PhD Program in Neuroscience

# *Novel competition test for food rewards reveals stable dominance status in adult male rats*

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*Doctoral Thesis presented by*

Diana Paula Ferreira da Costa

**Thesis Director:**

**Cristina Márquez Vega**

Universidad Miguel Hernández de Elche

- 2022 -

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# DOCTORAL THESIS BY COMPENDIUM OF PUBLICATIONS

To whom it may concern:

The doctoral thesis entitled: *“Novel competition test for food rewards reveals stable dominance status in adult male rats”,* has been developed by myself, Diana Paula Ferreira da Costa. This thesis is presented as a compendium of publications and includes the following publication in which I am the first author:

- *Costa, D.F., Moita, M.A. & Márquez, C. Novel competition test for food rewards reveals stable dominance status in adult male rats. Sci Rep* ***11,*** *14599 (2021).*

  **PMID**: 34272430

  **DOI:** 10.1038/s41598-021-93818-0

I declare that the publication has not been used and will not be used in any other thesis in agreement with my thesis director Cristina Márquez Vega.

Yours sincerely,

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Dr. *Cristina Márquez Vega* Director of the doctoral thesis entitled *“Novel competition test for food rewards reveals stable dominance status in adult male rats”*

**INFORM/S:**

That Mrs. *Diana Paula Ferreira da Costa* has carried out under our supervision the work entitled *“Novel competition test for food rewards reveals stable dominance status in adult male rats*" in accordance with the terms and conditions defined in her Research Plan and in accordance with the Code of Good Practice of the University Miguel Hernández of Elche, satisfactorily fulfilling the objectives foreseen for its public defense as a doctoral thesis.

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I sign for appropriate purposes,

at Sant Joan dÁlacant, 12 of  May of 2022

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**Universidad Miguel Hernández**
Instituto de Neurociencias

**Dra. Elvira de la Peña**
Coordinadora del Programa de Doctorado en Neurociencias

A la Comisión de Estudios de Doctorado de la UMH;

Reunida la CAPD de Neurociencias,

CERTIFICA: que la Tesis Doctoral titulada "Novel Competition test for food rewards reveals stable dominance status in adult male rats "

Ha sido realizada por D. DIANA PAULA FERREIRA DA COSTA, bajo la dirección de la Dra. CRISTINA MÁRQUEZ VEGA y que reúne los requisitos necesarios para su lectura y defensa.

En caso de que la lectura y defensa tuviera que ser telemática, esta comisión da su conformidad.

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Por lo que doy mi conformidad para que sea presentada a la Comisión de Doctorado de la Universidad Miguel Hernández.

San Juan de Alicante, 18 /05/2022

Dra. Elvira de la Peña García
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To whom it may concern:

The doctoral thesis entitled: *“Novel competition test for food rewards reveals stable dominance status in adult male rats”,* has been developed by myself, Diana Paula Ferreira da Costa. This thesis is presented as a compendium of publications. It is based on experimental studies undertaken at the Instituto de Neurociencias UMH-CSIC during the PhD program in Neuroscience of the Miguel Hernández University. This work has been partially supported by:

- Formación del Personal Investigador (FPI) grant with reference **BES-2016-076747** and a CSIC-Severo Ochoa grant of the Instituto de Neurociencias UMH-CSIC with reference **SEV-2013-0317-16-2**.

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# Acknowledgments

This monograph is the culmination of the work developed during my PhD in the Neural circuits of social behavior Lab. I am extremely grateful to several people who, directly and indirectly, helped me along this journey:

I want to thank my PI, Cristina Márquez. First and foremost, all the trust she placed in me when she asked me to follow her to a different country to do my PhD in her new lab. Starting a new project is both an exciting and challenging venture, so having someone that always trusted me to overcome wherever challenges a PhD can throw at you was always an important source of support. During these 9 years that we have been working together (almost a third of my life!), she always believed (often more than I did) in what I was capable achieving and to me that made all the difference.

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However, a laboratory is much more than a PI and I was lucky enough to have an exceptional group of people around me. To be surrounded by a group of people with such generosity, that supported me in all my (often crazy) ideas, and above all that were always present in the not so good moments of my PhD journey was truly something I'm extremely grateful for and always made me feel less far away from home. In particular, I want to thank Miki for his contagious good energy and for showing me that everything can have a positive side. Kevin for everything he taught me, for his support whenever it was needed, for his unforgettable Halloween parties and for being the best landlord ever (wink wink). To Adele and Helena (our "Pókemons") for their friendship, good disposition and for making me feel that I actually know what I'm doing most of the time (even when that's not really the case) Two very kind souls that brought a freshness of joy to our lab. I think that Adele's voice still echoes in the institute though! completely different personalities but with one thing in common, a huge curiosity and eagerness to learn. It was a real pleasure to share with you everything I know, specially to Helena who spend her time with me, side by side in my last month at the institute. And of course, to Joan Adrian the Drama Queen Esteve Agraz. None of this would have been the same without you and without your support, this PhD would probably not be a thing. You are the true righteous heir of my throne (desk, chair and everything that comes with it!) and I know that what I left unfinished couldn't be in better hands (good luck though). By the way, I'm still waiting for the trip to the *teteria...*

Being a scientist in Spain involves a lot more than siestas and eating paella, and I was extremely fortunate to be able to spend a good chunk of my time building all my behavioural setups from scratch. This, however, would not have been possible without the help of the staff from the Hardware platforms at the Champalimaud Foundation and Instituto de Neurociencias. A huge thanks to all of them and in particular to Victor Rodriguez for his patience and generosity in everything he taught me.

I also have to thank all the staff of Champalimaud Foundation *vivarium* and the technicians of the Instituto de Neurociencias for taking care of our animals and being available to help whenever needed. Without them I wouldn't have been able to do any of my experiments.

In my way of looking at science, I believe that collaborations are the way to go and unfortunately there are places that still show some resistance. However, I also believe that change can be made with small steps and luckily the Institute of Neurociencias has the privilege of having people guided in the right way. For this reason, I want to thank everyone at the Instituto de Neurosciencias who supported me and showed me that it is possible to do things in a different way. In particular, I would like to thank those that joined me as representatives of the PhD students and also Virtudes, for all her help with the fascinating world of Spanish bureaucracy.

A very special thanks to the "Brain Addicts", a group of very geek neuroscientists who quickly became good friends (even Adam!). Without you, my lunch hours would have been extremely boring. A special thanks to Elena, Maria and Raquel, you three are cool af. You are an example of intelligence, determination, and perseverance, but most important of true friendship, which I will carry for life.

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Throughout my decades of existence, I have had the fortune of meeting some wonderful people that I have religiously kept as close friends. Even though they were far away, you always found ways to be present and thus become a pillar in my life. I would have to write a whole new thesis just to thank you properly and as you know, writing is not my strong point. Having said that, I leave you the most clichéd acknowledgment in history of all acknowledgments: To all my friends, you know who you are, and a heartfelt thank you to Eli, Raqs and Oásis because #estátudovivo.

Finally, an enormous thank you to my family. To my parents for their unconditional support and for giving me the opportunity to pursue my goals, being well-aware of the uncertainty that comes hand in hand with a career in scientific research. I also want to thank the best brother I have. Every day I got a phone call from him to ask me if everything was okay (probably because no one wanted to put up with him), and even when my mood wasn't the best, he always managed to make me laugh. To my borrowed uncles, for whom I have the greatest affection in the world, Tio Álvaro, Tia Fátinha, Eduardo and Nené. For the love they have always showed me and for being proud of everything I did (even though they have no idea what it actually was most of the time).

And last, but definitely not the least, a very special thanks to Doutor Tozé and Aurizita. To Tozé for always being present and for his (often unrelentless) critical sense that always pushed me to do my best (even if he often had to fight through some very mild stubbornness…). For this, his patience (his overall very peculiar Zen approach to things actually), and a lot more that I don't know how to properly put into words, a BIG thank you. To Auri, for all the joy and happiness that she showed me whenever she saw me. Kevin's Halloween parties were good, but they pale in comparison to the summer festivals that Auri would throw every time I got home after months of being away. It is truly unfortunate that she also does this every time she sees a tennis-ball though…For all her shenanigans, the warmness she brings to my heart (and all other body parts in the winter) and being the sweetest dog in the world: Thank you!

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# Abstract

Social hierarchy is a potent modulator of behavior in many species including humans, which provides social structure and minimizes energy directed to agonistic interactions between individuals in a group. Thanks to recent work in rodents, we are now starting to understand the neural circuits responsible of the establishment of dominance behavior. Most of the available tasks for identification of hierarchy in rats rely on the quantification of agonistic interactions when unfamiliar animals, usually in deprived states or in isolation, need to compete for a resource that is scarce or to defend a territory. However, the establishment of a new hierarchy might not relay in the same neurobiological mechanisms than those maintaining stablished hierarchies between stable pairs of animals.

Here we present and validate a novel trial-based dominancy assay, the modified Food Competition test, where established social hierarchies can be identified in the home cage of non-food deprived pairs of male rats living together. In this task, we introduce a small conflict in the home cage, where access to a new feeder containing palatable pellets can only be gained by one animal at the time. This subtle conflict does not evoke aggressive encounters during the task, however induces interesting social dynamics while animals are gaining access to the positive reinforcers and results in asymmetric consumption favoring one of the animals of the pair. Detailed behavioral quantification of behaviors displayed in

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this task was performed and reliability as well as stability of social hierarchies was assessed by comparing to behaviors displayed in other social tasks where animals competed for access to sucrose solution, or to more standardized procedures, such as a food and water competition under deprivation states or the tube test.

We thus propose the modified Food Competition test as a robust and easy to implement tool for the evaluation of established social hierarchies in rats that will enrich and diversify the functional task available to study dominance in laboratory rats.

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# Resumen

La jerarquía social es un potente modulador del comportamiento en muchas especies, incluidos los humanos, que proporciona una estructura social y minimiza la energía dirigida a las interacciones agonísticas entre los individuos de un grupo. Gracias a trabajos recientes en roedores, estamos comenzando a identificar los circuitos neuronales responsables del establecimiento del comportamiento de dominancia. La mayoría de las tareas disponibles para la identificación de jerarquías en ratas se fundan en la cuantificación de interacciones agonísticas cuando animales no familiares, generalmente aislados o en estados de deprivación, necesitan competir por un recurso escaso o para defender un territorio. Sin embargo, el establecimiento de una nueva jerarquía podría no depender de los mismos mecanismos neurobiológicos que los que mantienen jerarquías establecidas entre pares estables de animales. El conocimiento actual en el campo se centra exclusivamente en los mecanismos del establecimiento de jerarquías, básicamente debido a una falta de paradigmas conductuales que permitan evaluar dominancia una vez ya es estable.

En esta tesis presentamos y validamos una nueva tarea de dominancia, la prueba de competición por comida, donde se pueden identificar jerarquías sociales establecidas entre parejas de ratas macho no privadas de alimentos, mientras viven en sus jaulas. En esta tarea, introducimos un pequeño conflicto en la jaula de las ratas, donde el acceso

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a un nuevo comedero que contiene recompensas de comida sólo puede ser conseguido por un animal a la vez. Este sutil conflicto no produce encuentros agonísticos durante la tarea, sin embargo induce interesantes dinámicas sociales mientras los animales acceden a los reforzadores positivos, que resulta en un consumo asimétrico que favorece a uno de los animales de la pareja. Realizamos una cuantificación conductual detallada de los comportamientos manifestados en esta tarea y evaluamos la fiabilidad y estabilidad de las jerarquías sociales observadas comparándolas con los comportamientos mostrados en otras tareas sociales en las que los animales compiten por el acceso a una solución de sacarosa, o con procedimientos más estandarizados, como el acceso a comida o agua en estados de privación o la prueba del tubo, esta última ampliamente utilizada en ratones de laboratorio.

Proponemos así esta prueba de Competición por comida como una herramienta robusta y fácil de implementar para la evaluación de jerarquías sociales establecidas en ratas que enriquecerá y diversificará las tareas funcionales disponibles para estudiar la dominancia en ratas de laboratorio.

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# Nomenclature

**blA** - Basolateral Amygdala

**CI** - Conflict Index

**CRI** - Conflict Resolution Index

**D** - Dominant

**DFC** - Diana Ferreira da Costa

**DI** - Dominance Index

**dlPFC** - Dorsolateral Prefrontal Cortex

**DRN** - Dorsal Raphe Nucleus

**fMRI** - Functional magnetic Resonance Imaging

**GR** - Glucocorticoid Receptor

**HPA** - Hypothalamic-pituitary-adrenal

**MDT** - Medio-dorsal Thalamus

**mFCD** - Modified Food Competition with food Deprivation test

**mPFC** - Medial Prefrontal Cortex

**mRNA** - Messenger Ribonucleic Acid

**NAc** - Nucleus Accumbens

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**OFA** - Oncins France Strain A

**mFC** - Modified Food Competition test

**PFC** - Prefrontal Cortex

**S** - Submissive

**SC** - Sucrose Competition test

**SCI** - Sucrose Competition with Intermittent access to resources

**TT** - Tube Test

**VBS** - Visible Burrow System

**VS** - Ventral Striatum

**WC** - Water Competition test

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# 1 | Introduction

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# Chapter 1 | Introduction

## 1.1 Social Behavior

We define as "social" all the interactions that occur between individuals. These interactions can be either agonist, common whenever there is competition for resources, or affiliative, if they bring benefits for both parties. The type of interactions that are established between individuals are influenced by multiple factors, such as the animal's age, sex, internal state and memories from previous interactions. If these interactions happen on a regular basis, a social relationship can be established (Scott, 1956).

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Some species form groups which may have a more or less permanent nature. Being part of a group provides social animals ample opportunity to use the information/cues provided by others to help guide their behavior. Living with others has costs and benefits, however animals can adapt their behavior to counter-weigh the costs of group living. Sharing a communal living space with others may increase the transmission rate of diseases and parasites however, some species such as impalas and non-human primates perform reciprocal allogrooming to lower parasite transmission and consequently spreading of diseases (Hart and Hart, 2018). In cases in which the shared space is restricted, living in groups intensify competition for resources, such us space, food or females. However group living will also improve the foraging proficiency of the group. By cooperating, animals will be better at find, collect and defend their food resources. Another example of the benefits of group cooperation is the cooperative breeding observed in some species of birds, fishes and mammals in which members of a group other than the actual progenitors, cooperate to raise the offspring (Koenig, 2017) increasing their chances of survival.

Importantly, communal living provides benefits such as protection and interactions that to our eyes might appear meaningless, such as those observed in some populations of chimpanzees who groom each other while holding hands (McGrew and Tutin, 1978), which may play an important role in group cohesion by reinforcing affiliative interactions (Fig.1.1).

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Fig. 1.1| **Chimpanzees showing Grooming hand-clasp behavior.** Each individual extends an arm overhead and then either one clasps the other's wrist or hand, or both clasp each other's hand and start grooming of the other.

However, behaviors displayed at an inappropriate time or with disproportionate intensity can have negative effects not only for the interacting individuals but also in the cohesion of the group. Adapting behavioral responses depending on the social status of the interacting partner can be cost-effective, but in some cases, a very adaptive strategy to survive. This organized social structure is commonly known as social hierarchy.

## 1.2 The Norway rat

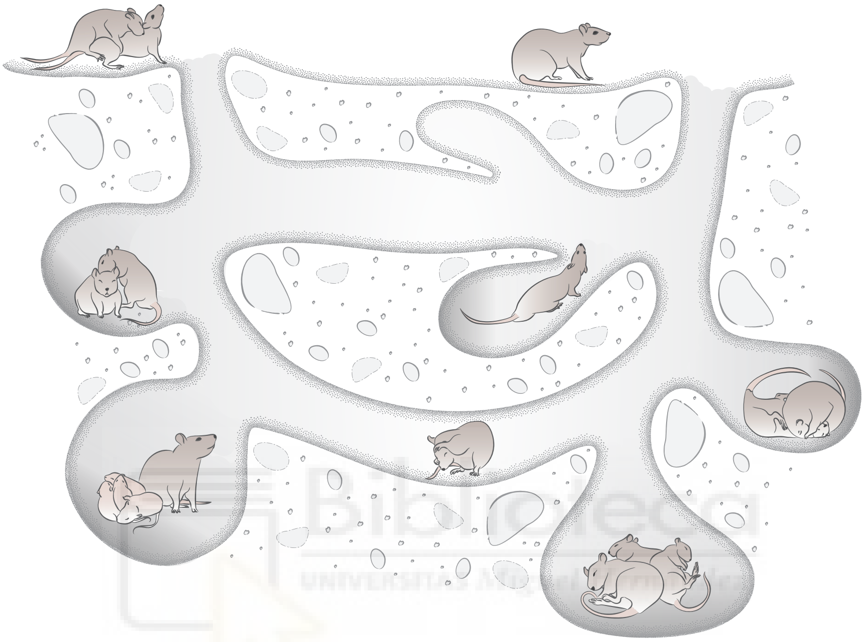

Fig. 1.2| **Social organisation of a rat colony.** In the wild, the Norway rat dig burrows and build extensive systems of tunnels and passages where they usually live in groups and reproduce. Juvenile rats tend to engage in play-fighting which can involve a combination of behaviors such as jumping and chasing, however, if living space becomes limited, rats may turn to aggressive behavior. Rats in the same group commonly groom each other and sleep together to keep warm. *Adapted from Schweinfurth,2020*

Norway rats live in complex social groups in the wild (Fig.1.2) and recent laboratory studies are placing this species as an excellent animal model for the study of complex social behaviors, displaying highly sophisticated social skills (see (Schweinfurth, 2020) for review), with the added value of being a model system amenable for neural circuits manipulations. We know now that rats display affective reactions to the distress of conspecifics (Scheggia et al., 2019; Knapska et al., 2006, 2010; Atsak et al., 2011), and that they can vicariously learn from others the imminent threat of a danger (Pereira et al., 2012; Pereira, Farias, and Moita, 2020; Cruz et al., 2020; Andraka et al., 2021). This vicarious fear learning is potentiated by previous experience with the threat (Atsak et al., 2011; Pereira et al., 2012) where auto conditioning of the self-experience is then recognized as alarm cues in the behavior of others (Cruz et al., 2020), in an exquisitely dynamic manner, where behavior of both animals feedback into each other (Han et al., 2019). Interestingly, this aversive emotional contagion leads rats to perform actions to avoid the pain of others (Hernandez-Lallement et al., 2020).

