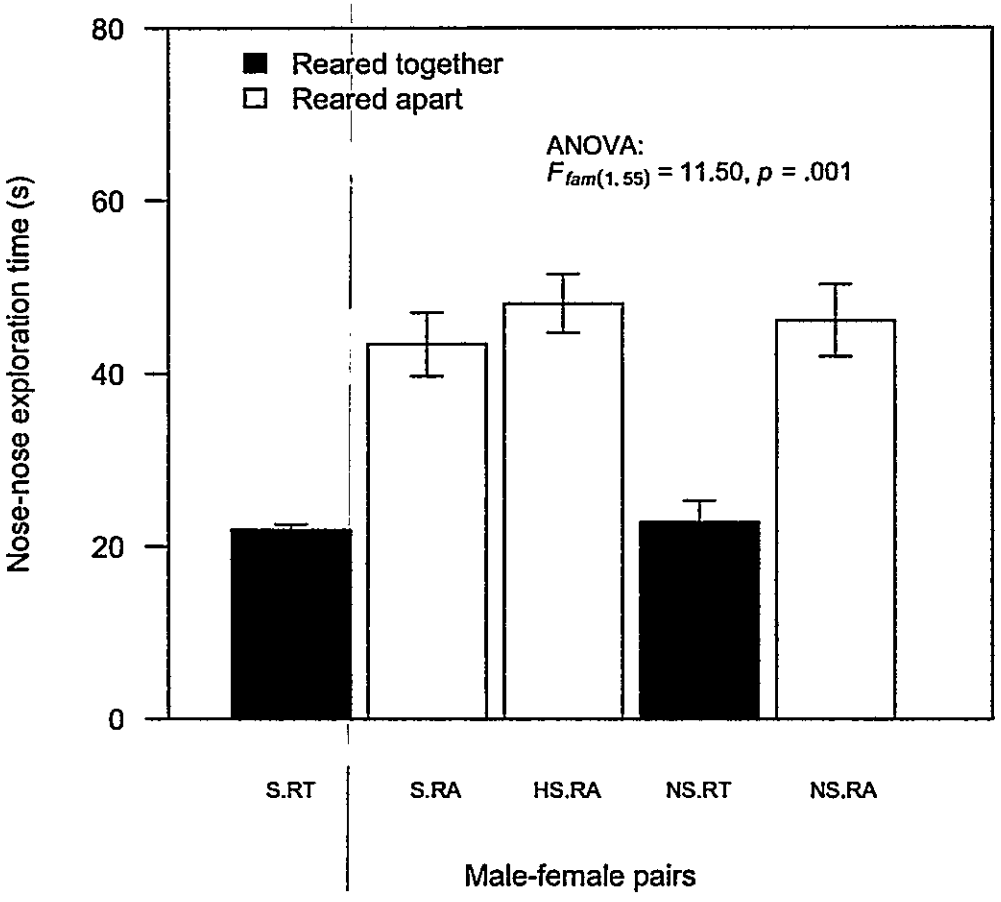

Figure 1.4

Figure 1.4. Effects of familiarity and genetic relatedness during dyadic encounters. Five experimental pairs were created to manipulate rearing condition (i.e., familiarity) and genetic relatedness: siblings reared together (S.RT), siblings reared apart (S.RA), half siblings reared apart (HS.RA), non-siblings reared together (NS.RT) and non-siblings reared apart (NS.RA). Exploratory behaviours were affected by familiarity (two-way ANOVA for transformed data).

We also found that tail wagging, an agonistic behaviour, was displayed significantly more by members of unfamiliar couples ($H = 5.83$, d.f. $= 1$, $p = 0.016$; Fig. 1.5 A), irrespectively of their degree of genetic relatedness ($H = 0.024$, d.f. $= 2$, $p = 0.88$) or interaction effects ($H = 0.59$, d.f. $= 1$, $p = 0.44$). Fights, in the

other hand, were rarely observed (only in 3 of 30 couples), and therefore not considered in the statistical analysis. On the other hand, and interestingly, sexual behaviours appeared to be more frequent between unfamiliar male-female couples, (with a 93% of confidence: $F_{[1,55]} = 3.35$, $p = 0.07$; Fig. 1.5 B).

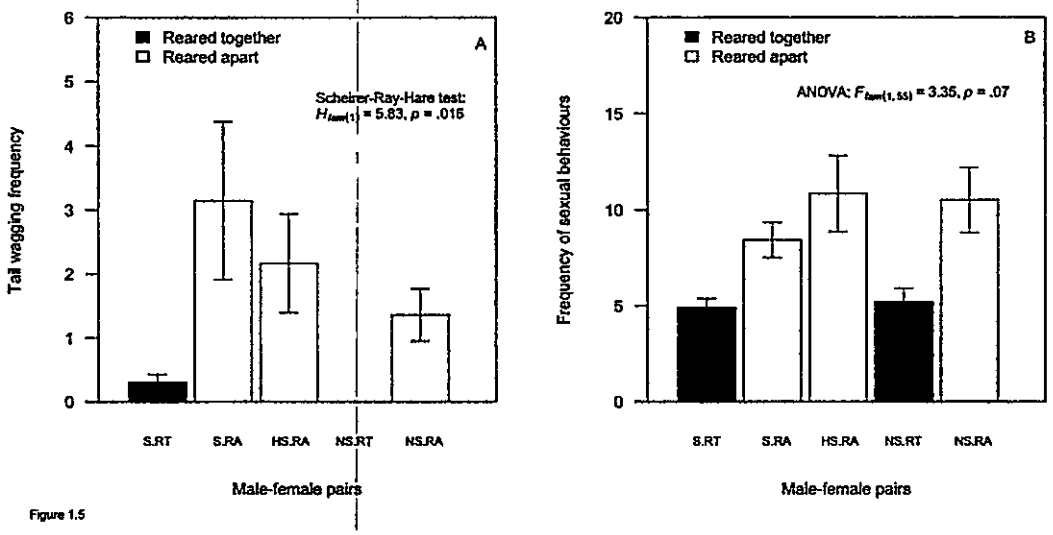

Figure 1.5

Figure 1.5. Effect of familiarity during dyadic encounters. (A) Tail wagging in agonistic context was affected by familiarity (Scheirer-Ray-Hare test). (B) Sexual behaviour tended to be more frequent in degus reared apart, though two-way ANOVA did not reach statistical significance.

The only behaviour found to be affected by genetic relatedness (Scheirer-Ray-Hare test: $H = 6.63$, d.f. $= 2$, $p = 0.01$ Fig. 1.6) and not by familiarity ($H = 0.77$, d.f. $= 1$, $p = 0.38$) was social contact. Interaction effects were also not found on this behaviour ($H = 1.41$, d.f. $= 1$, $p = 0.24$). Nonparametric multiple comparison using the Behrens–Fisher's approach showed that irrespectively of familiarity,

siblings pairs spend significantly more time in close contact than non-siblings pair ($p = 0.019$).

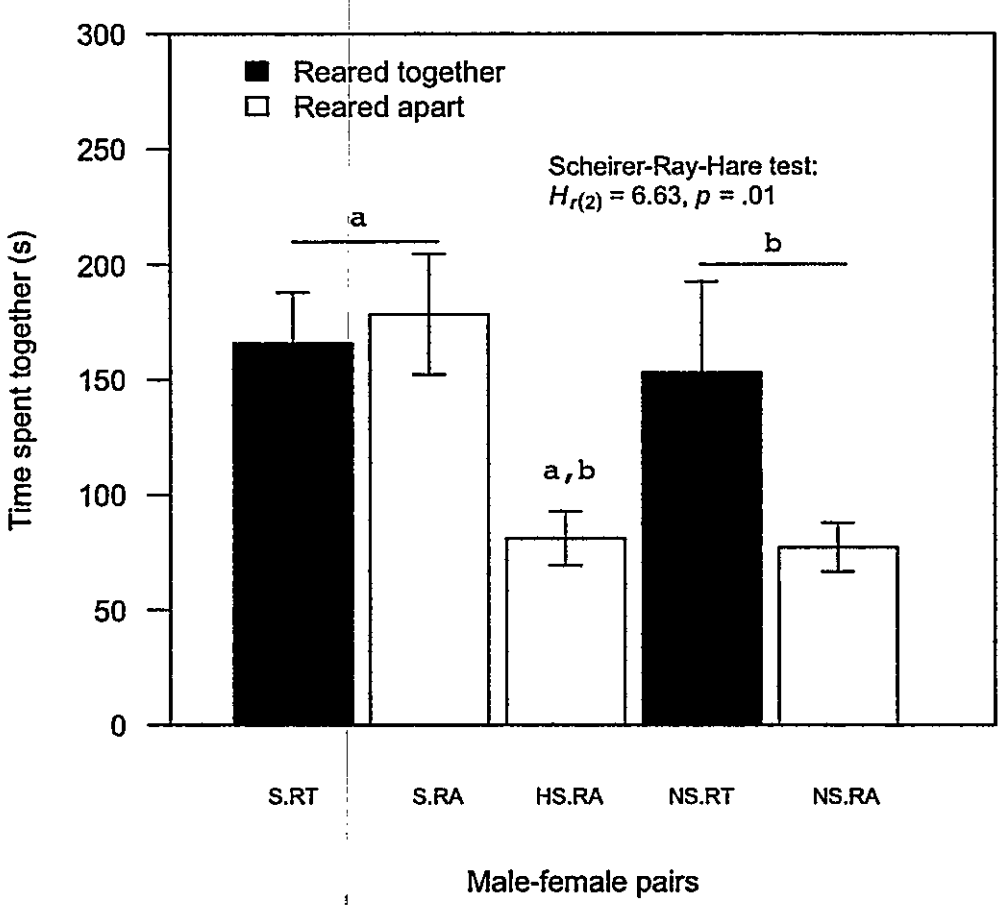

Figure 1.6

Figure 1.6. Effects of genetic relatedness on social contact during dyadic encounters. Different letters represent statistical differences using the Behrens–Fisher's approach of non-parametric multiple comparisons between groups with different coefficient of kinship (r) after Scheirer-Ray-Hare test.