The ability to perceive, react and decide upon information from conspecifics is not only observed in threatening-aversive contexts but, although less studied, mounting evidences show that rats do also perceive appetitive states from others (Kashtelyan et al., 2014), have been shown to cooperate in instrumental tasks testing for coordination (Daniel, 1942; Conde-Moro et al., 2019; Conde-Moro et al., 2022), direct and generalized reciprocity (Rutte and Taborsky, 2007, 2008) and to possess the cognitive capacity to engage in cooperation in the context of social dilemma games (Viana et al., 2010). Rats also perform prosocial actions to benefit conspecifics in aversive (Bartal, Decety, and Mason, 2011; Bartal et al., 2014, 2021) and appetitive situations, providing food to their partners (Márquez et al., 2015; Kentrop et al., 2020; Hernandez-Lallement et al., 2015; Gachomba et al., 2022) but are also able to precisely select when they want to engage in competition for food with others (Hillman and Bilkey, 2012).

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This new wave of studies that leverage on the design of new behavioral paradigms that embrace the complexity of social cognition and provide new knowledge on social behavior beyond the pure social investigation, have confirmed the richness of the social behavioral repertoire of the Norway rat, and are starting to allow for the dissection of the neural circuits underlying these processes (Hillman and Bilkey, 2012; Carrillo et al., 2019; Pereira, Farias, and Moita, 2020; Twining et al., 2017; Hernandez-Lallement et al., 2020; Andraka et al., 2021). A fundamental question now is how different brains will differently process and react to social information. Understanding the mechanisms of individual differences in social relations is key not only to further identify the factors that are relevant in normal healthy social behavior, but also to understand what can go wrong in the diseased brain.

Therefore, identifying how individuals tune their responses depending on the quality or quantity of the cues perceived from others would be of high interest. It is very likely that factors such as the social status, a major modulator of behavior that social animals use to structure in groups, might be playing an important role.

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## 1.3 Social Hierarchy

Social hierarchy is a multidimensional trait which has profound impact on emotion and cognition, not only for humans (Sapolsky, 2004; Cruz et al., 2018) but also other social species (see (Sapolsky, 2005) for review), having important consequences for social organization, survival, reproductive success and health of animals in a group.

One of the first attempts to study social hierarchy systematically was originally led by the Norwegian zoologist and comparative psychologist Thorleif Schjelderup-Ebbe. By the age of 10 he developed a special interest in domestic fowls and kept tracking patterns of behaviors in detailed annotations for many years until he published his work in 1922.

As a description of the social organization of the domestic fowl, he introduced the concept of the "peck-order" in which reliable patterns of agonist behaviors between two individuals were described that consistently favored access to resources of one animal of the pair. The highest-ranking fowl – despot – pecks and is not pecked back by the so called subordinate. This rank remains steady until the despot's position is defied by another individual in a combat. He proposed that such hierarchical organization reduces intense conflicts, saves energy, and promotes social stability (Schjelderup-Ebbe, 1922). This "pecking order" concept was introduced into the field of behavioral sciences known today as "social dominance".

Following these pioneers observations, the concept of social dominance immediately became object of study in many species. In 1939, Meredith Crawford published that social dominance had been studied in all vertebrates except fish and amphibia (Crawford, 1939) and later, several studies bridged that gap by incorporating not only fish (Braddock, 1945; Grosenick, Clement, and Fernald, 2007) and amphibia (Boice and Witter, 1969) but invertebrates too (Ewing, 1972; Cole, 1981).

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## 1.4 Measures of dominance hierarchy in laboratory rodents

The most established view is that social hierarchy is built upon aggressive interactions (see (Drews, 1993) for conceptual review), and that they are established as a mechanism for control of resources and minimization of energy expenditure by groups of animals, as once a hierarchy is established, the priority access to resources is organized and aggressive levels between the interacting animals are reduced (Vessey, 1981). Following this view, most of the behavioral paradigms available for measuring social hierarchy in laboratory animals are based in agonist interactions while defending access to resources, whether a sexual partner, food or water when they are scarce or the defense of a territory. Although classical studies were focused in rats, there are many recent alternatives to test for establishment of social hierarchies in mice, as a reflection on how the field is favouring the use of this species.

The **tube test** (Fig.1.3A), first developed in 1961 as a test to measure defense of a territory (Lindzey, Winston, and Manosevitz, 1961) and recently validated as a task that can evaluate the linearity and stability of social rank in mice (Wang et al., 2011), has become a potent test to measure hierarchy, both for the easiness of implementation, but specially because it reliably identify social rank when compared to other behavioral paradigms measuring social hierarchy in mice (Wang et al., 2011; Wang, Kessels, and Hu, 2014).

In this paradigm, each animal is placed at opposite ends of a tube divided in the middle by a transparent partition. Once the animals meet in the middle, the partition is removed, and the animals will fight for possession of the tube. The one who expels the partner from the tube is considered the winner of the trial. The tube test has also recently started to be used in rats to identify hierarchy in large groups or unfamiliar animals, with conflicting results (Cao et al., 2017; Jupp et al., 2016; Saxena et al., 2018). However, ecological differences between species should be taken into account when studying social behavior in different species, as rats and mice clearly differ in their social structure in the wild, where rats live in larger groups and tolerate better the presence of other in-group males, compared to mice (Schweinfurth, 2020).

**Whisker and/or hair barbering** (Fig.1.3B), commonly seen in laboratory rodents (Beare-Rogers and McGowan, 1973; Strozik and Festing, 1981; Gerold et al., 1997) is often used as another strong correlate of hierarchy in mice (Strozik and Festing, 1981; Kalueff et al., 2006; Wang et al., 2011). Excessive barbering occurs predominantly in submissive animals, either by disproportionate levels of self-grooming or by excessive grooming from more dominant males. Importantly, studies have found that the hierarchy status asserted by the degree of barbering correlates with that of the tube test (Wang, Kessels, and Hu, 2014; Wang et al., 2011; Zhou, Sandi, and Hu, 2018).

Sexually inexperienced male mice dominant in the tube test have also shown to emit significantly more 70kHz **ultrasonic vocalizations** when in the presence of a female stimulus compared to the subordinates (Wang et al., 2011; Nyby, Dizinno, and Whitney, 1976; Catanzaro and Ngan, 1983; Zhou, Sandi, and Hu, 2018) (Fig.1.3C). Ultrasonic vocalizations are produced by rodents and depending on their developmental stage might be involved in different aspects of social behavior such as parent-offspring interactions, courtship/matting behavior, aggression, alarm and territoriality (Nyby, Dizinno, and Whitney, 1976). It has been described that adult males begin emitting 70 kHz ultrasonic vocalizations in response to female stimulus while performing behaviors correlated with strong sexual motivation (Nyby, Dizinno, and Whitney, 1976).

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Another common test used to access dominancy is the **urine marking assay**. Amongst many mammalian species, animals use the chemical components of their urine to communicate with other individuals, giving of information about, for example, their health status, territoriality or receptivity (Ralls, 1971). The frequency and pattern of urine marking is strongly dependent of dominance status (Desjardins, Maruniak, and Bronson, 1973; Ralls, 1971; Wang et al., 2011; Zhou, Sandi, and Hu, 2018). In the urine marking assay, two male mice that have been previously isolated are paired together and, after a series of aggressive encounters, the dominant male covers the entire cage floor with drops of urine. On the other hand, subordinate males only leave pools of urine in the corners of the box (Desjardins, Maruniak, and Bronson, 1973; Wang et al., 2011; Zhou, Sandi, and Hu, 2018) (Fig.1.3D).

The **visible burrow system (VBS)** (Fig.1.3E), initially developed for rats, has been widely used in rodents to study formation of hierarchies in large groups of animals (Blanchard and Blanchard, 1989; Blanchard et al., 1995; Adams and Boice, 1989; Arakawa, Blanchard, and Blanchard, 2007; Wang et al., 2011; Davis et al., 2009). In mice, dominance in the VBS was found to be significantly correlated with rank in the tube test (Wang et al., 2011; Zhou, Sandi, and Hu, 2018). In this task, mix-sex groups living in a complex environment where food and water are difficult to access, compete chronically for territory and resources, facilitating the emergence and maintenance of social hierarchy, which is easily detectable by the wounds and severe weight loss of subordinates.

These studies using the VBS have provided strong evidence that body weight of the animals is a good indicator of social status, being bigger individuals the dominant ones in a group. However, this finding must be placed into the ecological context of this task, as these conditions are not the routine of most of laboratory studies where animals live in standard cages with free access to *ad libitum* food for most of their lives. Although the correlation of high rank and increased body weigh is a widely spread dogma, whether it can be generalized as a good indicator of social hierarchy in non-deprived animals that live in stable hierarchies is still an open question. The VBS generates very rich behav-

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ioral data sets but is difficult to implement in most laboratories, hence, other behavioral tasks are commonly used, where animals **compete for food** or **water** under deprivation states (Fig.1.3F).

The study of social dominance in pairs of rats has enormously benefitted by the use of these previously mentioned tasks (Timmer et al., 2011; Cordero and Sandi, 2007; Timmer and Sandi, 2010), where competition typically occur in neutral arenas or contexts that induce conflict between the pairs of animals. In all these tests, social isolation is performed prior to testing with variable durations as a mean to increase territoriality, favoring strong agonist interactions during the establishment of new hierarchies. Importantly, the development of these behavioral tools has been key in the advancement of the identification of the neural substrates of social rank and the study of how social status might be modulating cognitive function (Hollis et al., 2015; Timmer and Sandi, 2010; Timmer et al., 2011; Zhou, Sandi, and Hu, 2018).

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All the above mentioned tasks are thus evaluating how a new hierarchy is established between pairs of unfamiliar, frequently isolated animals, in neutral arenas to increase territoriality, where agonist behaviors performed to establish dominancy are very evident. However, the establishment of social hierarchy might not rely on the same mechanisms than the expression of dominancy when a hierarchy is already established. Indeed, recent reports indicate that this is the case in mice (Pallé et al., 2019, 2020).

To our knowledge, the study of the possible differences between the establishment and established social hierarchies in rats has been virtually unexplored. There is thus a need for new behavioral paradigms that evaluate social status of familiar animals living in stable dyads, that could allow to assess the effects of stable social rank in sophisticated cognition in rats, to understand how animals tune their social behaviors according to the rank of the interacting animals. Preferably, the evaluation of the hierarchy would not imply manipulations that could alter internal state in a long-term manner and affect how social interactions of interest are displayed. This is a challenge, as once hierarchies are established, agonist behaviors are minimized and the opportunities to observe dominance interactions are reduced and might be subtle.

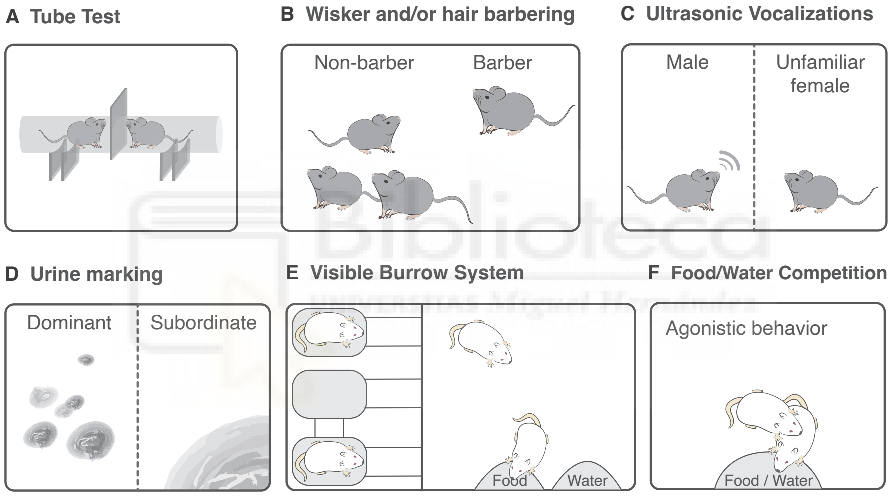

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Fig. 1.3| **Measures of dominance hierarchy in laboratory rodents.** **(A)Tube Test:** each animal is placed at opposite ends of a tube will fight for possession of it. The animal who consistently expels the partner from the tube is considered the dominant. **(B)Wisker and/or hair barbering test:** excessive barbering occurs predominantly in submissive animals, either by disproportionate levels of self-grooming or by excessive grooming from more dominant males. **(C)Ultrasonic vocalizations measurements:** quantification of 70kHz ultrasonic vocalizations emitted by males when in the presence of a female stimulus. According to previous studies, dominant male mice emit significantly more 70kHz ultrasonic vocalizations when in the presence of a female stimulus compared to subordinates. **(D)Urine marking assay:** two male mice previously isolated are paired together and, after a series of aggressive encounters, the dominant male covers the entire cage floor with drops of urine. On the other hand, subordinate males only leave pools of urine in the corners of the box. **(E)Visible Burrow System:** Study formation of hierarchies in large groups of animals. Animals compete for territory and limited resources, facilitating the emergence and maintenance of social hierarchy, which is easily detectable by the wounds and severe weight loss of subordinates. **(F)Food/Water Competition tests:** Animals are placed in neutral arenas and compete for food, water or palatable rewards. Measurements of agonist behavior are taken into account to determine social hierarchy. Adapted from *Wang et al.,2011*

## 1.5 Neural mechanisms of social dominance

Following Schjelderup-Ebbe's original descriptions on the establishment and maintenance of social hierarchies, recognizing where we position ourselves relative to others is essential for establishing social roles and creating successful social interactions such as avoidance, cooperative, aggressive or mating behavior. The complexity of those interactions begs the question if the brain processes social information and makes social decisions in a distinctive way and if different individuals will differently process social information. Unfortunately, most of the literature regarding the neural circuits of social behavior are obtained using basic behavioral tasks that only allow to understand how animals investigate others vs inanimate objects, or how they do discriminate a familiar vs unfamiliar animal. However, over the last years, there has been an effort in the field to increase the diversification of behavioral paradigms that will allow to further understand all the complexities of social behavior, that go beyond social interactions.

Several brain regions have been implicated in how an animal reacts during an interaction between a conspecific and recent studies have shown that when mice have social interactions with a novel conspecific, groups of cells from different neural circuits are activated to create a representation of a social memory – the social engram (Okuyama,

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2018; Okuyama et al., 2016; Hitti and Siegelbaum, 2014). In a future encounter with the same conspecific, this social memory engram is activated to recover the memory of this familiar social interaction. Importantly, a nonfamiliar interaction does not triggers this social memory retrieval (Okuyama, 2018).

Recent work hypothesizes that this social engram is formed in the hippocampus during sleep between the CA2 and CA1 layers and the information travels to other regions such as nucleus accumbens (NAc) and basolateral amygdala (blA) (Oliva et al., 2020; Okuyama, 2018). However, apart from recognizing another individual by retrieving a social memory, one should also be able to position himself regarding its social status which might involve different neural circuits.

Over the last years, a greater number of studies have suggested that the **prefrontal cortex** (PFC) has been involved in recognition and establishment of social hierarchies (Zink et al., 2008; Wang et al., 2011; Zhou et al., 2017; Davis et al., 2009).

A study in humans using functional magnetic resonance imaging (fMRI) technique, exhibited evidence that **dorsolateral prefrontal cortex** (dlPFC) is recruited when the participants performed a discrimination task while observing high and low-ranking players with a stable hierarchy. They also observed that additional regions such as **amygdala** and **medial prefrontal cortex** (mPFC) are specifically recruited while performing a similar task but in a context of unstable hierarchy (Zink et al., 2008). In a different study performed in non-human primates, researchers observed that high-ranking monkeys became completely submissive after lesions in the amygdala (Rosvold, Mirsky, and Pribram, 1954). The structural organization of PFC shows enormous variation across species. In rodents, the functional homologous region to the dlPFC and mPFC described in the earlier studies correspond to the dorsal mPFC (Uylings, Groenewegen, and Kolb, 2003; Franklin and Chudasama, 2012). Growing evidence have been pointing the mPFC as one of the areas linked to social hierarchy (Wang et al., 2011; Zhou et al., 2017; Davis et al., 2009).

As previously mentioned, in many species social hierarchy is established after individuals engage in a series of aggressive episodes. This behavior can be disadvantageous not only because they can, most probably, cause physical harm to those involved but can also draw unwanted attention from foreign competitors and put the group at risk (Hand, 1986). Therefore, a certain degree of risk-taking is necessary for the development of social hierarchies. However, the risk of engaging in these aggressive interactions will be outweighed by the benefits of establishing a hierarchical relationship. A study in rats using the VBS system to access the dominance status in the colony have shown that dominant individuals were more risk-takers and display an increased motivational state for food rewards. This evidence was supported by the evidence that same individuals presented increased orexin receptor mRNA in the mPFC (Davis et al., 2009). The hypothalamic orexin system was previously reported to stimulate synapses in the mPFC consequently enhancing cognitive performance (Lambe et al., 2005). More recently, an optogenetic study in mice led by Zhou and colleagues revealed that activation of **dmPFC**, specifically the synaptic input from the **mediodorsal thalamus (MDT)-dmPFC** circuit, induces winning while the animals were performing the Tube Test hierarchy assessment task (Zhou et al., 2017).

New studies in both humans and rodents have been also appointing **ventral striatum** (VS), more particularly the NAc as a brain area involved in the early stages of establishment of social hierarchies (Zink

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et al., 2008; Hollis et al., 2015). After being reported in humans an increased ativation of VS when individuals were winners in a competitive scenario (Zink et al., 2008), studies in rats not only observed that Dopamine receptor (D1) containing cells in the NAc were active during social competition scenario but also that inactivation of NAc reduces social dominance (Hollis et al., 2015). In the same study, when a high-anxious (later to became subordinate) rat was paired with a low-anxious (later to became dominant) rat in a social competition setup it shows to have reduced mitochondrial function in the NAc which suggests that acute stress increases the propensity to become subordinate when hierarchies are being established.