DISCUSSION

The present study addressed the influence of familiarity and genetic relatedness on olfactory preferences toward conspecifics of the opposite sex and male-female social interaction. By means of cross-fostering of newborn degus we were allowed to rear animals with siblings and non-siblings.

Familiarity was found to affect the exploratory interest that focal subjects had for cosnpecifics in the *y-maze* labyrinth, as revealed by differences on exploratory behaviour between subjects exposed to a sib and to a non-sib that differed in the familiarity with the SPs. Frequency analysis of exploratory behaviours showed that degus from both groups where a sibling with whom they had lived together was present explored the labyrinth significantly less than degus exposed to two unfamiliar conspecifics (Fig. 1.2). Interestingly, subjects exposed to a non-familiarized sibling and to a familiarized non-sibling (S.RA/NS.RT) showed no differences in exploration frequencies with the other three groups, representing an intermediate situation. Since *y-maze* protocol is an experiment with no movement restriction, and olfactory exploration is considered as the degree of interest toward a certain odour (Johnston et al., 1997), our results revealed that in degus unfamiliar olfactory stimuli (i.e., two

unfamiliar conspecifics) elicits more olfactory interest than familiar stimulus, at least when one of them is a sibling. In the intermediate situation, as discussed above, subjects exposed to S.RA/NS.RT pairs compared with the other group that were exposed to a familiarized and to a non-familiarized pair (S.TR/NS.RA) did not show significantly less interest than the S.RA/NS.RA group, suggesting in one hand an effect of the unfamiliar stimulus (S.RA) but also and an effect of the familiar stimulus (NS.RT) in the other, considering that exploration was not different from degus exposed to S.RT/NS.RA and from subjects exposed to S.RT/NS.RT

Our study also found differences in the exploration time between sexes. Exploration time was significantly higher in females than in males (Fig. 1.3 A). In the same sense, studies on the ontogeny of exploratory behaviour have reported that even though sex differences are not observed during early ontogeny, at the end of adolescent stages females explore more than males (Lynn and Brown, 2009). Furthermore, experiments assessing the role of the kind of stimuli presented (social vs. physical) have shown that both sexes are more interested in social stimuli, but females explore more than males in both cases (Cavigelli et al., 2011).

In addition, the MET exhibited by degus in each exploratory visit was negatively correlated with the visit frequency, showing that subjects exposed to S.RA/NS.RA visited more, but during shorter time periods both arms of the *y*-

maze. As a result their activity patterns showed a high frequency of shifts between arms.

Dyadic encounters (experiment 2) showed a strong effect of familiarity on exploratory and agonistic behaviours regardless of the genetic relatedness between individuals (Fig. 1.4 A and fig. 1.5 A), in the same sense familiarity affected sexual behaviours (Fig. 1.5 B). By contrast social contact was affected by genetic relatedness, siblings spent significantly more time together than non-siblings even though they had been separated at birth (Fig. 1.6). This experiment showed that familiarity accounts for some of the male-female social interaction quantified. Similarly Villavicencio et al., (2009) showed that kin discrimination in degus between same sex siblings was influenced by familiarity, even when they were unable to discard totally the influence of genetic relatedness.

Studies aimed to assess the two more accepted behavioural mechanisms underlying kin discrimination (i.e. familiarity or phenotype matching) have revealed for some species the importance of familiarity (e.g. thirteen-lined ground squirrels: Holmes, 1984; prairie voles: Paz-y-Miño and Tang-Martinez, 1999b) and for others the ability to recognize kin without previous experience (e.g. belding ground-squirrels: Holmes, 1986), suggesting that close related animals share phenotypic features that would allowed to recognize them as kin and behave differentially toward them. In a deeper analysis, it has been proposed that both behavioural mechanisms are indeed the reflect of learning (see Tang-Martinez, 2001 for a critical re-evaluation of kin recognition

mechanisms), and that in order to better understand behavioural bias toward kin the attention should be paid on the learning of distinctive signals (whether associated with kinship or not), and on how they participate in kin recognition (Tang-Martinez, 2001, Mateo and Holmes, 2004). Consequently, behavioural bias toward kin observed under natural conditions occurs in some species because they grow surrounded by kin and they learn their distinctive cues (i.e., visual and/or olfactory), while in others they learn this cues from their close related kin or from their own and later behave differentially with cosnpecifics that share these signals, even if they haven't met before.

Moreover, studies assessing mate preferences have revealed that familiarity have different effects depending on the social system of each species (Adrian and Sachser, 2011, Brandt and Macdonald, 2011). Females from highly social rodents tend to prefer unfamiliar males (Clarke and Faulkes, 1999, Tai et al., 2000), while in solitary species they tend to prefer males living near by and to which scent marking they have been exposed (Ramm et al., 2008). Furthermore, mandarin vole females prefer the odour of the species with whom she was fostered over odours from her cosnpecifics (Tai et al., 2000). The *y-maze* experiments here presented did not allow contact between males and females therefore can only reflect social interest not sexual preferences (Clarke and Faulkes, 1999).

Field studies reveal that degus' socials system consists of 1-2 adult males and 2-6 females (Fulk, 1976, Ebensperger et al., 2004, Quirici et al., 2011), and that

communal nesting is observed between genetically related females (Ebensperger et al., 2004). Furthermore young males disperse more than females (Ebensperger et al., 2009). 97.6% of female interactions above the ground consist of co-nesting encounters (all amicable). In contrast the scant agonistic interactions (less than 3%) occur only between females from different nests (Ebensperger et al., 2004). However, social groups are not exclusively formed by close related degus (Ebensperger et al., 2009), and has been reported that familiarity reduces agonistic behaviours between males after repeated encounters (Davis, 1975). Altogether we consider that familiarity plays a mayor role mediating degus' social interactions. Degus born in a commonly nesting burrow will probably share nests with close related kin (i.e., siblings, cousins) and also with no kin. Early social experience with other pups might be crucial in the learning of distinctive olfactory signals that later might be mediate in behavioural bias and social interest that could account for the influence of familiarity and genetic relatedness observed.

CAPÍTULO II

EARLY OLFACTORY ENVIRONMENT INFLUENCES SOCIAL BEHAVIOUR IN ADULT *OCTODON DEGUS*

ABSTRACT

To evaluate the role of olfactory learning in the context of sibling and mother-offspring interactions we manipulated early olfactory environment in newborn *O. degus*, scenting all litter members with *eucalyptol* during the first month of life. Sexually mature degus (5-7 months old) were tested in a *y-maze* labyrinth (experiment 1) against two different olfactory configurations: (i) a non-familiarized conspecific impregnated with *eucalyptol* (*eucalyptol* arm), and (ii) a non-familiarized conspecific without any artificial odour (control arm). Additionally, we assessed differential treatment influenced by differences in olfactory experience by observing dyadic interactions of a focal subject in an experimental arena with a non-familiarized conspecific artificially scented

(experiment 2). In experiment 1, naïve subjects exhibited neophobia, spending significantly less time in the arm with the artificial olfactory configuration (degu + *eucalyptol*). *Eucalyptol* experienced subjects did not show neophobia and did not spend different times in both arms of the labyrinth. In experiment 2 naïve males escaped and avoided their scented partner more frequently than *eucalyptol* experienced subjects, sex differences also influenced the behavioural differences mentioned above. No difference in exploratory behaviours was found between both groups, suggesting that even though the presence of *eucalyptol* in a non-familiarized conspecific accounts for a decrease in agonistic behaviour, degus might be able to discriminate the olfactory configuration produced by non-familiar conspecific impregnated with *eucalyptol* in relation to the one produced by a familiar sibling impregnated with the same odorant. These results indicate that olfactory cues learned within a social context such us suckling and kin interactions can influence olfactory-guided behaviours in sexually mature degus.

INTRODUCTION

Experience during early ontogeny determines a wide range of behaviours observed in adults. Studies in birds have shown how social attachment can be modified by isolating individuals in a very early age and exposing them to different moving objects within a critical time period (Lorenz, 1937, Gottlieb, 1961). Under these conditions goslings and ducklings could, for example, become attached to a moving duck decoy or to a human, and therefore, follow it as ducklings in nature follow their mother. Interestingly, experiments where the maternal assembly call was played inside the model during training revealed that, contrary to what was previously postulated, attachment to a familiar object could be modified (Johnston and Gottlieb, 1981, 1985). Additionally, duckling trained in brood conditions lack of any preference for the familiar model, evidencing the role of social environment on the establishment of visual preferences (Lickliter and Gottlieb, 1986)

Studies on early olfactory learning in mammals have focused in understanding how the ontogenetic olfactory environment could influence olfactory preferences in young and adult animals. Similarly to what it was found for

ducklings, Apfelbach (1986) showed that the olfactory environment during feeding behaviour of juveniles can influence the odour-food choices of adult ferrets, and that food-odour preferences are established during a critical period in early ontogeny. Within a social context, on the other hand, early olfactory experience also seems to play an important role. Studies on rats and guinea-pigs have shown that animals exposed to non-natural odours present in the mother's nipples during suckling, develop preferences towards conspecifics impregnated with the same odour. In contrast, animal raised in cages impregnated with artificial odours did not develop such preferences (Marr and Gardner, 1965, Marr and Lilliston, 1969, Carter and Marr, 1970). In addition, research on the effects of olfactory learning in the context of suckling have shown that neophobia and conditioned odour aversion normally present in naïve rats, is no longer observed in animals that have experienced the artificial odour scented on the mother's nipples (Sevelinges et al., 2009). The presence of an odorant in the genital area and nipples of the mother has also been shown to elicit differential sexual arousal toward non-familiarized females impregnated with the artificial scent (Fillion and Blass, 1986). Thus, olfactory experience during early ontogeny alone is not sufficient to determine behavioural preferences in adults, the olfactory learning must occur in a relevant social context (e.g. suckling period). Studies on odour conditioning in rat pups revealed that olfactory preferences can be established during a sensitive period from post natal (PN) day 1 to PN10, by presenting a non-natural odour along with tactile stimulation as replacement of contact from the mother (Sullivan et al., 1986, Sullivan and Leon, 1986).

On the other hand, olfaction also seems to be fundamental for conspecific and kin discrimination (i.e. behavioural bias toward kin) in many rodent species (Halpin, 1991; Hepper, 1991). In thirteen-lined ground squirrel sibling recognition seems to be based on familiarity (i.e. common living) rather than on genetic relatedness (Holmes, 1984). However, it has been found that kin recognition also occur in individuals separated at birth from their relatives using crossed-fostering technique (Holmes and Sherman, 1982, Hare, 1998, Villavicencio et al., 2009). Studies on kin recognition have focussed on two of the most widely accepted mechanisms: (1) recognition by prior association, which consider that behavioural bias is determined by familiarity, and (2) recognition by phenotype matching where individuals learn cues that are shared by family members, treating individual with this cues as kin (Holmes and Sherman, 1982; Tang-Martinez, 2001). Based on data collected over 25 years Tang-Martínez argued that indeed what underlies both mechanisms is learning, and that both mechanisms could together modulate kin recognition (Hepper, 1991; Tang-Martinez, 2001).

Despite the evidence showing the important role of social context on the formation of social attachment and olfactory preferences (Sullivan et al., 1986, Sullivan et al., 1989, Moriceau and Sullivan, 2005), the role of olfactory learning and early social context on the establishment of behavioural bias toward conspecifics has not been thoroughly studied. In order to explore this relationship, we artificially scented all litter members daily for one month. Olfactory preferences and possible behavioural bias were tested on 5-7 months

old *O. degus*, a diurnal and highly social semifossorial caviomorph rodent, endemic to central Chile (Fulk, 1976). Several social characteristics make degus a very interesting study model, they present communal nesting (Ebensperger et al., 2004, Jesseau, 2004, Jesseau et al., 2008) and it has been reported that degus can display differential behavioural treatment between kin and non-kin mainly based on direct familiarization despite the ability to discriminate kin based on olfactory cues (Villavicencio et al., 2009). Using *y-maze* technique we assessed olfactory preference for non-familiar conspecifics impregnated with an artificial odour to which they were exposed during early ontogeny (experiment 1; see below). In addition, we quantified behavioural differences during pair encounters in an experimental arena toward a non-familiar conspecific impregnated with the odorant (experiment 2).

MATERIALS & METHODS

Subjects

Males and females *O. degus* born and reared in our colony were used. Dams and litter were housed in metal cages (50 x 40 x 35 cm) with wood shaving, under natural photoperiod in two different air-conditioned rooms at the University of Chile. Animals were fed with alfalfa and rabbit pellet together with water provided *ad libitum*. Both maintenance and experimental procedures were approved by the ethics committee of the Faculty of Sciences of the University of Chile, and followed Chilean regulations.

Rearing conditioning

From postnatal day (PN) one the mother was rubbed on the anterior and ventral area with cotton balls (Simmond's, Chile) impregnated with *eucalyptol* (C80601, Aldrich) 3.3 µM 10-15 times. From PN2 to PN30, pups and dams were daily rubbed in the ventral and dorsal area with the artificial odour. After this exposure period dams and siblings remained together in their home cages and only experienced *eucalyptol* once again in the experimental session.

***Y-maze* (Experiment 1)**

Behavioural differences toward two different odour configurations were assessed using in a y-maze labyrinth (Fig. 2.1). Animals were carried in individual plastic cages from the housing rooms to the experimental rooms. The experiment consisted in 1 min of acclimatization and 3 min of test time. Subjects (Ss) used in behavioural tests were either from the *eucalyptol* exposed litters as described above, or from control litters reared in colonies housed in a different room to ensure that the animals had no experience with the odorant prior the test. In the *y-maze* each stimulus chamber contained either a non-familiarized degu (i.e. reared apart) impregnated with *eucalyptol*, or a non-familiarized degu without any artificial odour. The odorant was applied in the same way as in the *eucalyptol* rearing condition procedure. To avoid scent contamination between animals, we used different sets of gloves two manipulate animals in the maze. Position of the stimulus was randomized to control for labyrinth side preference. The *y-maze* was cleaned with 95° Ethanol to eliminate odour traces after each experiment. All experiments were video recorded (colour CCTV camera connected to a Sony video recorder) from above the *y-maze* system. An observer blind to the treatment and Ss sex analyzed from the recordings the different behaviours considered in our study using the JWatcher 1.0 software (Dan Blumstein, University of California, Los Angeles, U.S.A). The behaviours evaluated were direct division plate exploration time, arm exploration time and still time in each arm.

Fig.2.1

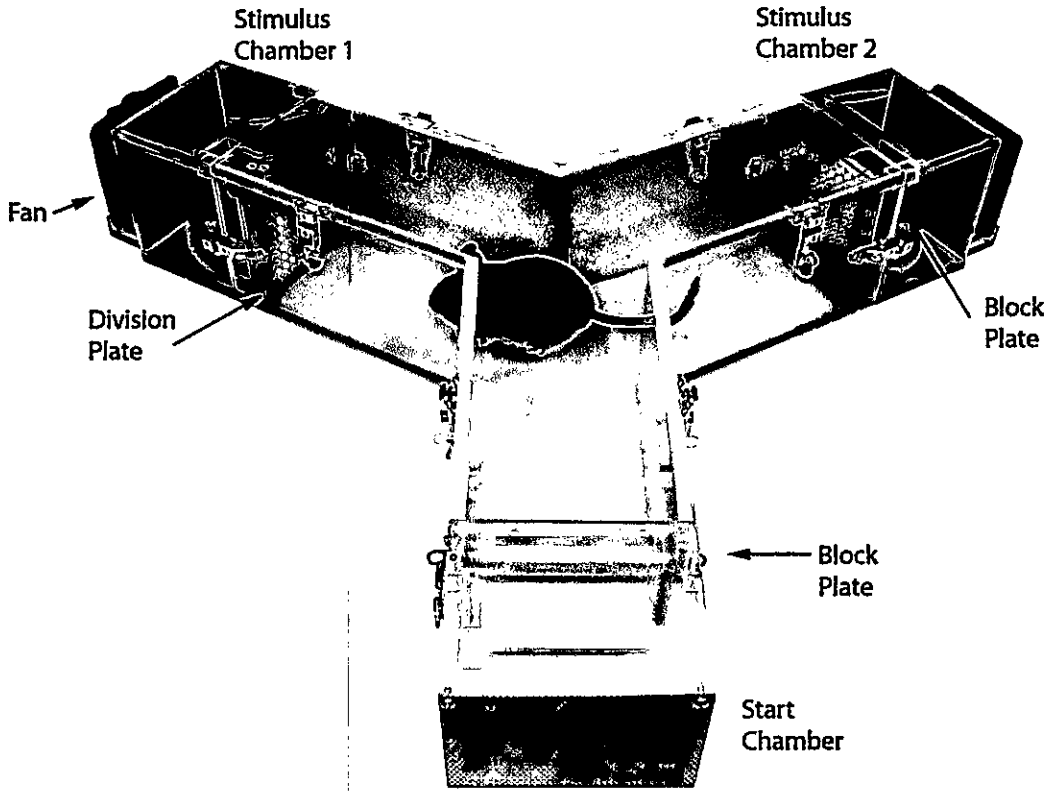

Fig.2.1 *The y-maze.* The system was designed to allowed a focal degu explore freely the three arms of the labyrinth (50 cm long x 15 cm with x 15 cm high Plexiglas arms forming angles of 120°). Stimulus Ss were maintained in chambers attached to the extremes opposite the start chamber. A perforated Plexiglas plate prevent from direct contact between focal and stimulus Ss, but allowed the airflow produced by a fan behind each stimulus chamber to circulate through the labyrinth. During acclimatization time a Plexiglas plate (Block plate) avoided the movement of the focal subject, and also prevented volatile odours from the stimulus chambers reach the start chamber. Once the experiment began the block plates were removed, and the fans next to the stimulus chambers switched on.

Statistics

To evaluate the effects of early experience, the measured behaviours described above were analysed with one-way ANOVA or Kruskal-Wallis test depending

on whether the data met parametric requirements or not. Intra-group comparisons were analysed using paired *t*-test or Wilcoxon Signed-Rank test depending on normality of dependent variables. The total time spent in each arm was also analysed. For all the analysis we used the software R (R Foundation for Statistical Computing, Vienna, Austria 2009).

Pair encounters (Experiment 2)

In order to assess behavioural discrimination during pair encounters we compared exploratory, social and aggressive behaviour exhibited by animals that differed in their previous experience with *eucalyptol*. Animals were tested with a non-familiarized conspecific impregnated with the artificial odour in an experimental arena during 10 min. From a total of 28 pairs of the same sex, 7 male and 7 female pairs were assigned to each treatment. Experiments were carried out as described by Villavicencio et al. (2009). Briefly, in two 80x80x50 cm metal arenas that could be divided by placing a division plate. The floor consisted in a removable white-painted metal plate that was cleaned with detergent between tests to remove any trace of scent that could have been left by previous pair. Animals were carried to the experimental rooms as described for the *y-maze* experiment, the focal subject was painted with non-toxic painting which allowed to distinguish between the two degus when analysing the experimental video recordings. Previous studies have shown no change in locomotion, vigilance (Vasquez et al., 2002) or exploratory behaviour (Villavicencio et al., 2009) due to marking. The stimulus Ss was impregnated with *eucalyptol* as described for experiment 1. After a 10 min acclimatization

period, the division plate was removed and when the focal subject started to explore the arena began behavioural quantification. All experiments were video recorded with a camera placed above each arena as described for the *y-maze* experiment and analysed with the JWatcher software. Three behavioural categories were defined based on behavioural descriptions of intraspecific interactions in degus (Wilson and Kleiman, 1974, Kleiman, 1975, Fulk, 1976) and mice (Baudoin et al., 1991): (1) olfactory exploratory behaviour consistent of exploratory approaches to the mouth, head, flanks and/or anogenital area of its partner; (2) cohesive behaviour, was considered when degus were in contact either side by side, on right angle to each other, grooming, or huddling one over the other, and (3) agonistic encounters of two kinds, (i) evasive, whenever a focal subject by turning aside or running away avoided the partner in hostile contexts, and (ii) aggressive, if the animal performed tail wagging, hindleg kick, foreleg push, chasing or fight against its conspecific.