To support these observations, a recent study in mice made by the same team found that mice with a deficiency of Glucocorticoid receptor (GR) in dopaminergic neurons in the NAC had a higher probability to became dominant (Papilloud et al., 2020). During a stressful situation, animals physiologically react with the activation of the Hypothalamic-pituitary-adrenal (HPA) axis and releases as final output the glucocorticoid hormones leading to increase levels in the bloodstream which was also observed to happen after a social defeat situation (Barik et al., 2013).

Furthermore, classical studies in lobsters reported increases in fight durations and reduction of the probability to retreat when animals were injected with serotonin (Livingstone, Harris-Warrick, and Kravitz, 1980). Since the **dorsal raphe nucleus** (DRN) is the main serotonergic nucleus in the brain, this region as also being pointed as an additional mPFC downstream target involved in the manifestation of social hierarchy. Further studies will be needed to dissect the contribution of this neuromodulator to the establishment and maintenance of social hierarchy in other species, such as mammals.

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# 2 | Objectives

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# Chapter 2 | Objectives

Social hierarchy is a potent modulator of behavior, that is typically established through overt agonistic interactions between individuals in the group. Once established, social ranks are maintained through subtler interactions allowing the redirection of energy away from agonistic interactions towards other needs. The available tasks for assessing social rank in rats allow the study of the mechanisms by which social hierarchies are formed in early phases but fail to assess the maintenance of established hierarchies between stable pairs of animals, which might rely on distinct neurobiological mechanisms. With this work we want to fill the gap in this field of behavioral neuroscience by developing and validating a novel trial-based dominancy assay, where established social hierarchies can be identified in the home cage of non-food deprived pairs of male rats.

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- Develop a novel behavioral paradigm that allows for the identification of stable social hierarchies in rats;
- Identification of behavioral patterns observed in this task;
- Study the reliability of the social hierarchy measured by the modified food competition task;
- Study the stability of the social hierarchy measured by the modified food competition task

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# 3 | Material And Methods

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# Chapter 3 | Material and Methods

## 3.1 Animals

40 three-months old male Sprague-Dawley rats (OFA, Charles-River, France) weighing 325-410g at the beginning of the experiment were used. Upon arrival from the commercial vendor (Charles River, France), rats were pair-housed and maintained with *ad libitum* access to food and water under a reversed light cycle (12 hours dark/light cycle; lights off at 10 AM) in controlled temperature conditions, and with a transparent red tunnel as environmental enrichment (8 cm diameter, Bio-Serv, #K3325). Animals were left undisturbed in their home cages for approximately three weeks, allowing rats to habituate to our Vivarium Facility and routines, and to reverse their circadian rhythm.

After this period, animals were handled four times every other day during one week. Body weight was controlled weekly and prior to each testing session. Experiments were performed during the dark cycle, waiting at least 2 hours after the lights were off to start with behavioral procedures. Animals were provided by a commercial company, thus previous social experience, social status and degree of relatedness between the animals was not known. Animal husbandry and all experimental procedures were approved by the Animal Care and Users Committee of the Champalimaud Neuroscience Program and the Portuguese National Authority for Animal Health (Direcção Geral de Veterinária), which is in strict compliance with the European Directive 86/6097EEC of the European Council. We confirm that the study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org/).

## 3.2 Experimental Procedures

Twenty pairs of animals were tested in the different behavioral paradigms (Sucrose Competition, Food Competition with and without food deprivation, Tube test and Water Competition, see below for description of each task) in order to identify social rank and study reliability of social status between them. Each pair of animals consisted of cage mates living in the same cage for 4 weeks before starting the behavioral procedures. The interacting animals were thus familiar and the same pairs were maintained throughout the entire duration of the experiment. To control for possible influences of the order of behavioral testing on the evaluation of social status, we divided the animals in two independent groups (n=10 pairs each) where the order of the tests that involved competition for positive reinforcers was counterbalanced (Fig.3.1). Those tests that required food or water deprivation, and thus were more stressful and/or could a priori induce strong aggressive behaviors, were performed towards the end of the experiment. All pairs were tested in all the tasks with a 2 to 6 days of interval between testing sessions.

At the beginning of the experiment, we randomly identified each rat of a cage as 'Animal A' and 'Animal B' and quantified their behavior and consumption of the resources for each of the behavioral tests. Before each habituation or test session the fur of the animals was marked using a black pen in order to enable clear identification of each animal for post hoc video-annotation analysis. All the tasks, except for the Tube Test, were performed in the animal's home cage with small modifications to the lid to accommodate a customized feeder/water bottle. During testing, standard chow and water bottles were removed from the home cage, to accommodate the modified lids, being replaced immediately after behavioral testing.

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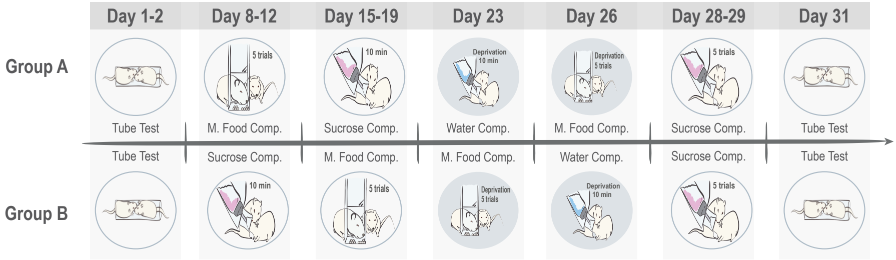

Fig. 3.1| **Timeline of experimental design.** After handling, two independent groups of animals were created where the order of the tests was counterbalanced. The order of the test is provided in the cartoon, and days indicate when they were performed. Note that in the case of the first evaluation of the Tube test, the modified Food Competition test without deprivation and the Sucrose Competition with continuous access to the bottle (10 minutes), these days include also the habituation sessions. All animals were tested in all competition tasks with inter-tasks intervals ranging between 2 and 6 days. Sucrose competition with intermittent access to the bottle (5 trials) was included towards the end of the experiment, after realization of the low amounts of drinking performed in the sucrose test with the continuous access to the bottle configuration. Shaded circles in the competition tasks at days 23 and 26 indicate that they were performed under deprivation states.

### 3.2.1 Modified Food Competition test (mFC)

Food competition for palatable pellets (Dustless Precision Pellets, 45mg, Rodent Purified Diet, Bio-Serv) was performed in the home cage of non-food restricted pairs of animals. For this test, the home cage lid was replaced by a modified laser-cut acrylic one that accommodated a fully transparent feeder (Fig.3.2A-C). The feeder was designed so only one animal could access the food pellets at a time, promoting conflict and competition for the reward. Moreover, the feeder accommodated a sliding door that prevented access to food pellets during the inter-trial interval, and an opening on the top to facilitate delivery of food pellets in each trial with minor interference from the experimenter. This customized lid was used during habituation and test sessions. Animals were exposed to palatable pellets in their home cages during the handling period for four days in order to reduce neophobic responses to the food. Then, during three consecutive days, all the animals went through a habituation period to the modified lid, where they were allowed to explore and consume the pellets individually without competition, while the partner would be kept in a separate cage. Specifically, during habituation days, the new lid holding the feeder was placed on the home cage containing 10 palatable pellets. The sliding door was closed, preventing access to the pellets. Two minutes after, the door was opened, allowing the rat to access the pellets for 2 minutes, after which the door was closed again and 10 new pellets were placed. In total, the animal was given 4 minutes' access to 20 pellets in a total session of 10 minutes. Next, food competition in a social context was performed for two consecutive days. Pairs of animals were re-marked, and the home cage lid was replaced by the modified one with the feeder and 10 palatable pellets. 1 minute after, the sliding door was opened allowing the rats to have access to the pellets for 2 minutes, after which the door was closed again for a 1-minute inter-trial interval and 10 new pellets were delivered. We repeated this procedure for 5 trials and a total session of 15 minutes and 50 pellets. After the session, the customized lid was replaced by their home cage lid.

### 3.2.2 Modified Food Competition with food deprivation (mFCD)

The modified Food Competition test was performed in the home cage of familiar animals as described above but, in this case, animals underwent only one session of social competition after a 24h period of food deprivation. After the test, the modified lid was replaced by the standard one and rats were allowed to eat and drink *ad libitum* for the rest of the cycle.

### 3.2.3 Sucrose Competition (SC)

Pairs of non-water-restricted cage mates competed for access to a bottle containing 1% sucrose solution placed in a modified lid on their home cages. The lid was designed so the bottle holders were prolonged with a transparent acrylic tube, in a manner that the tip of the bottle was surrounded by an extension that would allow the head of only one animal to drink at a time (Fig.3.2D-F). We performed three habituation sessions. In the first habituation day, animals were exposed to the new lid for 20 min where no bottle was available. In the two following days, the new lid was holding two bottles of 1% sucrose solution and animals were given free access to the sucrose solution for 20 minutes. Then, animals were re-marked and tested for sucrose competition in two consecutive days for 10 minutes, where the modified lid presented only one bottle of sucrose this time.

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### 3.2.4 Sucrose Competition with intermittent access to the resources (SCI)

In this test, access to the 1% sucrose solution followed an intermittent schedule. One minute after the beginning of the session, a bottle with sucrose solution was placed in the dispenser allowing the animals to access the solution for 2 minutes. After this time, the bottle was removed for 1 minute and put back again for 2 more minutes, performing a total of 5 trials. The SCI was performed over two consecutive days. After each session, the customized lid was replaced by their home cage lid.

### 3.2.5 Water Competition (WC)

Animals were water deprived for 24h and tested for competition for water in their home cage. At the moment of the test, the cage lid was replaced by a modified one where access to the bottle was only possible for one animal at a time. The duration of the test was 10 minutes, after which the standard lid was replaced and both rats had *ad libitum* water access.

### 3.2.6 Tube Test (TT)

We used a transparent Plexiglas tube with 60 cm length and 8 cm diameter, a size that allows an adult rat to pass through without reversing its direction, and, when two rats are placed in the tube, prevents one rat from crossing the tube by passing the other (Fig.3.2G-H). We performed one habituation session where animals individually explored the apparatus, allowing spontaneous entering and crossing of the tube for 5 minutes. During this habituation session, animals were placed initially in front of one of the ends of the tube, and freely allowed to enter the tube and explore the behavioral table. In our hands, rats immediately entered and crossed the tube, spontaneously performing 5 to 6 crossings during the habituation period, without the need to force exploration nor to push them, as they did not display extended periods of immobility. After the 5 minutes' habituation animals were returned to their home cages. During test days, each pair of cage mates rats was simultaneously placed into opposite ends of the tube and met in the middle. At this time, a partition placed at the center of the tube was removed. The rat that first retreated from the tube was designated as the 'loser' and the other as the 'winner'. After each trial, both rats were placed back into their home cages until the beginning of the next trial. From trial to trial, animals were released at either end of the tube alternately. We performed one testing session of the Tube Test with 10 trials at the beginning of the experiment and assessed stability of social rank within this test with another testing session at the end of the experiment. We quantified the amount of time that one of the animals took to push the other out of the tube and annotated the winner and loser of each interaction.

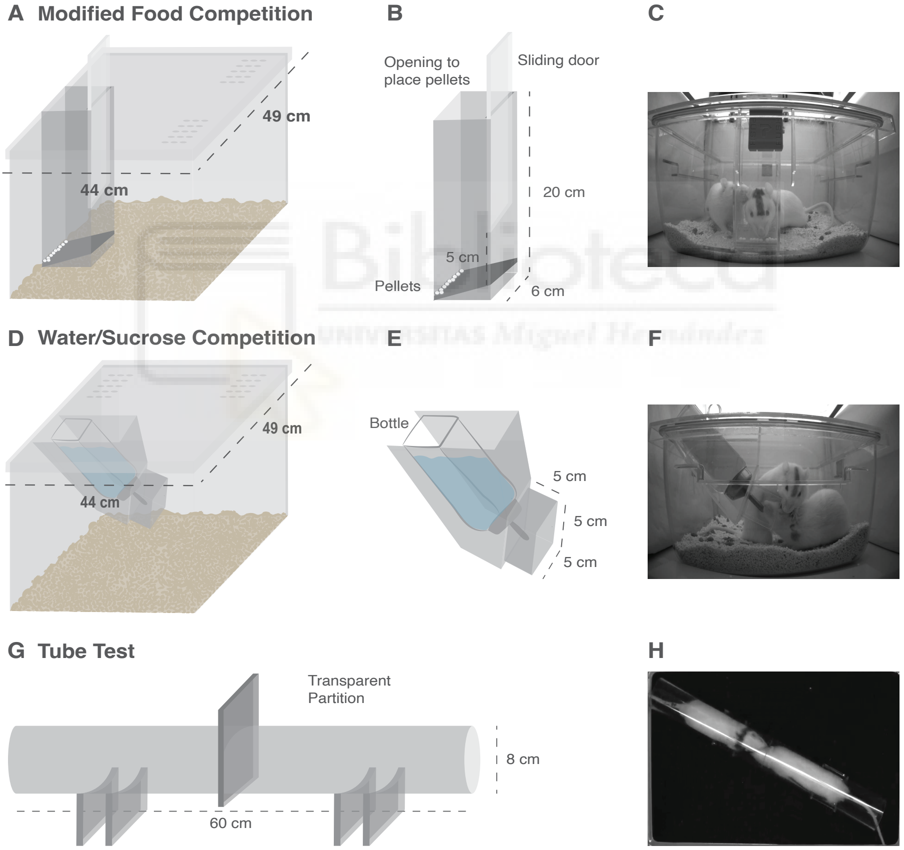

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Fig. 3.2| **Design and measurements of the behavioral tests used for identification of established dominance status.** **(A)** Schematic illustration of the transparent lid and feeder in the modified Food Competition apparatus. **(B)** Detailed schematic illustration of the feeder used in Food Competition protocols (with and without deprivation) including the measurements to fit the bottom part of a Rat IVC cage (Sealsafe PLUS Green Line ventilated cages, Techniplast). The sliding door can be opened leaving a 5 cm high access which only allows one animal to eat at a time, and allows a trial structure for the task. A small opening on the top of the feeder allows to refill new pellets during inter trial interval. If adaptation of measurements to another type of home cage is needed, we advise to leave 3–4 cm from the end of the feeder and the bottom of the home cage. This prevents bedding to go into the feeder, which difficults the visibility of the pellets while animals are consuming. **(C)** Two cage mates can be observed at the feeder area, where one is consuming the pellets while the other is pushing to get access to the food. **(D)** Schematic illustration of the lid and bottle holder for the Water and Sucrose Competitions protocols (with continuous or intermittent access) protocols. **(E)** Detailed schematic illustration of the bottle holder. A 5 × 5 cm restraining tube around the lick spout was created to prevent simultaneous access of both animals to the resource. **(F)** Two rats behaving in the Water Competition task, where one of the rats is drinking while the other is pushing to have access to the bottle. **(G)** Schematic of the Tube Test with measurements used in this task, the transparent partition in the middle of the tube is removed at the beginning of a trial once both animals reach this area. Laser-cut acrylic holders were used to give stability to the set up. **(H)** Two rats interacting inside the tube during the initial moments of a trial.

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## 3.3 Video acquisition and Behavioral Quantification

Compared to humans, rats have a reverse circadian cycle meaning that they are fully active during the night (Stephan 1983). In order to study their behavior during their active state, experiments were performed under the dark cycle of the animals and video recordings were obtained by a high resolution infra-red camera (PointGrey Flea3 -U3-13S2M CS, Canada) under infra-red illumination, capturing frames at 30Hz at 1280x960 pixel resolution.

Supervised offline frame by frame video annotation of behaviors of interest was performed by a trained blind experimenter (DFC) after confirmation of highly reliable quantifications. For each animal of the dyad we quantified frequency, latency, and duration of **(1) consumption of resources** (number of pellets eaten or time spent drinking water or sucrose); **(2) exploration of the feeder** (sniffing behavior inside and outside the feeders or bottle holders); **(3) self-grooming**; and **(4) pushing the other animal to gain access to the resource**.

In those tests with a trial structure, behaviors were aligned to time 0, i.e. the moment where the sliding door that gave access to food pellets was opened for food competition tests, or when the bottle was placed in the modified lid in the case of the Sucrose Competition Intermittent. For these tests with trial structure, intertrial interval (the time where reward was not present) was set to 60 seconds and the duration of a trial (where animals could access to the reward) to 120 seconds. However, due to the manual control of the timings of the experiment, some trials of some pairs of animals resulted with shorter durations than aimed. In order to ensure comparable behavioral profiles across trials and dyads across these tests, we decided to narrow our behavioral quantification window and focus our statistical analysis to the 40 seconds before and 80 seconds after time 0 (the moment where access to rewards was possible). This resulted in a total of 10 min duration test for both tasks with and without trial structure. However, note that consumption in tasks with trial structure was only quantified for 400 seconds (80 seconds during 5 trials in a day), while in the other tasks this could be possible during 600 seconds.

To take this difference into account, comparisons between consumption levels across tasks were performed with the percentage of time spent consuming relative to the duration of the session. Exploration of the feeder was measured during the whole session, and in those tests with a trial structure, we differentiated between exploration of feeder when reward was accessible and before that, when the sliding door was closed or there was no bottle there yet, as a proxy for anticipatory exploration. Pushing behavior was divided in two distinct categories depending on the outcome: Successful Pushing, if the animal managed to displace the partner from having access to the resource and Unsuccessful Pushing, when animals would attempt to get access to the resources but were unsuccessful to displace their partner from the reward area.

In the Tube Test, we quantified the number of wins for each animal and the duration of each trial as a proxy for the time the animals used to solve the territorial conflict.

Bonsai (Lopes et al. 2015) and Python Video Annotator (https://pypi.org/project/Python-video-annotator/), both open source computer vision software available online, were used to perform behavioral quantification.

First, digitally assigned behaviors were quantified with Bonsai, which created timestamps for the beginning and ending of each behavioral event. Then, the start and end of each behavioral bout was curated with frame-by-frame investigation using Python Video Annotator, which allows fine modification of the timeframes with subsecond resolution. Moreover, Python Video Annotator allowed easy post hoc categorisation of the two types of exploration of the feeder (anticipatory or during the presence of the resource) and pushing behavior (successful or unsuccessful) which can only be identified once the bouts of pushing behavior are finished and is thus not possible to analyse with online video analysis.