Statistics

The analyzed data did not reach parametric requirements, for this reason we employed the Scheirer-Ray-Hare test extension of the Kruskal-Wallis test for non-parametric data (Sokal and Rohlf, 1995)

RESULTS

Y-maze

No preference toward any particular arm of the *y*-maze was observed (paired *t*-test $t = 2.021$, $p = 0.06$). We found effects of *eucalyptol* early experience on exploration time spent in each arm. Naïve Ss showed significantly lower exploration time in the arm of the *y-maze* having a degu impregnated with *eucalyptol* in the stimulus chamber than *eucalyptol* experienced Ss degus (ANOVA: $F_{(1,22)} = 8.88$, $p = 0.007$; Fig. 2.2 A). Comparisons within groups show that *eucalyptol* experienced Ss explored equally both arms of the labyrinth (paired *t*-test: $t = 0.49$ $p = 0.64$), while naïve Ss explore significantly more the arm where the olfactory stimulus was a non-familiarized degu not impregnated with an artificial odour (control arm) (paired *t*-test: $t = -4.45$, $p = 0.001$; Fig. 2.2 A).

Effect of early experience on the total time spent by animals in both arms was also found. *Eucalyptol* experienced Ss spent significantly more time in the *eucalyptol* arm than naïve Ss animals (Kruskal-Wallis test: $H = 5.48$, $p = 0.02$). Moreover, naïve animals spent significantly more time in the control arm than *eucalyptol* experienced Ss (ANOVA: $F_{(1,21)} = 10.66$, $p = 0.004$; Fig. 2.2 B). Statistical

analysis comparing total time spent by naïve Ss revealed significant differences between both arms (paired *t*-test: $t = -3.78$. $p = 0.004$; Fig. 2.2 B). Both groups

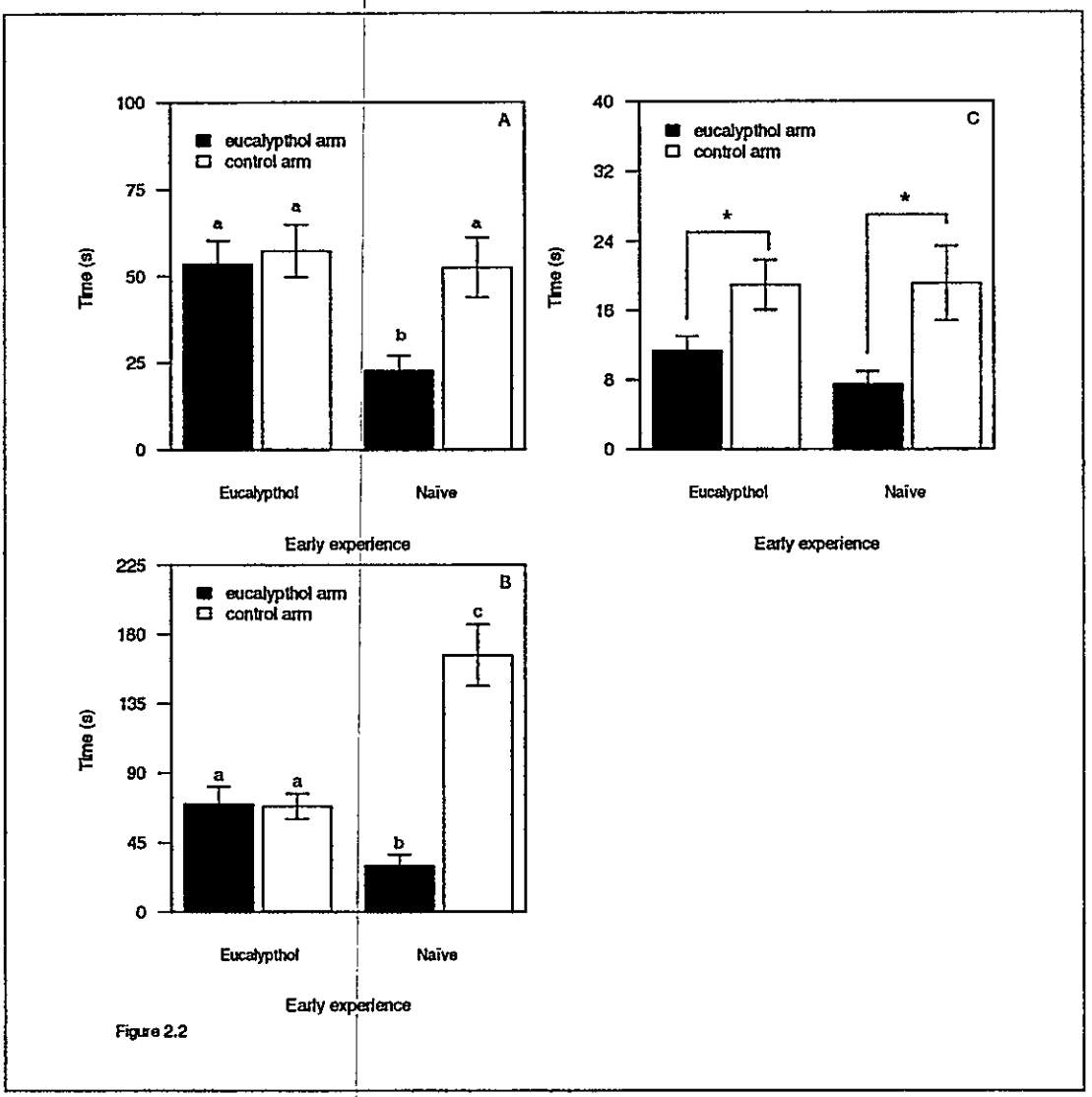

Figure 2.2

Figure 2.2. Effects of odour experience in *y-maze* experiment in *Octodon degus*. (A) Exploration time (mean ± SE) in each *y-maze* arm by animals with different olfactory experience during early ontogeny. Ss reared with *eucalyptol* explore equally both arms, while Naïve Ss explore significantly less the *eucalyptol* arm. (B) Total time (mean ± SE) spent by both groups in each arm. Naïve Ss remain significantly less time in the *eucalyptol* arm and spent significantly more time in the control arm than *eucalyptol* experienced Ss. (C) Time smelling directly division plate in both arms. *Eucalyptol* experienced and naïve Ss investigate significantly more the plate of the control arm. Different letters represent statistically significant differences between the groups in each arm. Asterisks represent significant differences in investigation time of division plate (paired *t*-test $p < 0.05$, see text for statistical values).

investigated more the control division plate (Wilcoxon signed-rank test: impronted group $V = 9$, $p = 0.01$; naïve group $V = 4$, $p = 0.02$) and no effect of early experience in plate investigation was found for either of the stimulus chambers (Fig. 2.2 C).

Paired encounters

Two animals were not considered in the statistical analysis because they remained still during the experiment and the repetition session. Scheirer-Ray-Hare test revealed that the effect of early experience on the evasive behaviour depends on the sex of the subject (sex: $H = 4.996$, d.f. $= 1$, $p = 0.025$; interaction: $H = 4.59$, d.f. $=1$, $p = 0.032$; Fig. 2.3 A). We found that males performed significantly more agonistic behaviours than females (sex: $H = 4.51$, d.f. $= 1$, $p = 0.033$; Fig. 2.3 B), but no effect of the interaction between sex and experience was found (interaction: $H = 1.75$, d.f. $=1$, $p = 0.19$; Fig. 2.3 B). Males also explored significantly more their partner than females in the anterior as in the anogenital region (anterior: $H = 7.25$, d.f. $= 1$, $p=0.007$; anogenital: $H=5.19$, $p=0.022$; Fig. 2.3C).

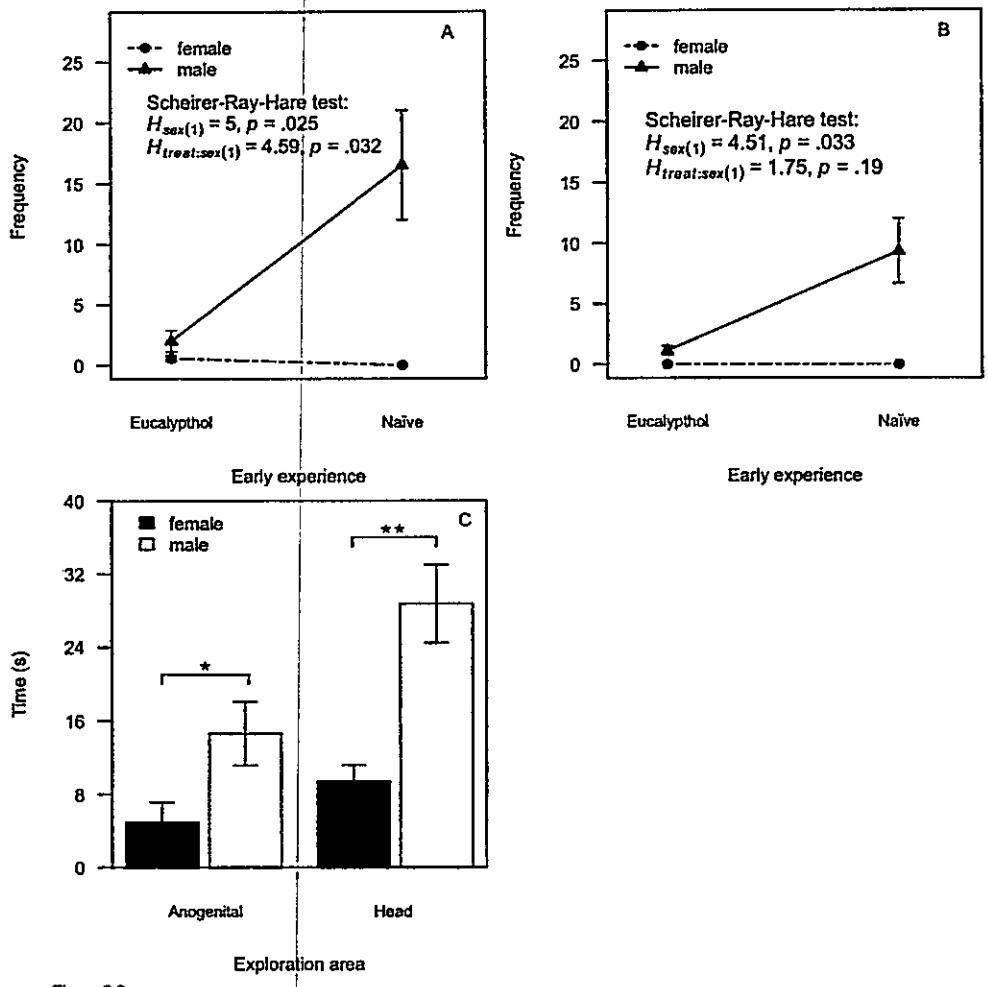

Figure 2.3. Male-male and female-female interactions during pair encounter experiments. Evasive (A) and aggressive behavior frequencies in agonistics behavior context (B). (C) Exploration time (mean ± SE) of anogenital and anterior regions, asterisks represent significant differences between males and females in Scheirer-Ray-Hare test (*$P < 0.05$ and **$P < 0.01$).

DISCUSSION

The present study revealed behavioural differences between degus developed in a social environment modified by the presence of an artificial odour (*eucalyptol*) and degus whose close olfactory environment was not perturbed. *Y-maze* experiments showed differences in exploratory behaviour depending on the experience with the artificial odorant. Naïve Ss animals showed neophobic behaviours toward the artificial olfactory configuration indicated by the avoidance of the *eucalyptol* arm. They smelled the division plate and explored the whole arm of the *eucalyptol* side significantly less than the control arm (see *y-maze* system Fig.2.1). In addition, animals remained still significantly more time in the arm of the conspecific without artificial odour.

Degus maintained in an artificially scented social environment, on the other hand, did not show avoidance for the arm containing an animal impregnated with *eucalyptol*, exploring both arms similarly. Previous studies have reported similar findings, where the presence of an artificial odour do not trigger neophobic behaviours in Ss that have been exposed to that odour in a social context (Sevelinges et al., 2009). In our case, the lack of neophobic behaviours in *eucalyptol* experienced Ss suggest a familiarity effect toward the conspecific

impregnated with *eucalyptol*. Familiarity could also explain the lack of interest in the investigation of the division plate of the *eucalyptol* arm by *eucalyptol* experienced animals.

Several studies show the tendency of rodents to explore in more detail unfamiliar olfactory stimulus, as in our case toward non-artificially scented degus (Davis, 1975, Johnston, 1993, Villavicencio et al., 2009).

The effect of early social olfactory experience in intraspecific interactions was also evaluated (experiment 2). Animals that had no previous experience with the artificial scent showed more evasive behaviours during agonistic encounters with partner scented than *eucalyptol* experienced Ss. This differences in behaviours within agonistic encounters resemble results on kin discrimination using cross-fostering, where less agonistic behaviours are observed toward conspecifics with olfactory signals similar to the signals from the nest mates (Holmes, 1986), suggesting that the learning of distinctive signals, whether associate or not with kinship, participate in kin recognition (Tang-Martinez, 2001, Mateo, 2004). In the case of degus, it has been reported, observing exploration in dyadic interactions, that kin discrimination is mainly influenced by familiarity and by phenotypic similarities (Villavicencio et al., 2009). Despite the effects of early experience with *eucalyptol* on agonistic behaviour, no differences in exploratory behaviours were found between naïve and eucalyptol experienced animals. This finding suggest that even though the presence of *eucalyptol* would account for decrease of agonistic behaviour, and degus might

be able to discriminate the olfactory configuration produced by non-familiar conspecifics.

Until now kin discrimination studies based on olfaction have been successful to prove behavioural bias toward siblings reared apart (S.RA), indicating that some olfactory features shared between genetically related conspecifics evoke behavioural preferences even though they had no previous contact (Holmes, 1986). Here we induced behavioural bias toward non-sibling reared apart (NS.RA) by impregnating them with an artificial odour present during early ontogeny in the subject's social environment.

The present study combined techniques used to assess the role of early social experience in later olfactory preferences (artificial scenting and *y-maze*) with behavioural experiments used to test kin discrimination (pair encounters arena). Our results support the idea that the learning of familiar signals would underlie kin discrimination as discussed by Tang-Martinez (2001).

The plasticity nature of social attachment and preferences, as illustrated by examples where social attachment or preference occur toward artificial stimulus, reveal that what is usually described as a normal or typical for a species (i.e. following response toward the mother in duckling (Lorenz, 1937, Gottlieb, 1961), might result from the repetition of the same epigenetic history (Gottlieb, 1991). If we consider epigenesis as a continuous process of structural transformation that occurs to the organism as a whole, and that is modulated by

its interactions with the environment (Oyama, 1985, Maturana-Romesin and Mpodozis, 2000), the study of such interaction during early ontogeny results fundamental to understand behaviour in adults. Gottlieb (1991) showed that the preference of duckling for the species maternal call was directly influenced by active vocalization of embryos before hatching. Devocalization of embryos resulted in the lost of preference. Furthermore, preference toward the chicken call could be induced by playing the chicken call to devocalized embryos. In this line, the evidence available on the structures of the nervous system that participate in the formation of olfactory preferences gives light to the role of somatosensorial stimulation in olfactory plasticity and, consequently in olfactory learning. If norepinephrine (NE) ß-receptor agonist is injected 30 min prior odour presentation olfactory preference is formed in the same way than under an odour-stroke protocol (Sullivan et al., 1989). Preference formation can be blocked if NE ß-receptor antagonist is injected prior to Odour-Stroke training (Sullivan et al., 1989). Interestingly locus coeruleus (LC) neurons, which represent the only NE input to the olfactory bulb (OB), respond to tactile stimulation only in young animals, from PN22 their response can no longer be observed (Shipley et al., 1985, Nakamura et al., 1987, Nakamura and Sakaguchi, 1990). These results have led to the idea that somatosensorial activation of LC neurons could modulate olfactory plasticity by increasing the levels of NE in the OB during early experience (Sullivan et al., 1989, Moriceau and Sullivan, 2005). If this was also the case in degus, the social context under which the artificial odour was applied in our study might be crucial for the formation of the behavioural bias described here.

In wild degus nesting communally, groups of two to four females with different degrees of kinship share underground nests (Ebensperger et al., 2004). Each female gives birth between three to eight pups (Fulk, 1976), therefore early social environment of degus is conformed by a large group of conspecific that differ in sex and kinship. Among ground female interactions, 97.6% represent co-nesting encounters (all amicable), in contrast to agonistic interactions (less than 3%) when females are from different nests (Ebensperger et al., 2004). We propose that cooperative or amicable behaviours in degus are mediated by olfaction, and they might be the result of learning olfactory signals present in conspecifics, with whom they have interacted since early ontogeny similar to what describes Holmes (2004) for Belding's ground squirrel. The development the olfactory system in degus might also be affected by somatosensorial stimulation. Thus, a great plasticity could be expected in a rich social environment as commonly occur in natural dens. Under these circumstances, conspecifics displaying similar olfactory cues would establish behavioural bias between them, even when no prior social association has occurred among them.

CAPÍTULO III

NEURONAL ACTIVITY IN THE OLFACTORY BULB OF *OCTODON DEGUS* EVOKED BY ODOURS OF CONSPECIFICS

ABSTRACT

The expression of the immediate-early genes *c-fos* has been used to describe patterns of neuronal activity in different systems. The specific circumstances under which *c-fos* is expressed are not yet fully understood, though recent studies coincide in the crucial role of stimulus novelty in the induction of high levels of *c-fos* expression. On the olfactory bulb, it has been shown distinctive patterns *c-fos* expression elicited by odours in agreement with maps observed using 2-Deoxiglucose labelling and optical imaging techniques. The expression of these patterns of activity depends on the novelty of the stimulus, repeated exposure to the same odorant resulting in a diminished expression of *fos* and therefore, characteristic olfactory maps are no longer observed. In this study we measured *c-fos* expression in the olfactory bulb of adults *Octodon degus* elicited by conespecifics' odours. Animals were exposed three consecutive days to a

non-familiarized conspecific (habituation trials), and during the fourth day the olfactory stimulus was either the sibling or an individual non-genetically related with the stimulus animal used in the 3-days habituation trials. We found that sex and treatment affects the expression of *fos*. Females exposed to a sibling pair expressed lower levels of *c-fos* in mitral/tufted cells than females exposed to a non-related pair. Males did not show differences on expression of Fos protein. Considering that *fos* expression is associated with the novelty of the stimulus, lower levels of *fos* indicate that two stimulus do not represent novelty. Our results suggest that at least for females the olfactory configuration of two sibling females is similar, so lower levels of *c-fos* expression were observed. However, differences between sexes should be further studied.