## 3.4 Data Analysis

Data was parsed and processed with Python (Python Software Foundation, v.2.7). In addition to comparing raw data obtained, we calculated several indexes to compare hierarchies across tests.

### 3.4.1 Dominance Index

The Dominance Index (DI) was calculated for each test, where the difference in resource consumption across partner animals was normalized by the summed resource consumption of the pair, following this formula:

$$DI = \frac{Consumption\ of\ Animal\ A - Consumption\ of\ Animal\ B}{Total\ Consumption} * 100$$

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Consumption corresponded to the number of pellets in the modified Food Competition tests, the duration of drinking in the Sucrose and Water Competition tests, and the number of wins displayed by an animal in the case of the Tube Test. The sign of this index would indicate whether animal A or B would consume more, i.e. positive values would indicate that animal A consumed more, and negative values that animal B consumed more. DIs close to 0 would indicate no differences in consumption between the animals of a pair. Differences of 5% to equal consumption, i.e. DI ranging from -10 and +10, were considered noise and indicative of no reliable hierarchy.

### 3.4.2 Conflict Resolution Index in the Tube Test

The Conflict Resolution Index (CRI) was calculated taking into account not only who won a trial, but also how long it took for the conflict to be resolved (i.e., the latency for one of the animals to be pushed out of the tube):

$$CRI = \frac{DI}{Time\ to\ solve\ the\ conflict}$$

### 3.4.3 Conflict Index

The Conflict Index (CI) was calculated by dividing the time animals spent pushing in a specific test by either: (1) the time spent consuming, in case of the Sucrose and Water Competitions, or (2) the latency to eat all rewards in the case of the modified Food Competition with and without food deprivation.

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### 3.4.4 Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics version 24.0 for Windows. The normality of the data was tested using Kolmogorov-Smirnov normality test, and when normality was not observed non-parametric tests were applied and median and 95% confidence interval were chosen to represent data in figures. Wilcoxon signed-rank tests with Bonferroni correction were used to study differences between counterbalanced groups in each protocol, of each behavior across the tasks, and to study differences between dominant and submissive animals on behavior. Paired t-test were performed to assess differences in the weights between dominant and submissive animals. One-Way ANOVA followed by post-hoc test Tukey was used to compare behaviors of interest across tasks. Here, when normality was not observed, a Kruskal-Wallis test with post-hoc Dunn-Bonferroni correction was performed. Bivariate Pearson Correlation was performed to measure the strength and direction of association between the Dominance Index of all the tasks and linear regressions for assessing predictive value of Food Competition and Tube Tests controlling for body weight. Statistical significance was set at $p<0.05$.

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# 4 | Results

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# Chapter 4 | **Results**

## 4.1 Behavioral profiles differ across the social competition tests used

To identify social status within pairs of cage mates we performed the modified Food Competition test and other behavioral tasks in which the animals needed to compete for resources, either for palatable pellets, sucrose solution, water or tube occupation (Fig.3.1 and 3.2). All the tasks, except for the Tube Test, were performed in the animals' home cage. Animals displayed different behavioral profiles depending on the configuration of the test, whether it had a trial structure, the amount of reward available, and their internal state (satiated vs deprived) (Fig.4.1 and Fig.4.2).

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In order to control for possible effects of winning history we created two independent groups where we counterbalanced the order of the tests. No differences were observed between the groups, suggesting that hierarchy was already established (Kruskal-Wallis test comparing the duration of consumption in the two counterbalanced groups: modified Food Competition test (mFC) Day1: $X^2(2)=0.026$, $p=0.871$; mFC Day2: $X^2(2)=0.007$, $p=0.935$; SC Day1: $X^2(2)=1.516$, $p=0.218$; Sucrose Competition with continuous access to reward (SC) Day2: $X^2(2)=1.904$, $p=0.168$; Sucrose Competititon with Intermittent access to reward (SCI) Day1: $X^2(2)=0.457$, $p=0.499$; SCI Day2: $X^2(2)=2.055$, $p=0.152$; mFCD: $X^2(2)=0.00$, $p=0.989$; Water Competition (WC): $X^2(2)=0.293$, $p=0.589$; Tube Test (TT) Day1: $X^2(2)=0.00$, $p=1.0$; TT Day2: $X^2(2)=0.00$, $p=1.0$). Data from both groups was thus merged for the rest of the analysis.

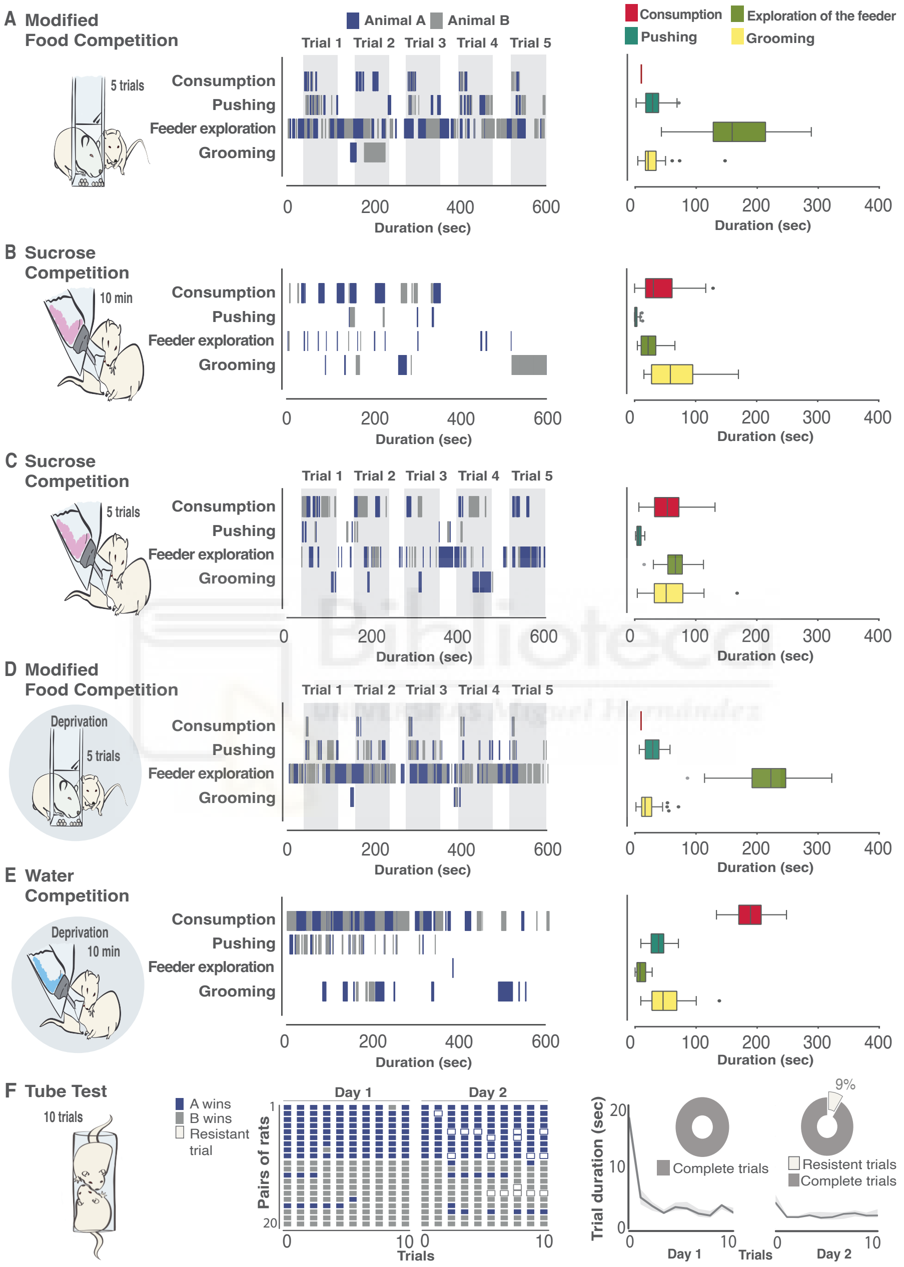

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Fig. 4.1| **Behavioral profiles change according to the specifics of each social competition test.** **(A)** modified Food Competition test. **(B)** Sucrose Competition with continuous access to the bottle. **(C)** Sucrose Competition with intermittent access to the solution, where animals could only drink during 2 minutes and the bottle was absent during 1 min inter-trial interval. **(D)** modified Food Competition test with deprived animals. **(E)** Water Competition test. **(F)** Tube test. For all tests, left panel shows the schematic representation of the task. Cartoons with a shaded circle as background indicate that tests were performed under deprivation. **A-F middle panel:** raster plots showing frequency and duration of behaviors of interest in an example pair of animals (animal A in blue, animal B in grey), except for **F** where the animal wining each trial is depicted for the 20 pairs of animals in all trials. White coloured trials in the Tube Test correspond to "resistant trials" where the loser of the pair resisted to enter the tube and trial could not be completed. In those tasks with a trial structure, **(A, C, D)** grey shaded areas indicate the moments where access to the resources where available, while intertrial interval, during which animals could explore the feeders but not access the food or sucrose bottle, are indicated with white background. **A-E right panel:** Box-plot representation of the duration of each behavior of interest for each individual animal. When more than one day of testing was performed, data represents average of the two days. For those tests with a trial structure, values represent behavior throughout the entire test period averaged over the 5 trials (or 10 trials, when two days of testing were performed), being one trial defined as the last 40 seconds of the intertrial interval and first 80 seconds of access to reward. Median, quartile 1 and 3 are represented, whiskers indicate minimum and maximum values and extreme values are signaled as a dot. Consumption in red, pushing in dark green, Exploration of the Feeder in light green, Grooming in yellow. **F right:** Trial duration in the Tube Tests in the two testing days. Black line represents the median duration that animals took to push their partner out of the tube, and grey shadows depicts 95% confidence interval. As an insert in each day, pie charts represents the percentage of trials that were completed. Note that 9% of trials in the second day of the Tube Tests were not completed as one animal (the loser) refused to enter the tube.

In the modified Food Competition task (Fig.4.1A, Fig.4.2), the limited number of available food pellets (10 per trial) led to a very fast consumption of resources which lasted a few seconds (Fig.4.2 B-C). Interestingly, although pellets were consumed in the first seconds of each trial, animals displayed high levels of exploration of the feeder and displayed notable amounts of pushing, suggesting high expectation of reward (Fig.4.2A).

On the contrary, during the Sucrose Competition task, exploration of the reward location and pushing levels were low (Fig.4.1B and Fig.4.2A), but exploration of the bottle location increased when access to the sucrose bottle was presented in an intermittent manner (Fig.4.1C and Fig.4.2A).

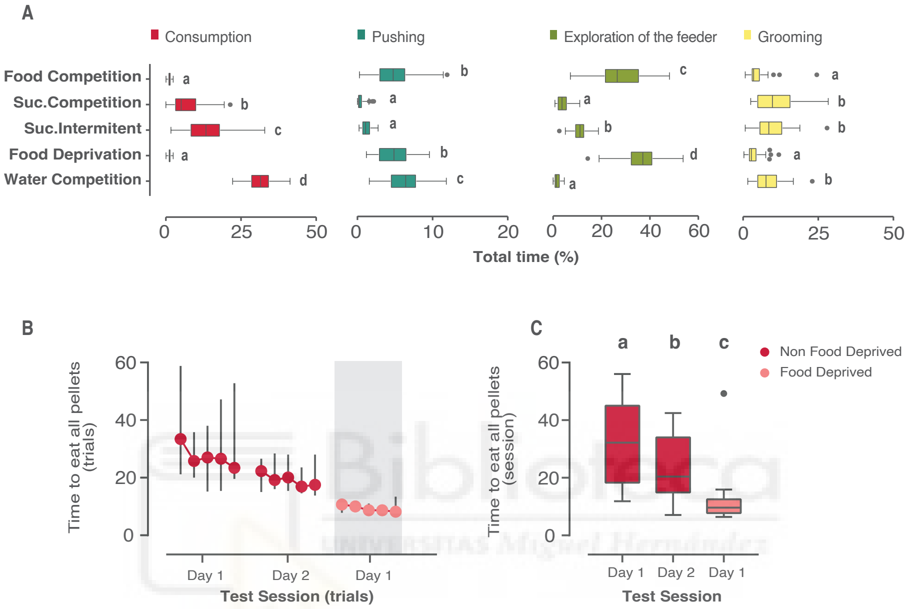

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Fig. 4.2| **Descriptives of the behavioral analysis for all social tasks involving competition for resources.** **(A)** Comparison of the behaviors of interest displayed by each animal across all behavioral tests. Data is presented as percentage of time performing a specific behavior related to the total duration of the task, enabling comparison between tests differing on duration. For those tasks with a trial structure, consumption corresponds to the first 80 seconds of reward availability for each trial, while pushing, exploration of the feeder and grooming duration correspond to the displays performed during the 40 seconds before and 80 seconds after reward availability. When more than one day of testing was performed, data represents average of the two days. Each competition test provided very different behavioral profiles, where the duration of consumption and exploration of the feeder or pushing behaviors to access the resources differed clearly over the tests. Note that pellets in the modified Food Competition test were consumed during the first seconds, but still high levels of pushing and exploration of the feeder where observed. However, the time spent consuming sucrose and the levels of pushing for accessing the bottle were low. Box-plots depict the median and quartile 1 and 3, whiskers indicate minimum and maximum values and extreme values are signaled as a dot. Consumption in red, pushing in dark green, Exploration of the feeder in light green, Grooming in yellow. Letters denote statistically significant differences between behavioral tests after one-way ANOVA with Tukey posthoc comparisons. Time spent grooming was not normally distributed, thus non-parametric analysis was performed. **(B)** Latency to eat all pellets for each trial is shown in the Food Competition tests, with or without deprivation where median and 95% CI for each trial are represented. Animals only took around 20 seconds to eat all pellets available, decreasing this latency when food deprived. Shaded background indicates session performed under food-deprivation. **(C)** Box-plots representing the average time to eat all pellets over testing days. Wilcoxon rank test revealed that latency to eat the pellets decreased on the second day of testing of the modified Food Competition test, and did further under deprivation state. **$p<0.01$, ***$p<0.001$.

As expected, modulation of internal state (food or water deprivation) affected the behavior of the animals. In the modified Food Competition task with deprivation (Fig.4.1D) animals consumed the pellets faster (Fig.4.2C, Wilcoxon signed rank tests against non-deprived Food Competition day 1: $z=-3.659$ $p=0.0003$; day 2: $z=-3.136$ $p=0.002$) and spent significantly more time investigating the feeder (Fig.4.2A), although the amount of pushing did not differ from that displayed in non-deprived animals (Fig.4.2A). When competing for access to water under deprivation (Fig.4.1E), animals dramatically increased the time they spent drinking compared to consumption displayed in the other tests (Fig.4.2A), performing long bouts of drinking and alternating between animals (Fig.4.1E). Levels of exploration of the water bottle were low compared to the rest of the tests. Surprisingly, although the motivation to drink was high, revealed by the long water consumption time, pushing levels did not increase proportionally (Fig.4.2A).

Finally, the dyads established a very stable winner/loser relationship in the Tube Test (Fig.4.1F), where most of the animals that would start winning in the first trials would continue doing so over the remaining trials. This winner/loser structure was maintained across the two testing days (Fig.4.1F middle panel, raster plot of winning history for all pairs). Interestingly, on the second day of testing, the loser partner of some pairs showed reluctance to enter in the tube (9% of the total trials), suggesting a strong subordination towards the partner. The time to solve the conflict in the Tube test, measured as the latency from the moment the partition at the center of the tube was removed until one of the rats was pushed out of the tube, rapidly decreased after the first trial, reaching then fast and stable latencies of around 3.5 seconds on average (Fig.4.1F, right panel).

## 4.2 Social hierarchy as priority access to resources

We categorized the animals of each pair as dominant (D) or submissive (S) according to the amount of resources they would consume within each test (pellets eaten in the Food Competition tests, time spent drinking in the Sucrose or Water Competition tests, and the number of wins in the case of the Tube Test). According to this criterion, as expected, animals categorized as dominant consumed significantly more resources than their partners in every task (Fig.4.3 A-E) (Wilcoxon signed-ranks for consumption in mFC: $z=-3.92$, $p<0.0001$; SC: $z=-3.82$, $p<0.0001$; SCI: $z=-3.92$, $p<0.0001$; mFCD: $z=-3.83$, $p<0.0001$; WC: $z=-3.92$, $p<0.0001$). In the same line, one animal always won more encounters than the other in the Tube Test (Fig.4.3 F, Wilcoxon signed-ranks for TT Day1: $z=-4.06$, $p<0.0001$ and for TT Day2: $z=-4.12$, $p<0.0001$), with the exception of one pair of animals in Day 1 and another in Day 2, where both animals of the pair won the same number of trials, thus no categorization as dominant or submissive was possible in these cases.

We decided to investigate how body weight would relate to dominance in established hierarchies of rats. Intriguingly, we did not observe differences in the body weight between dominant and submissive animals in any of the behavioral tests where animals would compete for food, sucrose or water (Fig.4.3 A-F; Paired Sample T-test for weights in mFC: t=1.208, p=0.242; SC: t=-0.309, p=0.761; SCI: t=-0.843, p=0.410; mFCD: t=0.522, p=0.608; WC: t=0.067, p=0.947). In contrast when they had to compete for territory for the first time in the Tube Test, a significant relationship between dominance and body weight was observed (Fig.4.3F; Paired Sample T-test for weights in TT-Day1: t=2.529, p=0.021). In conclusion, although no relation was observed between social hierarchy and body weight in the rest of the tasks, this was not the case in the Tube Test, where bigger rats had a higher probability of winning in the first encounters.

We then asked whether hierarchy following this criterion, amount of resources consumed, would also translate to differences in other behaviors within each test. General exploration of the resource location during the whole session did not differ between dominant or submissive animals when consumption in the same test was taken as criterion. However, we did observe that submissive animals would spend more time self-grooming in the modified Food Competition test (z=-2.016, p=0.044) and that time spent pushing was modulated by dominance in some tests. Dominant animals tended to display more pushing in the Sucrose Competition with intermittent access (z=-1.867, p=0.062) and surprisingly, submissive animals were the ones that pushed more in the Sucrose Competition with continued access to the bottle and the Food Competition under deprivation (SC: z=-2.722, p=0.006 and FD z=-3.472, p=0.0005). As this observation was unexpected, we next explored this further.