INTRODUCTION

The expression of the immediate-early gene *c-fos* has been widely used to characterize spatial patterns of neuronal activity in different systems (Sallaz and Jourdan, 1996, Iñzunza et al., 2000, Schettino and Otto, 2001, Staiger et al., 2002, Illig, 2005, Rojas et al., 2009). A transient expression of *c-fos* occurs after an episode of sustained neuronal depolarization (Morgan and Curran, 1986, 1988). Such episode results in an intracellular accumulation of Fos protein, which is followed by a drop, below basal levels in the *c-fos* expression. During this latter period neuronal firing is not sufficient to re-induce *c-fos* expression (Morgan et al., 1987).

It has been shown that patterns of odorant-evoked expression of *c-fos* in the olfactory bulb (OB) match olfactory maps evoked for monomolecular odorants using optical imaging techniques (Rubin and Katz, 1999, Uchida et al., 2000, Belluscio and Katz, 2001) and 2-Deoxiglucose labelling (Sallaz and Jourdan, 1996), but at a cellular resolution. Complex odour mixtures, such as urine from two haplotypes differing in the major histocompatibility complex (MHC), also evoke distinctive *c-fos* patterns of expression (Schaefer et al., 2001, Schaefer et al., 2002).

Even though the specific circumstances under which *c-fos* is expressed remain unknown (Rojas et al., 2009), recent studies coincide in the crucial role of the stimulus novelty in the induction of higher levels of *c-fos* expression (Anokhin et al., 1991, Montag-Sallaz et al., 1999, Schettino and Otto, 2001, Jenkins et al., 2004, Rojas et al., 2009). Animals repeatedly exposed to an olfactory stimulus exhibit lower levels of *c-fos* expression in the OB compared to animals exposed to the same odorant for the first time. Furthermore, consistently repeated exposure to a certain odorant followed by exposure to a different one, results in high levels of *c-fos* expression that are similar to the ones exhibited by naïve animals (Montag-Sallaz and Buonviso, 2002). Thus, *c-fos* expression in the OB after a habituation/dishabituation protocol could represent a measure of similarity between two olfactory stimuli.

Studies focused on the olfactory signals involved in social interactions have shown that distinctive olfactory cues present in the urine are related to genetic differences (Boyse et al., 1987, Schaefer et al., 2002). Urinary proteins have been postulated to act as familiar distinctive signals that could be determinant in conspecific or kin recognition (Yamazaki et al., 2000, Beynon and Hurst, 2004). In the same sense, recently it has been reported that individuals of *O. degus* a highly social rodent endemic of central Chile (Fulk, 1976), can recognize their siblings (i.e., showing less olfactory exploration) whether they were reared together or never met before (Villavicencio et al., 2009). This suggests that kin recognition in degus can be mediated by distinctive familiar signals.

In this study we examined whether the olfactory cues produced by *Octodon degus* siblings are similar enough to produce an effect on *c-fos* comparable to the one obtained after repeated exposure to the same odorant. Focal degus were exposed during three consecutive days to a non-related conspecific as olfactory stimulus (habituation trials). At the fourth day (dishabituation) the olfactory stimulus was changed either by a sibling of the stimulus animals (kinship coefficient $r = 0.5$) or a non-related animal ($r = 0$). If the olfactory signals of two siblings are alike, we expect lower levels of *c-fos* expression in animals exposed during the habituation/dishabituation trial to siblings ($r = 0.5$ group) than animals exposed to two genetically unrelated conspecifics ($r = 0$ group).

MATERIAL AND METHODS

Subjects

Subjects used to quantify *c-fos* expression were adult males and females *O. degus* born and reared in our colony at the University of Chile. Animals were housed together with their mother and brood mates in metal cages (50 x 40 x 35 cm) that had wood shavings soil, food and water provided *ad libitum*. All experimental procedures were approved by the ethics committee of the Faculty of Science of the University of Chile, and followed Chilean regulations.

Olfactory stimuli and apparatus

Olfactory stimulation was carried out in a sealed Plexiglas apparatus consistent in two contiguous boxes connected by fine perforations. A constant pure airflow (6.0 l/min; AGA, Chile) was passed through an odourless silicone (peroxide-cured) tubing (Cole-Parmer, USA) to the smaller box, where the olfactory stimulus was placed (stimulus chamber), to finally reach the focal subject in the larger box (experimental chamber).

A preliminary experiment was carried out to determine first if as observed in mice and rats, repeated exposure to an artificial odorant result in a diminution of Fos levels

in the caviomorph rodent *O. degus*. Previous to olfactory stimulation, subjects ($n = 2$) were placed in the experimental apparatus with a constant airflow for five minutes. Afterwards cotton balls impregnated with *eucalyptol* (C80601, Sigma-Aldrich) were placed in the stimulus chamber for 10 min. The animals were kept in the experimental chamber with the airflow on for sixty more minutes. This procedure was repeated four consecutive days. In the control group ($n = 2$), experiments were carried out in the same way, with the difference that no *eucalyptol* was presented during the initial three habituation days, only a constant pure air flow (6,0 l/min). In the dishabituation trial (day four) the animals experienced *eucalyptol* for 10 min. Additionally, in one animal *c-fos* expression was measured after four consecutive days of pure air stimulation. In the next experiment, olfactory stimulation was carried out following the stimulation protocol described above. Briefly, each focal subject was exposed to a different pair of conspecifics that were not genetically related with him/her. First, for three consecutive days the same conspecific was placed in the stimulus chamber (habituation trails). In the fourth day the olfactory stimulus was a different conspecific, that could be either the sibling of the animal used as olfactory stimulus in the habituation trials ($r = 0.5$ group; $n = 6$) or an animal not genetically related to the stimulus animals used during the habituation sessions ($r = 0$ group; $n = 6$).

Immunocytochemistry

After the fourth trial degus were perfused with 0,1 M phosphate-buffered saline (PBS) (pH = 7.4) followed by 4% paraformaldehyde in 0,1 M. Both OB were collected and post-fixated at least one day. Prior sectioning the OBs were transferred to 30%

sucrose in phosphate buffer until they sunk. Using a freezing microtome, 30 μm coronal and saggital sections were obtained and collected in PBS. Free floating sections were first incubated in H_2O_2 0,3% in PBS for 30 minutes followed by the incubation in normal goat serum 5% and 0,4% Triton X-100 in PBS (PBST) and then incubated with *c-fos* antibodies (sc-253, Santa Cruz Biotechnology, USA; diluted 1:200) overnight at 4°C. Thereafter, sections were incubated with biotinylated secondary antibody in PBST for one hour and then in avidin-peroxidasa in PBST for another hour (Vector Laboratories, USA). Finally the sections were reacted with diaminobenzidin (Sigma), mounted, dehydrated and coverslipped.

Quantification of *c-fos* marked neurons

We divided the OB in four regions: dorsolateral (DL), ventrolateral (VL), dorsomedial (DM) and ventromedial (VM) (coronal sections), and ventroanterior (VA), ventroposterior (VP) and dorsoanterior (DA) (sagittal sections), the dorsoposterior (DP) area of the quantified sections corresponded to the accessory olfactory bulb. Immunoreactive Granular (Gr), mitral/tufted (M/T) and periglomerular (PG) cells (see Fig 3.1) were estimated with the software StereoInvestigator (MicroBrightField Inc., USA) on a Zeiss Axioplan microscope using a 20X objective. Each cellular layer was delineated within its quadrant and then each cell type was visually identified and marked. Four sections separated 120 μm with each other, containing the same area of interested were analysed per subject.

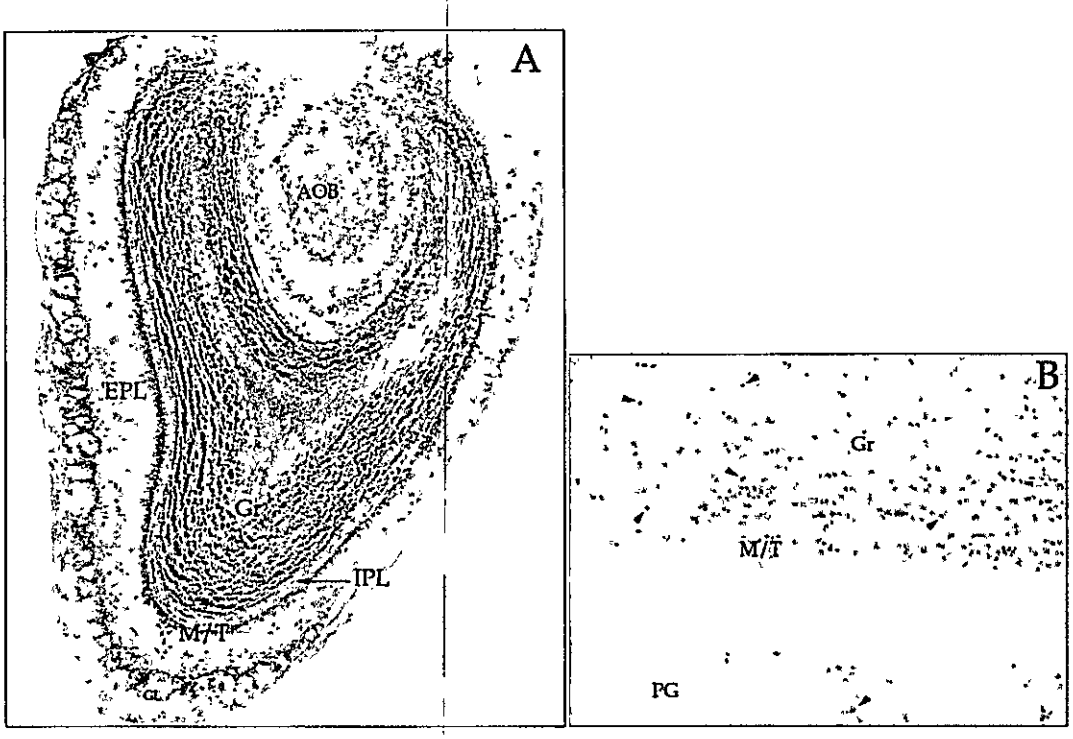

Figure 3.1. Photomicrograph of coronal section of *Octodon degus'* olfactory bulb (OB). (A) Nissl staining shows the layered structure of the OB, the glomerular layer (GL) is the most external one followed by the external plexiform layer (EPL), mitral layer (M/T), internal plexiform layer (IPL) and granular layer (Gr). In this section it is also present the accessory olfactory bulb (AOB). (B) *c-fos* expression in degus' OB, arrowheads indicate *fos* immunoreactive cells.