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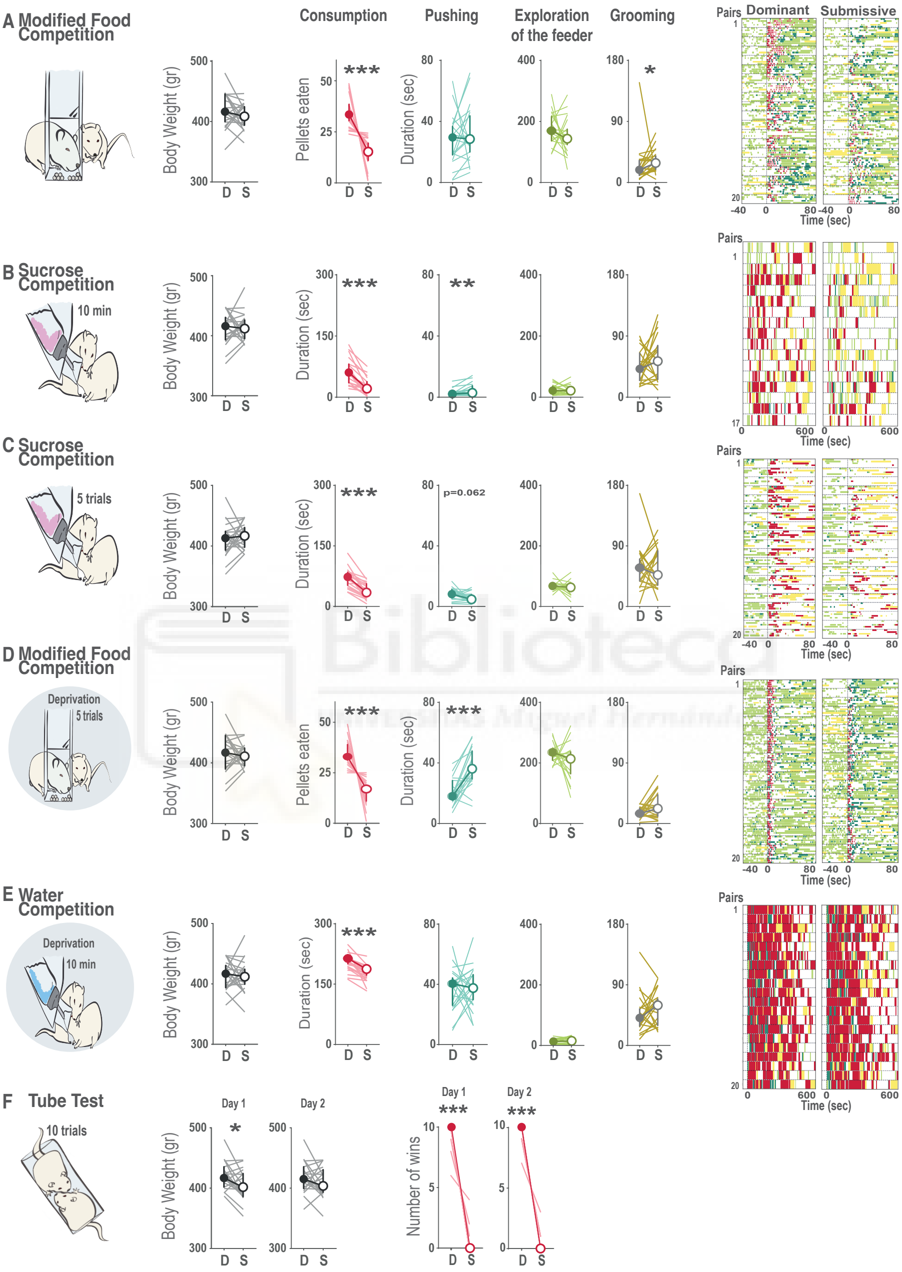

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Fig. 4.3| **Categorization of animals as dominant and submissive according to behavior within each task.** **(A-E)** Behavioral profiles when consumption of the resources within each test is used to define dominant and submissive animals. For all panels, a schematic cartoon with experimental design is provided. Cartoons with shaded background indicate that tests were performed under deprivation. For each test, differences between dominant and submissive animals are represented regarding body weight, time spent consuming the resource (or number of pellets eaten in the case of the food competition tasks), duration of pushing, exploration of the feeder and grooming is provided, where median and 95% interval confidence are displayed and individual values are showed with light lines. Color coded raster plots of behaviors of interests display raw data in a testing session, where pairs of animals are sorted according to the stronger differences in hierarchy for each test. For those tests with a trial structure, the five trials of each pair are plotted in separate lines, all aligned to time 0 (when access to reward was possible) and reflecting the behavioral data of the 40 seconds before and 80 seconds after that moment. When more than one day of testing was performed, data represents average of the two days and rasters display data of day 2. In those cases where the animals of one pair had identical values in the categorizing criteria (same amount of pellets eaten, duration of consumption or trials won in the Tube Test), no hierarchy was assumed for this specific task, and these pairs were removed from this analysis (one pair for the Sucrose Test, one for the Tube Test and one for the Food Competition under deprivation). For all graphs and rasters, consumption is represented in red, pushing in dark green, exploration of the feeder in light green and Grooming in yellow. Time of consumption in each test was significantly different between animals defined as dominant or submissive, and in some cases, these differences also were translated to a differential amount of grooming or pushing behavior. Interestingly, no differences in body weight were found in these tests, indicating that priority access to resources in established hierarchies is not influenced by the size of the animals. (F) In the Tube Test, the amount of winnings was clearly different between dominant and submissive rats, and was related to differences in body weight, which reached significance in the first days of testing, being bigger animals those more likely to win. *$p<0.05$, **$p<0.01$, *** $p<0.001$.

## 4.3 Dominant rats are more efficient displacing their partners to gain access to resources

Although the time animals spent pushing their partner should be a good measure of amount of conflict between the interacting animals, qualitative differences might be more informative of dominance status. One possibility is that even if a dominant rat pushes less often, its thrusts may be more successful in removing the partner from the resource. Thus, we calculated for each animal the percentage of successful pushing from the total number of pushing bouts, i.e. the fraction of pushing epochs that actually displaced the partner and allowed access to the resource.

Strikingly, dominant animals were more successful in displacing their partners in the modified Food Competition under deprivation, while submissive animals would push often but failed to displace their partner (Fig.4.4, Wilcoxon signed rank test z=-1.979 p=0.048). However, this was not observed in the Water Competition test (WC: z=-0.821 p=0.411) nor in the tasks not involving deprivation (mFCD: z=-0.933 p=0.351; SC: z=-1.014 p=0.310; SCI: z=-0.563 p=0.573).

The lack of differences in successful pushing between dominant and subordinate rats in the water competition test, could result from a limited window within the test where asymmetric interactions are apparent. To investigate this possibility, we identified the epochs with highest conflict in the Water Competition test, i.e., those where the most drinking and pushing behavior was observed for each pair of animals (Supplementary Fig.S1A).

We then classified the animals as dominant and submissive according to the duration of drinking in that epoch and quantified pushing displayed by either dominant or subordinates. This new categorization led to a change in the hierarchy in 55% of the pairs (11 out of 20). Here, although submissive animals spent longer time pushing their cage mate (Fig.4.4 E middle plot, z=-3.920, p<0.0001), again dominant animals displayed higher efficiency in displacing their subordinates, being successful practically 100% of the times (Fig.4.4 E plot on the right, z=-3.627 p=0.0002).

This fine grained behavioral analysis, where pushing is categorized into successful or unsuccessful, thus revealed that although in some tasks submissive animals displayed higher duration of pushing, they rarely managed to get the access to the resource, being dominant rats more successful to displace their cage mates.

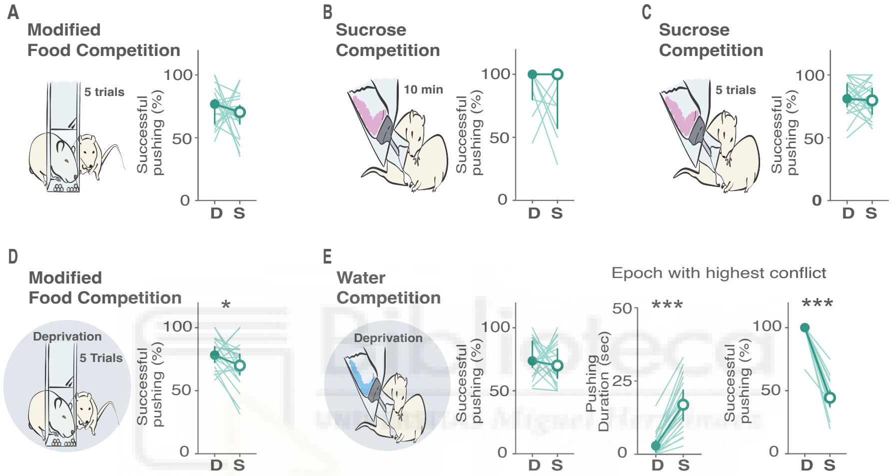

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Fig. 4.4| **Dominant animals are more successful in displacing their subordinate partners to gain access to resources when in deprivation states.** Dominant animals showed a higher percentage of successfully pushing away their subordinates while gaining access to the resources in moments with high conflict. Percentage of successful pushing did not reach significance in the modified Food Competition **(A)**, Sucrose competition with continuous **(B)** or Intermittent access to the bottle **(C)** but was significantly different in the Food Competition under deprivation **(D)**. No differences were observed in the efficiency of pushing behavior in the Water Competition task **(E)**. However, when assessing these differences in the moments with highest conflict in this test, defined as the bout where intense drinking was displayed and high levels of pushing behavior were observed, submissive animals spent more time pushing, but dominant animals were almost always successful to displace their partners in every bout of pushing. D: dominant, S: submissive according to the consumption in each test. Median, 95% CI and individual values for all animals are represented. *$p<0.05$, *** $p<0.001$.

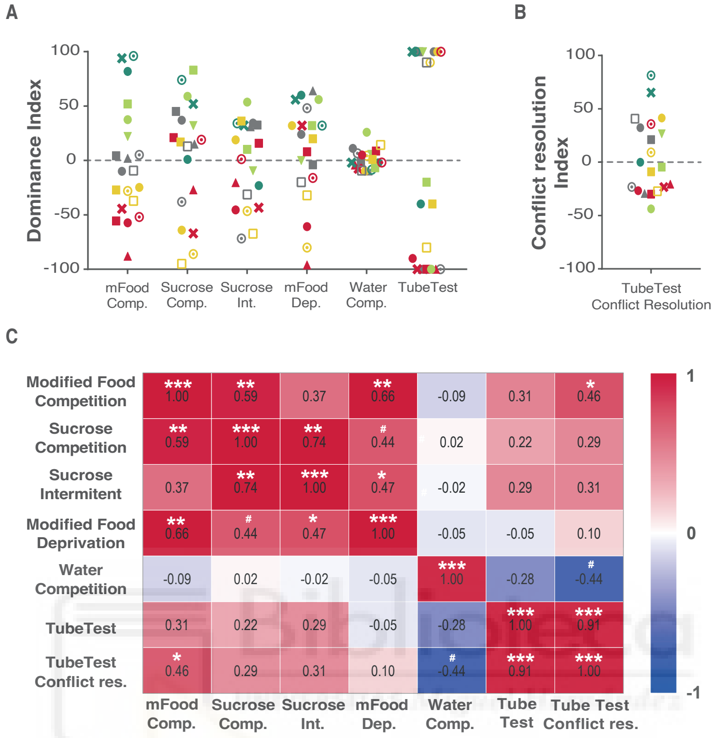
## 4.4 Social hierarchy as a stable trait between tests

To examine whether social hierarchy is a stable trait in familiar animals, we analyzed reliability across the performed tests. To this end, we computed the Dominance Index (DI) for each test where the difference in resource consumption across partner animals was normalized by the summed resource consumption of the pair. A DI close to 0 means that animals did not have a strong hierarchy. Positive values indicate that animal A consumed more, while negative values indicate that animal B was the one having priority access to resources or won more trials in the Tube Test. Provided that behavioral measures were stable across testing days (Supplementary Fig.S2) data was averaged for this analysis.

DI for positive reinforcers led to a highly variable distribution across dyads, where in some dyads animals would strongly differ in their consumption and in others differences were subtle (Fig.4.5 A). This was not the case in the Water Competition, where DI was mostly around 0 for all animals, indicating that both animals drank very similar amounts of water during the test. In contrast, the Tube Test gave very polarized DIs, where most of the pairs had one animal winning almost 100% of the trials. To refine our understanding of the hierarchy in the Tube Test, we computed a Conflict Resolution Index (CRI), which would take into account not only who won a trial, but also how long it took for the conflict to be resolved (i.e., the latency for one of the animals to be pushed out of the tube). The conflict resolution index revealed a more continuous and fine-grained measure of hierarchy strength in each pair (Fig.4.5 B).

If social hierarchy was a stable trait, then dominance indexes should be correlated across tests. We found DIs from tests with competition for positive reinforcers were positively correlated (Fig.4.5 C). Moreover, the modified Food Competition test correlated with the DI index in the Tube Test, when conflict resolution time was taken into account. Water competition DI was not correlated with any of the other tests. Water deprivation could have challenged the homeostasis of the interacting animals bringing them to a very different internal state that disrupted the stable hierarchies revealed by the other tests. Alternatively, computing the DI for the water competition test using the whole test duration, may have diluted differences in water consumption across animals within the pair. Hence we re-calculated DI for different time windows, just as we observed for the pushing behavior (see above). No correlation was found between water competition test and the other test when other time windows were used to compute DI (Supplementary Fig.S1B). Still, we have shown that during this test, the dominant animals accessed the water bottle in a qualitatively different manner, by successfully displacing their subordinates (Fig.4.5 E).

This highlights the importance of considering multiple behaviors simultaneously and suggests that social status in the Water Competition task could be better assessed by finer behaviors rather than water consumption.



| | mFood Comp. | Sucrose Comp. | Sucrose Int. | mFood Dep. | Water Comp. | Tube Test | Tube Test Conflict res. |
|---|---|---|---|---|---|---|---|
| Modified Food Competition | ***<br>1.00 | **<br>0.59 | 0.37 | **<br>0.66 | -0.09 | 0.31 | *<br>0.46 |
| Sucrose Competition | **<br>0.59 | ***<br>1.00 | **<br>0.74 | #<br>0.44 | 0.02 | 0.22 | 0.29 |
| Sucrose Intermitent | 0.37 | **<br>0.74 | ***<br>1.00 | *<br>0.47 | -0.02 | 0.29 | 0.31 |
| Modified Food Deprivation | **<br>0.66 | #<br>0.44 | *<br>0.47 | ***<br>1.00 | -0.05 | -0.05 | 0.10 |
| Water Competition | -0.09 | 0.02 | -0.02 | -0.05 | ***<br>1.00 | -0.28 | #<br>-0.44 |
| TubeTest | 0.31 | 0.22 | 0.29 | -0.05 | -0.28 | ***<br>1.00 | ***<br>0.91 |
| TubeTest Conflict res. | *<br>0.46 | 0.29 | 0.31 | 0.10 | #<br>-0.44 | ***<br>0.91 | ***<br>1.00 |

Fig. 4.5| **Reliability between dominance measurements between tests.** **(A)** Dominance index (DI) based in consumption of resources for each behavioral test is represented being each pair of animals identified with a specific color/symbol. In those cases where two days of testing was performed, values plotted correspond to the average DI of the two days. Competition for positive reinforcers produced highly variable DI indicating detectable differences in the strength of social hierarchy between the pairs. Water Competition, however produced DI close to 0 indicating that the animals of most of the dyads drank very similar amounts during this test. The Tube Test produced a very polarized distribution of DI, where one animal of each pair would win most of the trials. **(B)** These differences in hierarchy became less polarized when taking into account the time the animals took to solve the conflict in the tube (DI in the Tube Test /latency to finish the trial). **(C)** Correlation matrix between DI from all tests. Competition for positive reinforcers DIs were correlated across tests, and DI from the modified Food Competition test correlated with the Tube Test, when conflict resolution time was taken into account. However, no significant correlations were observed with the Water Competition tests. #p<0.10, *p<0.05, **p<0.01, *** p<0.001.

## 4.5 Modified Food Competition tests as a tool to measure stable hierarchies

Last, we calculated for each task a Conflict Index as a measure of the degree of conflict that our manipulations introduced in the home cage, by dividing the time animals spent pushing in one test by either the time spent consuming in case of the Sucrose and Water Competitions, or latency to eat all rewards in the case of the modified Food Competitions. Food competition with and without deprivation were the tasks with higher conflict, as animals displayed high amounts of pushing and the time available to eat the resources in each trial was very short (Supplementary Fig.S3A). Since the modified Food Competition test yielded significant levels of conflict but did not involve food deprivation, we next evaluated whether attributing dominance within dyads using this test, would allow correct identification of the dominant rat in the other tests. Specifically, animals were classified as Dominant or Submissive according to their DI in the second day of testing in this task, as the conflict index was higher in this day (Supplementary Fig.S3B). Those pairs where the difference of the number of pellets eaten was small (less than 5% difference compared to equal consumption between the animals) were considered to have an unstable or unclear hierarchy (n=4) and were not included in this last analysis. As expected, dominant animals consumed more pellets in the Food Competition test (Fig.4.6 A, Wilcoxon signed-ranks of average Consumption in mFC of both days z=-3.362, p=0.001). Interestingly, they also successfully pushed their partner away from the feeder more (z=-2.275, p=0.023), explored the feeder more during inter trial interval, where the pellets were present but not accessible (z=-2.844, p=0.004), and groomed less than their submissive pairs during the trial period (z=-2.275, p=0.023). Moreover, dominant animals according to the modified Food Competition did consumed more sucrose, both when access was continuous (Fig.4.6 B, SC z=-2.499, p=0.012) or intermittent (Fig.4.6 C, SCI z=-2.275, p=0.023). Attributing dominance found in the modified Food Competition, to the same test run under food deprivation, revealed similar dominance interactions (Fig.4.6 D, Consumption in mFCD z=-2.619, p=0.009; Successful Pushing in mFCD z=-2.534, p=0.011; Anticipatory exploration of the feeder in mFCD z=-2.902, p=0.034, Anticipatory grooming in mFCD z=-1.992, p=0.046). Moreover, dominant animals also successfully pushed away their partner more in the Water Competition test (Fig.4.6 E, z=-2.379, p=0.017). In the case of the Tube Test, the amount of wins did not differ between dominant and submissive animals, as determined by the modified food competition test (Supplementary Fig.S3C).

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In figure 4.2 we show that differences in body weight affects the probability of winning in the Tube Test, especially on the first day. This was however not the case for the other tests. Thus, weight may dominate the outcome of the tube test, overshadowing the dynamics of social interactions within stable pairs. Therefore, we decided to examine the relationship between the hierarchy in the modified Food Competition and the Tube Test while controlling for the effect of the body weight. To this end, we first regressed the number of pellets eaten against the animals' body weight and calculated the residuals. Next, we calculated, in the same manner, the residuals when regressing out body weight from the conflict resolution index (see above) of the first day of the Tube Test. Interestingly, the linear regression of these residuals was statistically significant (p=0.010) indicating that indeed, when correcting for the effect of body weight, consumption in the modified Food Competition predicts who will win in the first interactions of the Tube Test (Fig.4.6 F). Thus, dominant animal in the modified Food Competition test also won more trials in the Tube Test when the influence of the body weight was controlled for.

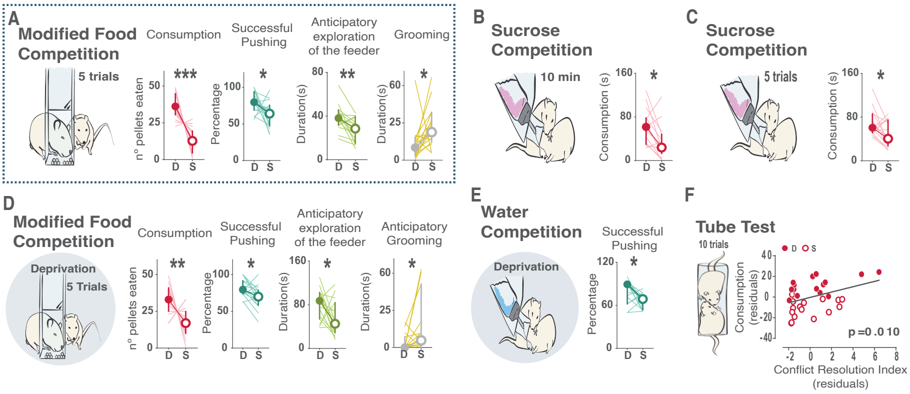
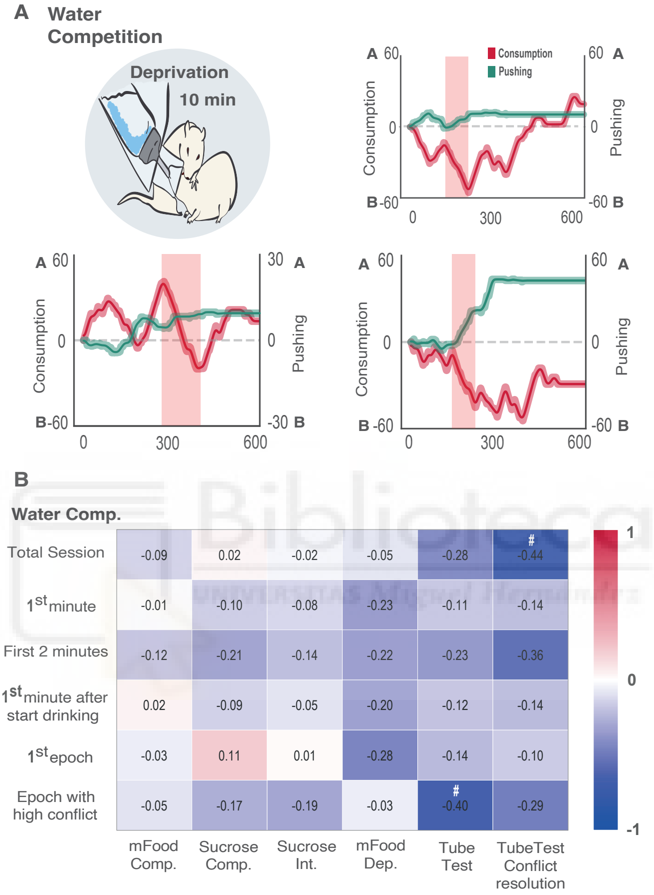


Fig. 4.6| **Food competition in the home cage is a simple and reliable measure of established social hierarchies in rats.** Animals displaying differences in the amount of pellets eaten in the modified Food Competition test were classified as dominant or submissive animals, and their behavioral profiles studied in the rest of the tests. **(A)** When considering average behaviors of both testing days in the modified Food Competition test, dominant animals significantly ate more pellets, were more successful to displace their partners from the food magasin during the competition and explored more the location of the resource when access was still prevented, and groomed less time throughout the session (considering the last 40 seconds of intertrial interval and first 80 seconds of reward exposure of each trial). Moreover, they **(B)** drank more in the Sucrose Competition test with continuous, and **(C)** with intermittent access to the bottle. **(D)** Similar behavioral profiles were displayed in the Food Competition under food-deprivation, where differences in consumption, successful pushing, anticipatory exploration of the feeder and grooming were also observed. We defined the anticipatory window as the last 40 seconds of the intertrial interval, just before the bottle was placed in the lid thus consumption was still not possible **(E)** Dominant animals according to the modified Food Competition test, were also more successful to displace their submissive in the Water Competition test, indicating that although no differences in the amount of water drank were observed, the quality of the interaction was significantly different. **(F)** The amount of pellets eaten in the modified Food Competition test significantly predicted the probability of wining in the first interactions in the Tube Test, when latency to win was taken into account, and values were regressed out of the influence of body weight. Dashed line around panel A indicates that consumption in second day of testing in the modified Food Competition test was the criteria to evaluate differences in all the tasks. Median, 95% CI and individual values for all animals are represented. * $p<0.05$, **$p<0.01$, ***$p<0.001$.

## 4.6 Supplementary Figures



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| | mFood Comp. | Sucrose Comp. | Sucrose Int. | mFood Dep. | Tube Test | TubeTest Conflict resolution |
|---|---|---|---|---|---|---|
| Total Session | -0.09 | 0.02 | -0.02 | -0.05 | -0.28 | # -0.44 |
| 1st minute | -0.01 | -0.10 | -0.08 | -0.23 | -0.11 | -0.14 |
| First 2 minutes | -0.12 | -0.21 | -0.14 | -0.22 | -0.23 | -0.36 |
| 1st minute after start drinking | 0.02 | -0.09 | -0.05 | -0.20 | -0.12 | -0.14 |
| 1st epoch | -0.03 | 0.11 | 0.01 | -0.28 | -0.14 | -0.10 |
| Epoch with high conflict | -0.05 | -0.17 | -0.19 | -0.03 | # -0.40 | -0.29 |

Fig. S1| **Identification of moments with highest conflict in the Water Competition tests and lack of correlation with Dominance Index (DI) in any other test.** **(A)** Graphical representation of dynamics of consumption (red, left axis) and pushing behaviors (green, right axis) and selection of the bout with highest conflict (shaded red rectangle) in three example pairs of animals during Water Competition test. Drinking and pushing raw data was transformed into modified cumulative plots and smoothed using convolution with a Gaussian filter of 30ms standard deviation. In this plots the direction of the cumulative graph indicated which animal was drinking or pushing over the session, either animal A or B. In this way, increases in this cumulative plot would indicate that animal A would be drinking, decreases that animal B would drink, and flat stable lines that no animal was drinking. The same applied for pushing data (in green). For example, for the example pair 1 animal B would start drinking while animal A was pushing for around 100 seconds, then they would alternate for a brief period of time, followed by another alternation, where a long bout of drinking was performed again by animal B while animal A continued pushing. After that, no significant pushing was performed by any of the animals, and although some alternations in drinking would be observed, now animal A would take over and drink more. In the different example graphs we can observe that dynamics between pairs are different over time but that animals mostly alternate in their drinking times, and pushing behavior decreases around half of the session. In order to select the bouts of highest conflict, we first defined epochs of consumption and pushing displayed by the pair by identifying the turning points that marks the moments when significant changes in the behavior occurs ($x[n+1] - x[n] < 0$). For each epoch we calculated the duration of both consumption and pushing behaviors and selected the epoch with the highest value of both consumption and time pushing the partner from the water dispenser. **(B)** No significant correlation was observed between Dominance Indexes (DI) of Water Competition when calculated with the drinking duration in the whole session, nor in the first min or the two first minutes, nor when taking into account the moment when animals started drinking. We then identified the epochs with longest drinking, as they could be not necessarily in the early moments of the test, but no correlation was observed with other behavioral tasks either. We then selected the epochs with higher conflict (see A for representative examples of the identification of the epochs), but again, no correlation was observed. Values in the correlation matrix correspond to Pearson correlation r values. #p<0.10.

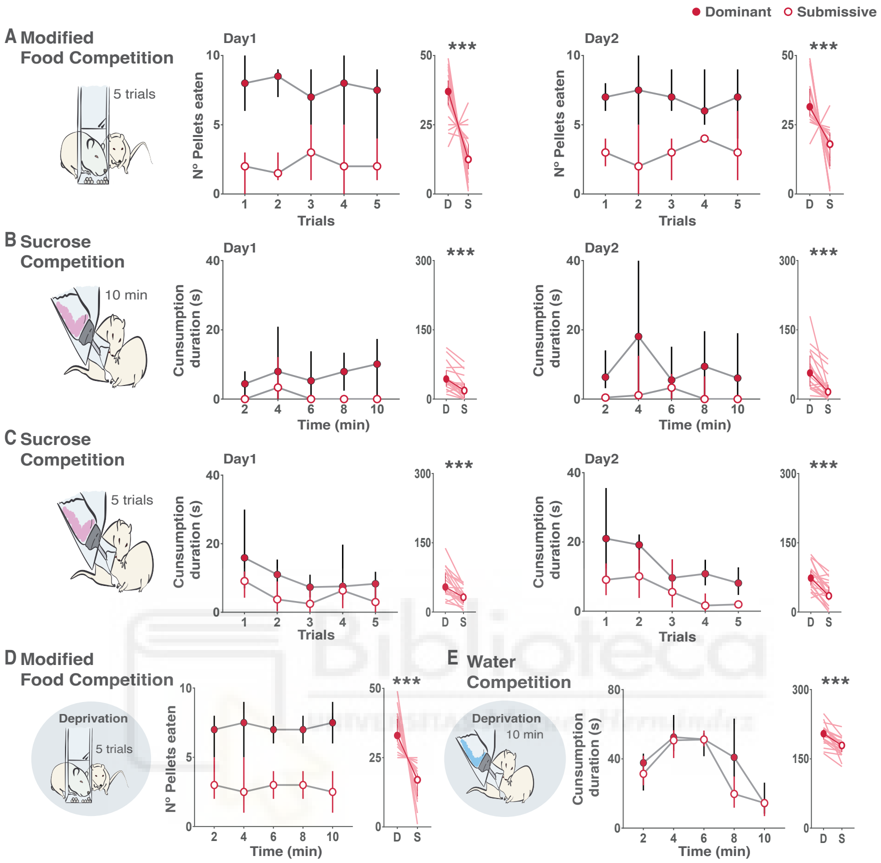

Fig. S2| **Social hierarchy measures are stable across time.** Consumption of resources is plotted over time or trials within a session and across days. In all cases the total consumption of all testing days was taken as the criteria to define dominant and submissive animals. In those tasks with a trial structure, results for each day are first presented by trials and then as the average of consumption in that day. For the sucrose competition with continuous access and the water competition, results are first presented in 2 min blocks and then the average consumption of that day. Statistics evaluating differences between dominant and submissive animals were performed in the average consumption of a day **(A)** consumption in the modified Food Competition on each of the two days of testing when the criteria to define hierarchy was the total consumption of the two days. Dominant and submissive animals clearly differed in their consumption across time. **(B)** Similar for the Sucrose Competition with continuous access to the bottle. Note the low levels of consumption, especially in day 1. **(C)** Similar for the Sucrose Competition with intermittent access, **(D)** the modified Food Competition under deprivation and **(E)** the Water Competition. Median, 95% CI and individual values for all animals are represented. ***p<0.001 after non parametric Wilcoxon test.

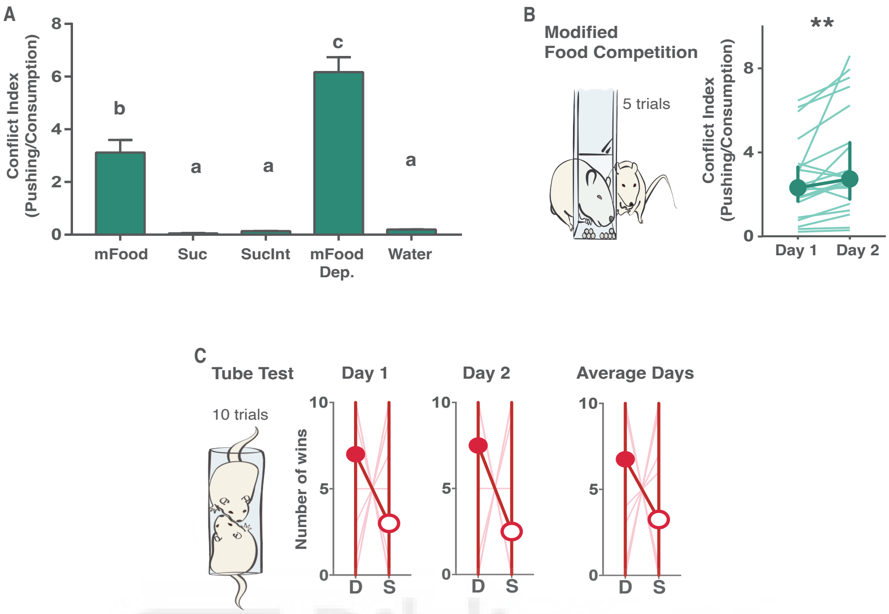

Fig. S3| **Food Competition tests reflect higher conflict.** **(A)** We calculated a conflict index for each test that involved consumption of resources by dividing the duration of pushing performed by the time the spent consuming (as the duration to consume 1 pellet would be few milliseconds in the case of the Food Competition tests, we considered the latency to eat the 10 pellets available in each trial). Due to the short duration of pellet availability and high pushing levels observed in the Food Competition tests, these tests showed higher conflict indexes than the sucrose tests or water competition (F(4, 98)=64.805 p<0.0001 followed by Tukey posthoc), which was more marked when animals were under deprivation. Average and SEM are represented, and letters denote statistically significant differences between behavioral tests after one-way ANOVA with Tukey posthoc comparisons. **(B)** Conflict index was higher in the second day of testing in the modified Food Competition test (paired t-test t(19)=-2.985 p=0.008). Median, 95% CI and individual values for all animals are represented. ** p>0.01. **(C)** Dominant animals defined according to their behavior in the second day of modified Food Competition test did not differ in the amount of winnings in the tube test in either of the days tested nor when the average winnings of the two days were considered (Wilcoxon signed-ranks for TT Day1: z=-0.774, p=0,44; TT Day2: z=-0.503, p=0,62; TT AVG Days = z=-0.699, p=0,49). Median, 95% CI and individual values for all animals are represented.

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# 5 | Discussion

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# Chapter 4 | Discussion

Due to its influence on behavior, social hierarchies have increasingly been studied over recent years (see (Drews, 1993; Wang, Kessels, and Hu, 2014; Zhou, Sandi, and Hu, 2018) for review). In most species, including rodents, these hierarchies are established mostly through a series of aggressive encounters (Drews, 1993). However, once established, the mechanisms that support them over time might not necessarily involve repeated hostile behaviors (Vessey, 1981), and instead might rely on a combination of more subtle signals that have been labelled status signals (Dwortz et al., 2022). Through multispecies studies, researchers observed that these status signals arise in extensive and often bundle together forms (from pheromones to complex behavioral displays) and the detection of these signals turn out to be more complex than the perception of a particular signal (Dwortz et al., 2022).

In this monograph, we developed and validated a new tool (Modified Food Competition Task) that allows us to investigate already established hierarchies in pairs of rats. One of the main benefits of this new tool is that it can be utilized in the animal's home cage, without the need to induce aggressive behaviors or to deprive the animals from resources.

In our task, cage mates with a stable hierarchy competed for access to positive reinforcers. The introduction of a small conflict in the home cage, where consumption of appetitive food is only possible for one animal at a time, led to subtle competition which translated into increased consumption by the dominant rat. This measure reliably predicted differences in other behaviors observed during the competition test. These differences held across the different social tasks evaluated, such as competition for sucrose solution or food and water competition under deprivation. Importantly, testing was performed in the home cage of the animals, thus minimizing the influence of the experimenter in the social interactions displayed, and interference by other factors such as anxiety or exploratory behaviors usually displayed in novel environments. Conflict in the modified Food Competition Task increased from day 1 to day 2, which gave us a clearer picture of the established hierarchy. The novelty of the conflict situation on day 1 likely contributed to the differences of conflict observed between both days of the mFC. In future experiments, it might be desirable to include more testing days to confirm whether conflict continues to build-up across days or rather it remains stable after the second day.

Interestingly, although competition was observed and hierarchy could be identified, no agonist behaviors (biting, boxing, keep down, lateral threat) were observed between the cage mates in our task. This is in accordance with previous reports indicating that once social hierarchies are established, the number of agonist encounters decreases (Vessey, 1981). Moreover, the fact that no food deprivation was required, nor aggressive behaviors were observed, can be considered as an added value of our task. Aggressive behaviors induce robust stress reactions in rodents (Márquez et al., 2013), and food deprivation, although widely used in neuroscience to increase motivational salience during behavioral testing, it modifies the animal's internal state (Kennedy and Shapiro, 2009) and social behavior (Reppucci et al., 2020). Minimizing the possible long term effects of these manipulations on the internal state of tested animals is particularly important for studies regarding the impact of social hierarchy on other behavioral, physiological and brain functions.