Statistics

The analyzed data did not reach parametric requirements, for this reason we employed the Scheirer-Ray-Hare test, an extension of the Kruskal-Wallis test for non-parametric data, multiple comparisons where carried out with Wilcoxon two sample test (see Sokal and Rohlf, 1995). For all the analysis we used the software R (R Foundation for Statistical Computing, Vienna, Austria 2009).

RESULTS

Artificial odour

Because the expression of *c-fos* differed significantly between cellular types ($H = 4.39$ $p = 0.04$, $n = 5$), further analyses were carried out separately for each cellular type. The OBs of degus that were exposed four consecutive days to *eucalyptol* (cin/cin group) expressed in all cellular types the same levels of *c-fos* as the group stimulated for four days with pure air (air/air group) (Gr $p = 0.7$; M/T $p = 0.9$; PG $p = 0.55$). Similarly to what was reported by Montag-Sallaz and Buonviso (2002), olfactory novelty (experienced *eucalyptol* after three day of pure air exposure, (air/cin group), only evoked massive levels of expression of *c-fos* in Gr cells (air/cin vs. cin/cin: $W = 0$, $p = 0.02$; air/cin vs. air/air: $W = 36$, $p = 0.002$ Fig. 3.2 A). Figure 3.2 B shows that M/T cells of air/cin group have a tendency to higher levels of *fos* immunoreativity, but this difference is not statistically significant (air/cin vs. cin/cin $W = 29.5$, $p = 0.078$). PG neurons in the other hand were observed to have similar levels of expression for all treatments ($H = 1.15$, $p = 0.28$; Fig 3.2 C).

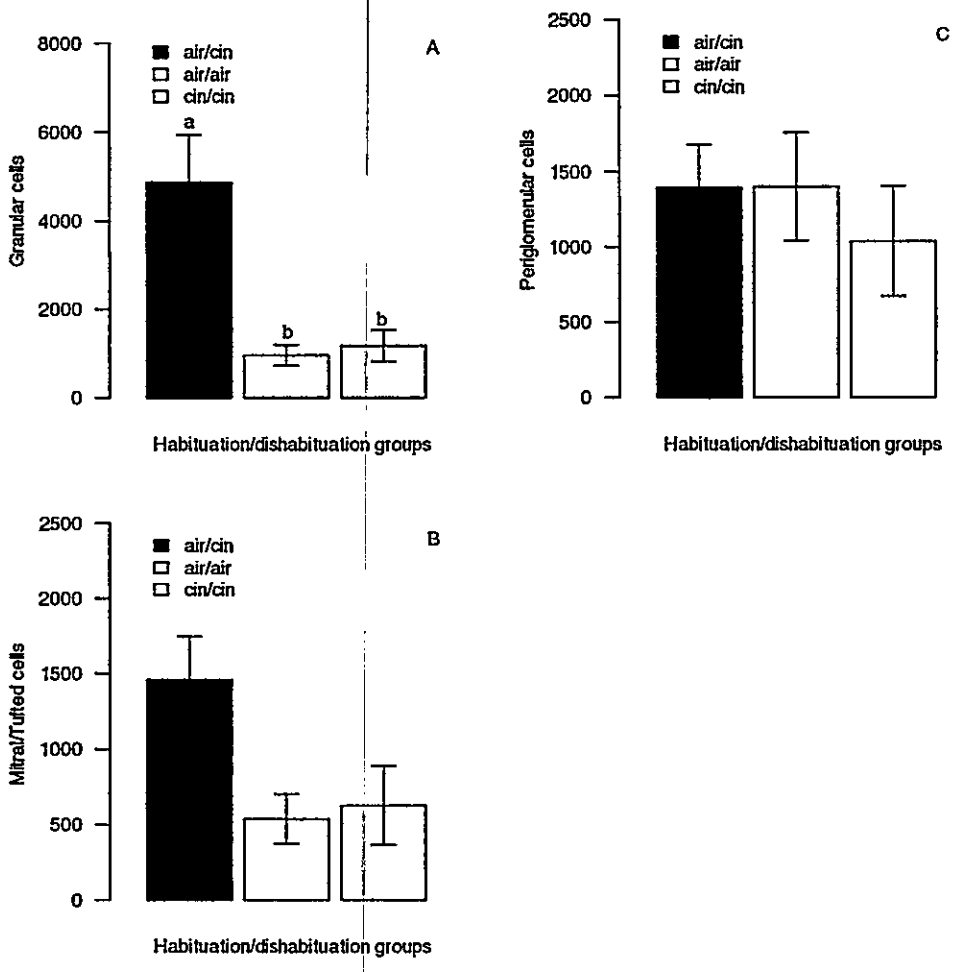

Figure 3.2. Estimated *c-fos* immunoreactive cells (mean ± SE, *n*=5) in the olfactory bulb of adult *Octodon degus*. Animals exposed three consecutive days (habituation trials) to pure air and then to *eucalyptol* (cineole) the fourth day (dishabituation trial) represent the group experiencing a novel stimulus (black). The control group was exposed to pure air in all trials (white), finally the group representing olfactory habituation experienced *eucalyptol* during habituation and dishabituation trials (gray). (A) *c-fos* expression in granular cells, different letter represent statistical significance $p < 0,05$ (see text for exact *p*-value). (B) Mitral/Tufted cells and (C) periglomerular cells.

Conspecifics' odours

Two animals were not considered in the statistical analysis due to immunohistochemical failures and subsequent sample lost. Statistical analyses were

performed for each cellular type due to significant difference of *c-fos* expression between cell types ($H = 58.05$ $p < 0.0001$). Contrary to what was found in the previous experiment, granular cells expressed the same levels of *c-fos* in both experimental groups (Fig. 3.3). Interestingly, expression levels of Fos protein in M/T and PG cells were affected by experimental groups and sex. Female's M/T neurons from $r = 0$ group expressed significantly more Fos than all other groups (females $_{r=0.5\ group}$ $W = 3$, $p = 0.03$; males $_{r=0\ group}$ $W = 64$, $p = 0.003$ and males $_{r=0.5\ group}$ $W = 43.5$, $p = 0.02$). Finally, statistical significant differences of *c-fos* expression in PG cells were observed between the r=0 female group and both male groups (males $_{r=0\ group}$ $W = 62$, $p = 0.005$ and males $_{r=0.5\ group}$ W=30, p=0.016).

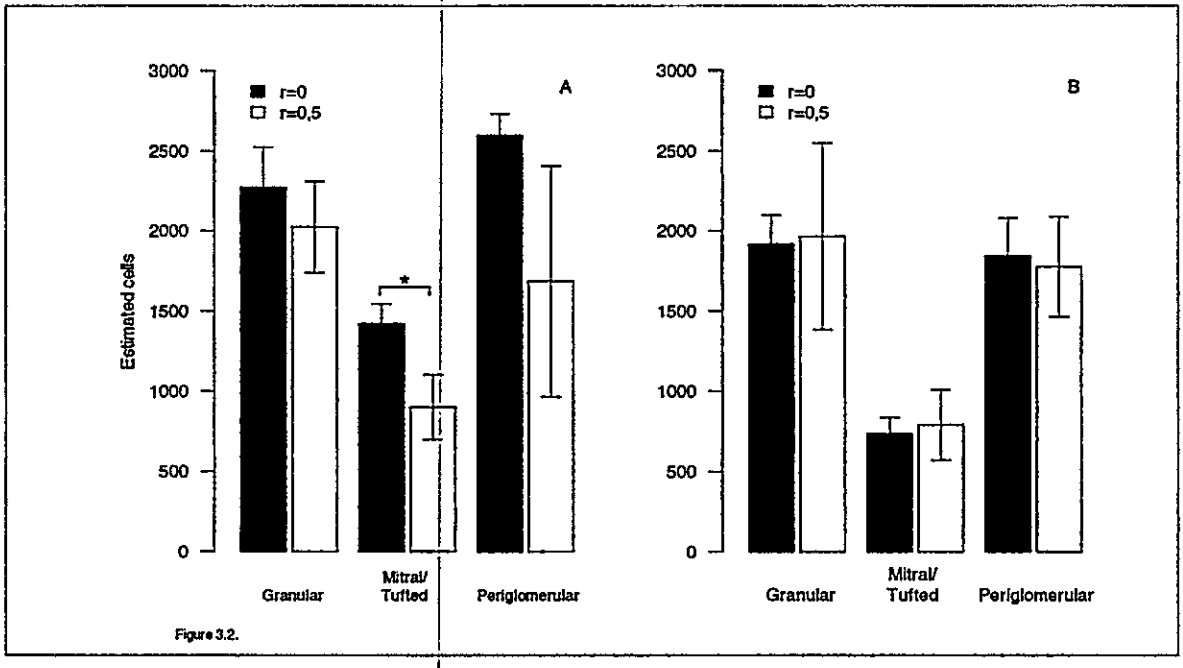

Figure 3.3. Estimated *c-fos* positive marked cells (mean ± SE, n=10) in females (A) and males (B) *Octodon degus'* OB. Subjects were exposed to a non-related conspecific three consecutive days (habituation trails), the fourth day they were expose either to a non-genetically related conspecific (r = 0; black) or to a sibling ($r = 0.5$; white) of the animal used as stimulus in the habituation trials. Asterisk indicates significant difference $p < 0.05$.