Moreover, the fine behavioral characterization across the different social tasks used, allowed us to identify very interesting social patterns, that to our knowledge have not been reported before. In this direction,

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we describe that social hierarchy tasks with a trial structure (modified Food Competition tasks and Sucrose Competition with Intermittent access), where access to resources was presented in a repeated and intermittent manner, promoted competition. Moreover, we describe that when measuring conflict, the time spent pushing by the animals is not indicative of dominance, but rather how efficient an animal is to displace its partner while pushing. Although the dominance index in tasks that involved competition for positive reinforcers reliably revealed the strength of social hierarchy within a pair, this was not the case in the Water Competition test under deprivation, where the animals drank around the same amount of water (dominance index around 0), nor in the case of the Tube Test, where very polarized results were observed. In the Water Competition task, animals tended to perform long bouts of drinking, alternating consumption until both animals were satiated, which resulted in very similar final consumption levels in both animals. We asked whether an analysis with finer temporal resolution could unveil structured dynamics of water consumption in this task, such that asymmetries across rats in the dyad would emerge during bouts of conflict, and these asymmetries would correlate with other social competition tests. However, to the extent that we could quantify, we did not observe such a pattern. Strikingly, we found that although consumption between animals was very similar, other behaviors displayed while approaching the water bottle were clearly different. Although submissive animals in this task spent more time pushing their cage mate in moments of high conflict, they were rarely successful in accessing the water if the dominant was already drinking. Indeed, the ability of dominant rats to successfully displace their partners from the resource location was not limited to the Water Competition, being reliable in those tests where more conflict (pushing) was observed.

In the case of the tube test, we showed that computing conflict resolution index which takes into account multidimensional behavioral measures, such as the winner of a trial and conflict duration (latency for one of the animals to be pushed out of the tube) revealed a more granular view of the strength of social hierarchy in this test.

In addition, this conflict resolution index correlated to the social hierarchy observed in the modified Food competition test, especially when taking into account the body weight of the animals. It is important to note that the final output of the tube test (i.e. who was the winner) did not correlate with other measures of social hierarchy. Although this test is widely used in mice (see (Zhou, Sandi, and Hu, 2018) for review), our data indicates that in rats this test might not be an appropriate tool to measure established hierarchies, as who manages to push out of the tube its partner is largely affected by the body weight of the interacting animals. However, the conflict displayed inside the tube in the first encounters (incorporating the latency to win in the conflict resolution index), might be a better measurement of established hierarchies for rats.

These results thus underscore the necessity of including multidimensional analysis of behavior and the importance of taking into account qualitative measurements when describing social interactions. Furthermore, although it might be surprising that the tube test is not a reliable measure of established hierarchy in rats, important differences in social behavior between mice and rats are starting to be reported (Reppucci et al., 2020; Netser et al., 2020). Rats are more socially tolerant, and less hierarchical compared to mice. This might be related to their natural behavior in the wild, where rats are often observed in larger groups (Schweinfurth, 2020). These ecological differences should be carefully taken into account when borrowing tools from one species to the other

(Genzel, 2021).

Finally, body weight has been largely assumed to be a good indicator of social hierarchy in rats (Macdonald, Berdoy, and Smith, 1995), although this has not been replicated in mice (Pallé et al., 2020; Wang, Kessels, and Hu, 2014). This view was inspired by classic works (Hoyenga and Rowe, 1969) and the seminal contributions in the field upon the development of the visible burrow system (Blanchard and Blanchard, 1989). However, our data, obtained in animals maintained with *ad libitum* access to food and water, did not support this observation, as dominant and submissive animals showed no differences in body weight, with the notable exception of the Tube Test. It is important to take into account that in our experiments the difference in body weight between the animals of a pair was lower than 10%, as they were age-matched. It is possible that larger body mass differences would indeed influence social rank in these tests, as previously shown in mice (Kim et al., 2015; Bartos and Brain, 1994) and rats (Macdonald, Berdoy, and Smith, 1995). However, when using animals with marked body weight asymmetries, differences in age, and thus social experience, should be then taken into consideration as possible modulators of hierarchy. In our conditions, only the Tube Test was affected by body weight, where bigger animals had indeed more chances to win in the first encounters in the tube, probably indicating that in rats, as opposed to mice (Zhou et al., 2017), body mass does affect the pushing behavior and the output in this task.

One limitation of our work was the use of a very specific population for our study: male adult Sprague Dawley rats. Although we do not anticipate major problems in using the modified Food Competition test in other rat strains, behavioral differences have been already reported in several social and nonsocial behaviors between Long-Evans, Wistar and Sprague–Dawley rats (Netser et al., 2020). It could be possible

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that different rat strains would react differently to the subtle conflict we induce in the cage during the modified Food Competition test, and that acute aggressive encounters could be observed depending on the strain. Future studies could also expand our task to other developmental ages (such as peripuberty or late adolescence), where play-fight behavior and aggressive profiles are being acquired (Márquez et al., 2013).

Our task is also well suited for the study of social hierarchy in female rats, as dominance is evaluated not according to the quantification of agonist behaviors, which female rats do not typically display, but on the behavioral response towards a subtle conflict, in this case access to appetitive reinforcers. Non-published work from our lab, using the mFC with female rats, has shown that similarly to males, females also establish social hierarchies, but they do not become stable until late adulthood (Bortolozzo-Gleich and Márquez, unpublished). The neural mechanisms of female dominance and aggression have been surprisingly overlooked, and most of the knowledge in this direction has been obtained in the context of maternal aggression (Caughey et al., 2011; Jurek and Neumann, 2018; Bosch et al., 2004), with some notable exceptions (Moura Oliveira et al., 2021; Cordero et al., 2012), moment where females display robust, clear and strong aggressive episodes. However, as we previously mentioned, established social rank, contrary to *de novo* establishment of social hierarchies, is not based on aggressive encounters. The paradigm described here will help us understand if the brain mechanisms involved in the maintenance of social hierarchy in female rats are the same as those previously described in aggressive encounters, such as maternal aggression.

Moreover, although not the aim of the present work, our task can be easily scalable to larger groups of animals living in standard home cages or more naturalistic environments. A collective evaluation of established

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hierarchies by introducing subtle conflicts during discrete periods in the home cage might provide very interesting information of complex social structures, and would suppose a clear advantage when evaluating groups of animals, as compared to repeated testing across multiple pairs, as the round-robin design currently used in the tube test in mice.

While most of the available tests for the evaluation of social hierarchy in rats are based on the identification of dominant animals during the establishment of a new hierarchy (Zhou, Sandi, and Hu, 2018; Timmer et al., 2011; Timmer and Sandi, 2010; Macdonald, Berdoy, and Smith, 1995; Hollis et al., 2015) it is uncertain whether becoming the dominant in a first encounter will translate into keeping the same rank when the hierarchy becomes stable. Indeed, previous reports indicate that repeated encounters are needed for two unfamiliar animals to establish a stable social rank (Timmer et al., 2011; Timmer and Sandi, 2010; Cordero and Sandi, 2007). By studying social status only during the early phases of its development, we are losing a huge aspect on the richness of social behavior and how it might be impacting brain function, in health and disease.

The differences between the establishment of a hierarchy and its maintenance are largely unexplored, and the field would vastly benefit from new behavioral tools to address this fascinating question. Our new behavioral task opens the possibility for the study of how social status affects different aspects of an individual cognitive, behavioral and physiological functions in the context of various social interactions, regarding which very little is known.

Our modified Food Competition task provides a simple, robust, and unintrusive means of assessing established social hierarchy that can be readily incorporated into future studies, with the notable advantage of

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not inducing aggressive behaviors between the interacting individuals, nor having to manipulate internal state (deprivation), and being performed in the home cage.

Intense efforts in the last years have highlighted the Norway rat as a very interesting animal model to identify the proximate mechanisms and neural circuits of complex social functions (Atsak et al., 2011; Pereira, Farias, and Moita, 2020; Márquez et al., 2015; Kentrop et al., 2020; Hillman and Bilkey, 2012; Cruz et al., 2020; Han et al., 2019; Hernandez-Lallement et al., 2020; Kashtelyan et al., 2014; Conde-Moro et al., 2019; Schuster and Perelberg, 2004; Viana et al., 2010; Bartal, Decety, and Mason, 2011). We believe that many of these behaviors are modulated by the manner in which an individual processes status signals emitted by others, and therefore, the existing social hierarchy. However, the circuits underlying the processing and interpretation of these signals and how they are used to influence behavior is still largely unknown. How a dominant and a submissive individual utilize the same social signals to generate markedly distinctive behaviors, and whether these rely on the same neural circuits remains one of the most interesting aspects to be resolved.

On the other hand, social status appears to influence the ways in which individuals gather social information. For example, in a study with Brown Capuchin Monkeys (*Cebus apella*) the authors reported that subordinate animals direct more visual attention towards dominant animals, rather than the other way around (Pannozzo et al., 2007). In another study, low-and medium-ranked Chimpanzees were found to learn how to perform a specific task by copying from the dominant members of the group (Kendal et al., 2015). Similarly, in a recent study from our lab (Gachomba et al., 2022), we have implemented the mFC to study the influence of social hierarchy in prosocial choice, i.e the propensity

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to make actions that benefit others. Interestingly, we have found that contrary to gender or the degree of familiarity, social hierarchy was a potent modulator of prosociality. Briefly, in a two-choice task where rats can provide rewards to a conspecific in the absence of self-benefit, dominant individuals showed not only faster but also higher levels of prosocial choices towards their submissive counterparts. By performing a very detailed and sophisticated quantification of behavior, we have also found that the behavior displayed by the submissive individuals has a strong impact on the prosocial decisions of the dominant animals. These results are a good example of the effectiveness of the task described here (mFC) at studying interactions between individuals within already established hierarchies. Moreover, the use of our modified food completion test to classify our animals' social status, which contrary to other dominance test does not induce agonistic interactions, did not have an impact on the normal development of helping behavior in the prosocial choice task. These studies and the development of tools such as the one described here pave the way for future work aimed at understanding the neural mechanisms by which animals recognize the social status of other individuals and modulate their actions accordingly.

Understanding the circuits behind the establishment and maintenance of social hierarchies, together with behavioral studies targeted at evaluating how social rank can affect the immune system, anxiety and depression will provide a unique opportunity to develop new therapeutic approaches to some conditions. Particularly, these might be especially relevant at treating some of the more common social impairments in our society nowadays, such as autism spectrum disorders.

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# 6 | Conclusions

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# Chapter 6 | **Conclusions**

Here we present a tool that allows, for the first time, to identify established social hierarchies in rats. After the precise description and validation of the modified Food Competition test presented here, this task is very easy and cheap to implement in any behavioral laboratory, which we expect will substantially help accelerate discovery on the effects of established social hierarchies on brain function.

1. We developed and validated a new tool that allows us to investigate already established hierarchies in pairs of rats in their home cage and without the need to induce aggressive behaviors or to deprive the animals from resources.
2. The introduction of a small conflict in the home cage, where consumption of appetitive food is only possible for one animal at a time, led to subtle competition which translated into increased consumption by the dominant rat and allowed us to identify behavioral patterns observed during the task that, to our knowledge, have not been reported before.
3. This detailed behavioral quantification of behaviors performed in this task allowed us to compare them to behaviors displayed in other social tasks where animals competed for access to sucrose solution, or to more standardized procedures, such as a food and water competition under deprivation states or the tube test and evaluate reliability across the different protocols.

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4. Social hierarchy evaluated through the modified Food Competition tests is a reliable trait in pairs of animals
5. Body weight is not predictive of social status
6. The number of wins in the tube test is not a good predictor of social status in rats

These results thus underscore the necessity of including multidimensional analysis of behavior and the importance of taking into account qualitative measurements when describing social interactions.

Our work adds to recent efforts towards the development of ethologically relevant paradigms (Winiarski et al. 2021), where rich information of the social interactions of rodents can be obtained with minimal intervention of the experimenters but in controlled and highly quantifiable laboratory settings.

Although we acknowledge that the general tendency in the field is to favor the use of mice as a model species, due to the large advantages related to its genetic tool box, cross-species validation is of utmost necessity, and validation of tools in different species an urgent need in Neuroscience. On the other hand, rats display much more sophisticated social and non-social behaviors compared to mice, and the development of tools such as CRISPR/Cas9 and state-of-the-art viral approaches, are making more accessible the precise monitoring and manipulation of neural circuits in other species. Taking all together, we foresee a drift towards a diversification of the species used in Neuroscience in the following years, and validation of ethologically relevant behavioral tools within the ecology of each species is needed.

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# 7 | Conclusiones

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# Chapter 7 | Conclusiones

Presentamos una herramienta que permite, por primera vez, identificar jerarquías sociales establecidas en ratas. Proporcionamos la descripción y validación precisas de la nueva prueba de competencia por comida, una tarea de fácil y económica implementación en cualquier laboratorio. Esperamos que este hecho ayude sustancialmente a acelerar el descubrimiento de los efectos de las jerarquías sociales establecidas en la función cerebral.

1. Hemos desarrollado y validado una nueva herramienta que permite investigar jerarquías ya establecidas en parejas de ratas, evaluadas dentro de su propia jaula y sin necesidad de inducir comportamientos agresivos o privar de recursos a los animales.
2. La introducción de un pequeño conflicto en la jaula, donde el consumo de comida sólo es posible para un animal a la vez, condujo a una competencia sutil que se tradujo en un mayor consumo por parte de la rata dominante y que nos permitió identificar patrones de comportamiento que, hasta donde sabemos, no se han descrito antes.
3. Esta cuantificación conductual detallada de los comportamientos realizados durante la tarea nos permitió compararlos con los comportamientos en otras tareas sociales en las que los animales competían por el acceso a la solución de sacarosa, o con procedimientos más estandarizados, como una competencia de comida y agua en estados de privación o el test del tubo, para poder evaluar la fiabilidad en los diferentes protocolos.

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4. La jerarquía social evaluada a través de la prueba de competición por comida es una medida fiable y estable en pares de animales
5. El peso corporal no es predictive del estatus social
6. El número de veces que un animal gana en el test del tubo no es un buen predictor del estatus social en ratas

Estos resultados subrayan, por tanto, la necesidad de incluir un análisis multidimensional del comportamiento y la importancia de tener en cuenta medidas cualitativas a la hora de describir las interacciones sociales.

Nuestro trabajo se suma a los esfuerzos recientes hacia el desarrollo de paradigmas etologicamente relevantes (Winiarski et al., 2021), donde se puede obtener información rica de las interacciones sociales de los roedores con una intervención mínima de los experimentadores pero en entornos de ambientes controlados y altamente cuantificables.

Aunque reconocemos que la tendencia general en el campo es la de favorecer el uso de ratones como especie modelo, debido a las grandes ventajas relacionadas con sus herramientas genéticas, la validación de herramientas y procesos en diferentes especies una necesidad urgente en Neurociencia. Por otro lado, las ratas muestran comportamientos sociales y no sociales mucho más sofisticados en comparación con los ratones, y el desarrollo de herramientas como CRISPR/Cas9 y enfoques virales de última generación están haciendo más accesible el monitoreo y la manipulación precisas de circuitos neurales en otras especies. En conjunto, prevemos una deriva hacia una diversificación de las especies utilizadas en Neurociencia en los próximos años, y la validación de herramientas conductuales etológicamente relevantes dentro de la ecología de cada especie, es un punto necesario.

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# A | Appendix

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# Novel competition test for food rewards reveals stable dominance status in adult male rats

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**Social hierarchy is a potent modulator of behavior, that is typically established through overt agonistic interactions between individuals in the group. Once established, social ranks are maintained through subtler interactions allowing the redirection of energy away from agonistic interactions towards other needs. The available tasks for assessing social rank in rats allow the study of the mechanisms by which social hierarchies are formed in early phases but fail to assess the maintenance of established hierarchies between stable pairs of animals, which might rely on distinct neurobiological mechanisms. Here we present and validate a novel trial-based dominancy assay, the modified Food Competition test, where established social hierarchies can be identified in the home cage of non-food deprived pairs of male rats. In this task, we introduce a small conflict in the home cage, where access to a new feeder containing palatable pellets can only be gained by one animal at a time. We found that this subtle conflict triggered asymmetric social interactions and resulted in higher consumption of food by one of the animals in the pair, which reliably predicted hierarchy in other tests. Our findings reveal stable dominance status in pair-housed rats and provide a novel tool for the evaluation of established social hierarchies, the modified Food Competition test, that is robust and easy to implement.**

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Social hierarchy is a multidimensional trait that has a profound impact on emotion and cognition, not only for humans[1,2] but also other social species (see[3] for review), having important consequences for social organization, survival, reproductive success, and health of animals in a group[4]. Indeed, adapting behavioral responses based on the social status of the interacting partner can be cost-effective and, in some cases, a crucial survival strategy. The most established view is that social hierarchy is built upon aggressive interactions[5], and serve as a mechanism of resource management and minimization of energy expenditure by groups of animals: once a hierarchy is established, priority access to resources is organized allowing the reduction of aggressive levels between the interacting animals[6]. Following this view, the behavioral paradigms available for measuring social hierarchy in laboratory animals are based in the nature of agonistic interactions while defending access to resources, whether a sexual partner, food or water when they are scarce, or the defense of a territory (see[3] for review).

Of note, most recent advances on the identification of the neural circuits underlying the establishment of social hierarchies have been performed in mice, as a reflection of a general tendency in the field which favors the use of this species due to the exceptional genetic tools available[7,8]. However, important contributions have been also performed using rats[9–11], and importantly, Norway rats live in complex social groups in the wild. This, together with the fact of being a model system amenable to monitoring, mapping and perturbation of neuronal circuits, has motivated a wave of recent laboratory studies uncovering the diversity and sophistication of rat's social skills (see[12] for review). Regarding their social status, the visible burrow system (VBS) has been widely used to study the formation of hierarchies in large groups of animals, where mixed-sex rat groups living in a complex environment compete chronically for territory and resources[13]. The VBS generates very rich behavioral data sets but is difficult to implement in most laboratories, hence, other behavioral tasks are commonly used, where animals compete for food or water under deprivation states[14–17]. Typically, social isolation of variable durations is performed prior to testing as a means to increase territoriality, favoring strong agonistic interactions during the establishment of new hierarchies. Therefore, these tasks evaluate how a new hierarchy is established between pairs of unfamiliar, frequently isolated animals, in neutral arenas where subjects display very evident agonistic behaviors to establish dominance.