DISCUSSION

The present study shows that levels of expression of *c-fos* in the OB of *Octodon degus* evoked by an artificial odorant were influenced by the novelty of the stimulus. Degus naïve to *eucalyptol* (air/cin group) exhibited high levels of *c-fos* expression in Gr cells, while individuals repeatedly exposed to this odour (cin/cin group) showed the same levels of immunoreactivity than the control group (air/air group). Though M/T neurons of naïve animals tended to express more Fos protein, PG cells expressed basal levels of *c-fos* in all treatments. This result is similar to the findings in rats where a novel stimulus evokes higher levels of *c-fos* only in Gr neurons (see Montag-Sallaz and Buonviso, 2002)

Unlike the results discussed above, no difference of *c-fos* expression in Gr cells was found between degus that after consecutive exposure to a conspecific (habituation trials) were exposed to either a sibling or a non-sibling of the stimulus animal experienced earlier. Nevertheless, females whose stimuli pairs were genetically unrelated conspecifics ($r = 0$ group), expressed higher levels of *c-fos* in M/T cells than the $r = 0.5$ female group and also than both male groups. Furthermore, the $r = 0$ female group expressed higher levels of Fos in PG neurons than both male groups. In our experiments animals were exposed to pairs of conspecifics from the same sex, this

might account for the sex differences found in *c-fos* expression, however, a study determining *c-fos* response in each sex to both male and female should be conducted to better address this possibility.

As can be observed in figure 2.3 the OB is a highly layered structure. In the glomerular layer (GL) olfactory sensory neurons (OSNs) and the dendrites from M/T make synaptic contact (Shepherd and Greer, 1998). PG cells are placed around the glomeruli (see Fig. 2.3), their activation result in the inhibition of mitral cells as well as neighboring glomeruli that can be up to 20 glomeruli away (Aungst et al., 2003) M/T cells have also reciprocal synapses with Gr cells in the external plexiform layer (EPL), the activation of Gr neurons inhibit mitral cells (Westecke and M.E, 1970). Centrifugal fibers are found to modulate glomeruli directly in the GL (Pinching and Powell, 1972) and also to modulate Gr-M/T synapses in the EPL (Shepherd and Greer, 1998). Consequently, neuronal activity in the OB is not only the result of sensorial input, distinctive activity patterns rise from a rather complex neuronal circuits where intrinsic and extrinsic components are involved.

Interestingly, both sectioning unilaterally the olfactory peduncle and treating pharmacologically with ß-adrenergic antagonist, reduces drastically *c-fos* levels in the rat OB (Sallaz and Jourdan, 1996). This evidence suggests that *c-fos* expression induced by novel olfactory stimulus in Gr cells is mediated by centrifugal fibers. Consistently, olfactory novelty induces *c-fos* expression in cortical areas receiving input from the OB (Montag-Sallaz and Buonviso, 2002, Illig, 2007).

Here we have presented results that resemble previous studies in other rodent species. However, when animals instead of being exposed to an artificial odour are exposed to a conspecific the findings differ observing changes in other cellular types. This observation could be in part due to differences between stimulus configurations, but most importantly to the difference elicited by the olfactory stimulus due to a particular context. We consider, that *c-fos* quantification can give lights of the role of experience in the activity evokes by odours that are present as part of the environment of individuals.

An example describing the mechanisms underlying social attachment in rats, have shown that plasticity of the OB during early ontogeny is modulated by centrifugal fibers from the *locus coeruleus* (LC) (Sullivan et al., 1986). Somatosensorial stimulation activates the LC in young animals (Shipley et al., 1985, Nakamura et al., 1987, Nakamura and Sakaguchi, 1990). Odours experienced during tactile stimulation or in presence of systemic ß-adrenergic agonists are learned and preferred (Sullivan et al., 1989). Thus, under natural conditions tactile stimulation from the mother can facilitate the learning of olfactory signals present within this social context and might determine behaviours in adults.

We certainly do not know yet how intraspecific interactions are modulated by sensory experience or even by other social interactions, though olfaction is central in many aspects in the way individuals relate to their environment (Gosling, 1987, Hepper, 1991, Raghurm et al., 2009). Understanding the neurobiological mechanisms of olfaction, paying special attention to the circumstances under which neural activity

occurs, can provide another approach in order to elucidate how nervous system structures, olfactory signal and experience are related and can therefore determine social behaviour.

DISCUSIÓN GENERAL

Esta tesis abordó el reconocimiento de parentesco haciendo énfasis en el papel de la familiaridad y del parentesco genético en posibles sesgos conductuales hacia parientes cercanos (i.e., hermanos) (Capítulo I). Además se profundizó en aspectos del aprendizaje temprano de señales olfatorias en dichos sesgos conductuales (Capítulo 2). Finalmente se determinó si la configuración olfativa de individuos emparentados genéticamente es lo suficientemente similar para producir diferencias a nivel de actividad neuronal en el bulbo olfatorio respecto de la configuración olfativa de dos individuos no emparentados genéticamente (Capítulo 3).

Los resultados aquí presentados revelan diferencias conductuales hacia conepecíficos del sexo opuesto mediados principalmente por la familiaridad (i.e., experiencia previa). Individuos expuestos a un hermano y a un no-hermano en el laberinto de *Y* investigaron más cuando ambos eran desconocidos para el sujeto focal, mientras que las parejas de estímulo donde había al menos un hermano familiarizado evocaron un menor interés olfatorio. Además se encontró que las hembras exploraron mayor tiempo que los machos a las parejas de estímulo presentadas. Por otra parte, los encuentros en la arena experimental entre machos y hembras mostraron que el tiempo de exploración entre individuos estaba influenciada por la familiaridad y no

por el parentesco genético, al igual que la conducta de amenaza (i.e., movimiento de la cola) dentro de un contexto antagónicos. Sin embargo, el contacto social fue influenciado por el parentesco genético.

Adicionalmente, los resultados aquí presentados revelan diferencias conductuales entre degus que se desarrollaron en un ambiente social modificado con la presencia de un odorante artificial (*eucalyptol*) e individuos que se desarrollaron en un ambiente olfatorio no perturbado (Capítulo II). Animales sin experiencia previa el odorante *eucalyptol* presentaron conductas neofóbicas hacia el brazo del laberinto de Y que tenía al conespecífico impregnado con dicho odorante, mientras que individuos que habían tenido experiencia con *eucalyptol* durante su ontogenia temprana no presentaron conductas que dieran cuenta de neofobia olfativa. Por otro lado, esta experiencia olfativa también influenció conductas evasivas y de escape de machos naïve frente a conespecíficos impregnados con *eucalyptol* en encuentros de parejas. Hasta ahora estudios de discriminación de parentesco basado en claves olfativas habían probado sesgos conductuales hacia hermanos criados aparte, indicando que en individuos emparentados genéticamente compartirían rasgos fenotípicos que evocarían dichas preferencias conductuales. En esta tesis se ha inducido sesgo conductuales hacia conespecíficos no emparentados ni familiarizados manipulando su entorno social temprano.

El estudio sobre los niveles de expresión del gen temprano *c-fos* reveló que no hay diferencia entre machos que huelen un hermano del sujeto al que fueron habituados (grupo $r = 0.5$) y machos que son expuestos a un no-hermano del sujeto al que fueron

habituados (grupo $r = 0$) (Capítulo III). Interesantemente, el estudio mostró también que el grupo de hembras $r = 0$ mostró mayores niveles de expresión de Fos en células mitrales que hembras el grupo de hembras $r = 0.5$. Estos resultados sugieren que dos hembras hermanas tienen olores similares puesto que no son discriminadas olfatoriamente por una tercera hembra no emparentada con ellas. Los machos en cambio parecieran no encontrar semejantes los olores de dos hermanos no emparentados con ellos. Una debilidad de nuestro experimento fue que los individuos estudiados fueron expuestos a parejas de conespecíficos de su mismo sexo. Por lo tanto, se requerirían experimentos donde se estudie para cada sexo las respuestas en el bulbo olfatorio a conespecíficos de distinto sexo para evaluar un posible sesgo sexual.

En resumen, esta tesis ha demostrado el papel fundamental del aprendizaje temprano dentro de un contexto social en la formación de sesgos conductuales en *Octodon degus* y también sugiere que individuos emparentados comparten señales olfativas distintivas. Además, los resultados apoyan otros estudios que evalúan la importancia del aprendizaje en la ecología y reconocimiento de recursos en esta especie (Vasquez et al., 2006).

Finalmente, los resultados aquí presentados complementan el estudio de Villavicencio et al. (2009) donde no se pudo descartar un efecto de parentesco genético en el reconocimiento de parentesco y donde se encontró que gran parte de los sesgos conductuales observados eran explicados por familiaridad. Sin embargo, debido a la naturaleza de la crianza cruzada donde los individuos crecían con

hermanos y no hermanos, no se puede descartar que el efecto observado del parentesco genético en el tiempo de asociación en encuentros macho-hembra sea debido al efecto del aprendizaje de señales características de los hermanos con quien compartió.

Considerando además que en la naturaleza, las hembras *O. degus* con distinto grado de parentesco pueden utilizar madrigueras comunalmente y que los encuentros agresivos entre hembras que comparten una madriguera son muy raros (menos del 3%) (Ebensperger et al., 2004), proponemos que el reconocimiento de parentesco en degus es el resultado de compartir un ambiente común durante el desarrollo temprano. Además, si animales genéticamente emparentados comparten señales olfatorias particulares, se reforzará el aprendizaje de dicha señal. Si bien es muy probable es que dentro del ámbito de hogar de los degus, particularmente en el caso de las hembras, convivan parientes (Ebensperger et al 2004), en eventuales encuentros entre parientes sin previa familiaridad, podrían ocurrir sesgos conductuales mediados por señales olfatorias compartidas.

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