However, there are no tools available enabling to assess already established hierarchies. Focusing on the early establishment of a hierarchy is neglecting a very important and rich part of this type of social interactions:

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how are they maintained in stable conditions. The establishment of social hierarchy might not rely on the same mechanisms as the expression of dominance when a hierarchy is already established, and recent reports in mice indicate that this might be indeed the case[18,19]. However, to our knowledge, the study of the possible differences between de novo and already established social hierarchies in rats has been virtually unexplored. To this end new behavioral paradigms that evaluate social status of animals living in stable dyads are urgently needed. Preferably, the evaluation of the hierarchy would avoid manipulations that could induce long-term alterations in internal state (i.e. isolation, deprivation) that, in turn, could affect how social interactions of interest are displayed. This is a challenge, as once hierarchies are established, agonistic behaviors are minimized and the opportunities to observe dominance interactions are reduced and likely subtle.

With this aim, we developed the modified Food Competition task, where a small conflict for access to a discrete number of palatable pellets was introduced in the homecage of non-deprived adult male Sprague–Dawley cage mate rats. We decided to focus our study on this specific population as most of the available literature on dominance and aggressive behavior in rats has been performed in adult males[13–17] being Sprague Dawley rats among the most used laboratory strains in behavioral neuroscience. In order to validate this new tool, we compared the dyads' behavior to that observed in other competition tests: (1) competition for 1% sucrose solution, (2) modified standard tests used in the field, that involve deprivation, where animals compete for food or water and (3) the Tube Test. We performed a detailed analysis of behavior in each test, and although no aggressive interactions were observed, our results indicate that stable hierarchies in rats are indeed detectable by the modified Food Competition test which is especially suited for their identification, based on its trial structure and the degree of conflict induced.

## Results

### Behavioral profiles differ across the social competition tests used.

To identify social status within pairs of cage mates we performed the modified Food Competition test and other behavioral tasks in which the animals needed to compete for resources, either for palatable pellets, sucrose solution, water or tube occupation (Fig. 1 and Supplemental Figure 1). All the tasks, except for the Tube Test, were performed in the animals' home cage. Animals displayed different behavioral profiles depending on the configuration of the test, whether it had a trial structure, the amount of reward available, and their internal state (satiated vs deprived) (Figs. 2 and 3). In order to control for possible effects of winning history we created two independent groups where we counterbalanced the order of the tests. No differences were observed between the groups, suggesting that hierarchy was already established (Kruskal–Wallis test comparing the duration of consumption in the two counterbalanced groups: modified Food Competition test (mFC) Day1: $X^2(2) = 0.026$, $p = 0.871$; mFC Day2: $X^2(2) = 0.007$, $p = 0.935$; SC Day1: $X^2(2) = 1.516$, $p = 0.218$; Sucrose Competition with continuous access to reward (SC) Day2: $X^2(2) = 1.904$, $p = 0.168$; Sucrose Competititon with Intermittent access to reward (SCI) Day1: $X^2(2) = 0.457$, $p = 0.499$; SCI Day2: $X^2(2) = 2.055$, $p = 0.152$; mFCD: $X^2(2) < 0.0001$, $p = 0.989$; Water Competition (WC): $X^2(2) = 0.293$, $p = 0.589$; Tube Test (TT) Day1: $X^2(2) = 0.00$, $p = 1.0$; TT Day2: $X^2(2) < 0.0001$, $p = 1.0$). Data from both groups was thus merged for the rest of the analysis.

In the modified Food Competition task (Fig. 2A, Fig. 3, Movie 1), the limited number of available food pellets (10 per trial) led to a very fast consumption of resources which lasted a few seconds (Fig. 3B-C). Interestingly, although pellets were consumed in the first seconds of each trial, animals displayed high levels of exploration of the feeder and displayed notable amounts of pushing, suggesting high expectation of reward (Fig. 3A). On the contrary, during the Sucrose Competition task, exploration of the reward location and pushing levels were low (Fig. 2B and Fig. 3A), but exploration of the bottle location increased when access to the sucrose bottle was presented in an intermittent manner (Fig. 2C and Fig. 3A).

As expected, modulation of internal state (food or water deprivation) affected the behavior of the animals. In the modified Food Competition task with deprivation (Fig. 2D) animals consumed the pellets faster (Fig. 3C, Wilcoxon signed rank tests against non-deprived Food Competition day 1: $z = -3.659$ $p = 0.0003$; day 2: $z = -3.136$ $p = 0.002$) and spent significantly more time investigating the feeder (Fig. 3A), although the amount of pushing did not differ from that displayed in non-deprived animals (Fig. 3A). When competing for access to water under deprivation (Fig. 2E), animals dramatically increased the time they spent drinking compared to consumption displayed in the other tests (Fig. 3A), performing long bouts of drinking and alternating between animals (Fig. 2E). Levels of exploration of the water bottle were low compared to the rest of the tests. Surprisingly, although the motivation to drink was high, revealed by the long water consumption time, pushing levels did not increase proportionally (Fig. 3A).

Finally, the dyads established a very stable winner/loser relationship in the Tube Test (Fig. 2F), where most of the animals that would start winning in the first trials would continue doing so over the remaining trials. This winner/loser structure was maintained across the two testing days (Fig. 2F middle panel, raster plot of winning history for all pairs). Interestingly, on the second day of testing, the loser partner of some pairs showed reluctance to enter in the tube (9% of the total trials), suggesting a strong subordination towards the partner. The time to solve the conflict in the Tube test, measured as the latency from the moment the partition at the center of the tube was removed until one of the rats was pushed out of the tube, rapidly decreased after the first trial, reaching then fast and stable latencies of around 3.5 s on average (Fig. 2F, right panel).

### Social hierarchy as priority access to resources.

We categorized the animals of each pair as dominant (D) or submissive (S) according to the amount of resources they would consume within each test (pellets eaten in the Food Competition tests, time spent drinking in the Sucrose or Water Competition tests, and the number of wins in the case of the Tube Test). According to this criterion, as expected, animals categorized as dominant consumed significantly more resources than their partners in every task (Fig. 4A–E) (Wilcoxon signed-ranks

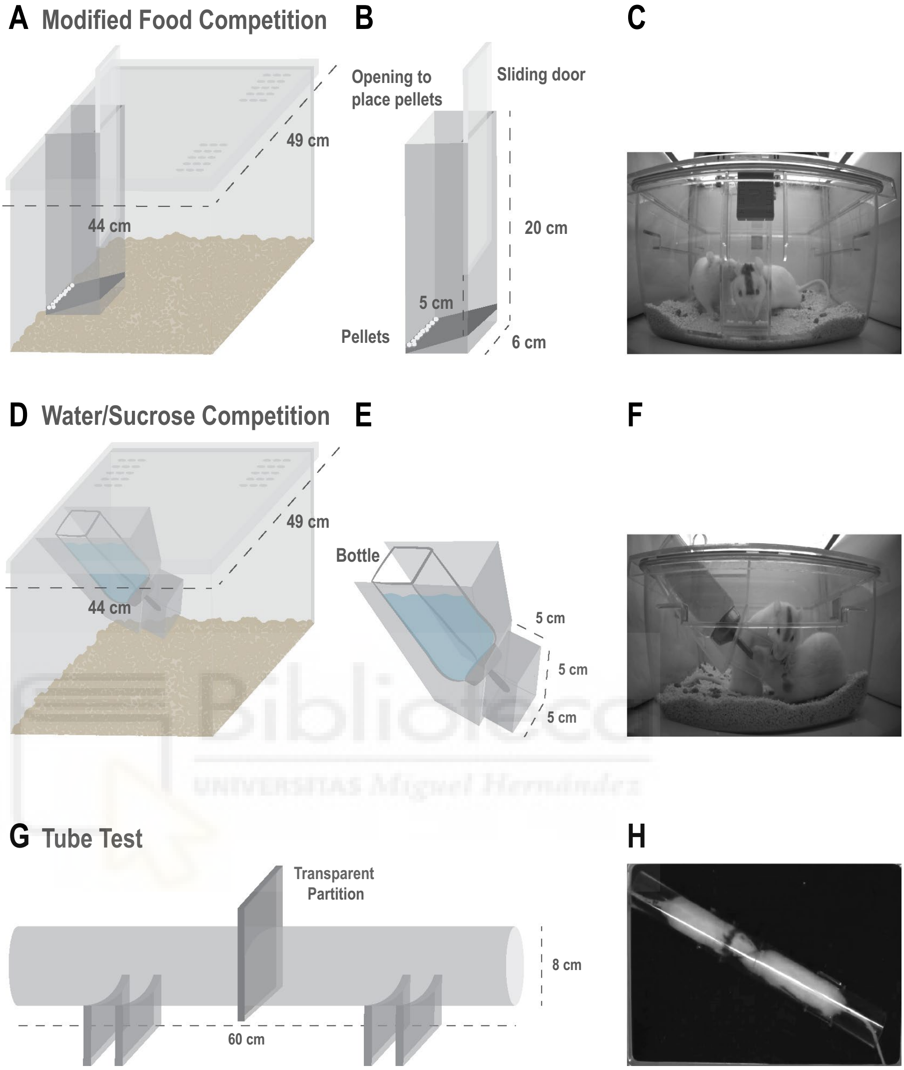

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**Figure 1.** Design and measurements of the behavioral tests used for identification of established dominance status. (**A**) Schematic illustration of the transparent lid and feeder in the modified Food Competition apparatus. (**B**) Detailed schematic illustration of the feeder used in Food Competition protocols (with and without deprivation) including the measurements to fit the bottom part of a Rat IVC cage (Sealsafe PLUS Green Line ventilated cages, Techniplast). The sliding door can be opened leaving a 5 cm high access which only allows one animal to eat at a time, and allows a trial structure for the task. A small opening on the top of the feeder allows to refill new pellets during inter trial interval. If adaptation of measurements to another type of home cage is needed, we advise to leave 3–4 cm from the end of the feeder and the bottom of the home cage. This prevents bedding to go into the feeder, which difficults the visibility of the pellets while animals are consuming. (**C**) Two cage mates can be observed at the feeder area, where one is consuming the pellets while the other is pushing to get access to the food. (**D**) Schematic illustration of the lid and bottle holder for the Water and Sucrose Competitions protocols (with continuous or intermittent access) protocols (**E**) Detailed schematic illustration of the bottle holder. A 5 × 5 cm restraining tube around the lick spout was created to prevent simultaneous access of both animals to the resource. (**F**) Two rats behaving in the Water Competition task, where one of the rats is drinking while the other is pushing to have access to the bottle (**G**) Schematic of the Tube Test with measurements used in this task, the transparent partition in the middle of the tube is removed at the beginning of a trial once both animals reach this area. Laser-cut acrylic holders were used to give stability to the set up (**H**) Two rats interacting inside the tube during the initial moments of a trial.

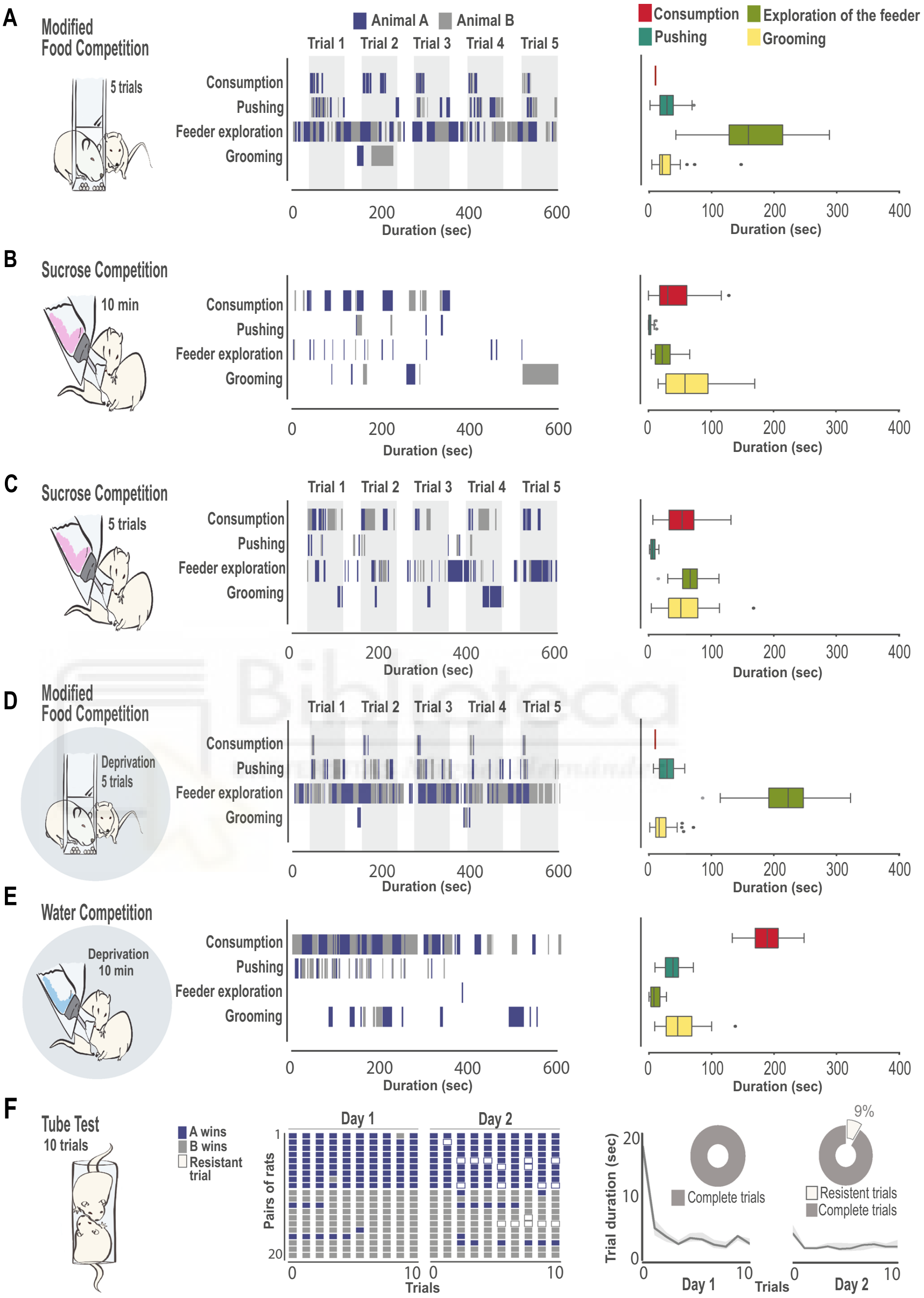
A
Modified Food Competition
5 trials
Animal A
Animal B
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Consumption
Pushing
Feeder exploration
Grooming
0
200
400
600
Duration (sec)
Consumption
Exploration of the feeder
Pushing
Grooming
0
100
200
300
400
Duration (sec)
B
Sucrose Competition
10 min
Consumption
Pushing
Feeder exploration
Grooming
0
200
400
600
Duration (sec)
0
100
200
300
400
Duration (sec)
C
Sucrose Competition
5 trials
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Consumption
Pushing
Feeder exploration
Grooming
0
200
400
600
Duration (sec)
0
100
200
300
400
Duration (sec)
D
Modified Food Competition
Deprivation
5 trials
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Consumption
Pushing
Feeder exploration
Grooming
0
200
400
600
Duration (sec)
0
100
200
300
400
Duration (sec)
E
Water Competition
Deprivation
10 min
Consumption
Pushing
Feeder exploration
Grooming
0
200
400
600
Duration (sec)
0
100
200
300
400
Duration (sec)
F
Tube Test
10 trials
A wins
B wins
Resistant trial
Day 1
Day 2
1
20
Pairs of rats
0
10
0
10
Trials
Trial duration (sec)
20
10
0
Complete trials
9%
Resistent trials
Complete trials
0
10
0
10
Day 1
Trials
Day 2

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**Figure 2.** Behavioral profiles change according to the specifics of each social competition test. (**A**) modified Food Competition test, (**B**) Sucrose Competition with continuous access to the bottle, (**C**) Sucrose Competition with intermittent access to the solution, where animals could only drink during 2 min and the bottle was absent during 1 min inter-trial interval; (**D**) modified Food Competition test with deprived animals, (**E**) Water Competition test, (**F**) Tube test. For all tests, left panel shows the schematic representation of the task. Cartoons with a shaded circle as background indicate that tests were performed under deprivation. (**A**–**F**) middle panel: raster plots showing frequency and duration of behaviors of interest in an example pair of animals (animal A in blue, animal B in grey), except for (**F**) where the animal wining each trial is depicted for the 20 pairs of animals in all trials. White coloured trials in the Tube Test correspond to "resistant trials" where the loser of the pair resisted to enter the tube and trial could not be completed. In those tasks with a trial structure, (**A**, **C**, **D**) grey shaded areas indicate the moments where access to the resources where available, while intertrial interval, during which animals could explore the feeders but not access the food or sucrose bottle, are indicated with white background. (**A**–**E**) right panel: Boxplot representation of the duration of each behavior of interest for each individual animal. When more than one day of testing was performed, data represents average of the two days. For those tests with a trial structure, values represent behavior throughout the entire test period averaged over the 5 trials (or 10 trials, when two days of testing were performed), being one trial defined as the last 40 s of the intertrial interval and first 80 s of access to reward. Median, quartile 1 and 3 are represented, whiskers indicate minimum and maximum values and extreme values are signaled as a dot. Consumption in red, Pushing in dark green, Exploration of the Feeder in light green, Grooming in yellow. (**F**) right: Trial duration in the Tube Tests in the two testing days. Black line represents the median duration that animals took to push their partner out of the tube, and grey shadows depicts 95% confidence interval. As an insert in each day, pie charts represents the percentage of trials that were completed. Note that 9% of trials in the second day of the Tube Tests were not completed as one animal (the loser) refused to enter the tube.

for consumption in mFC: $z = -3.92$, $p < 0.0001$; SC: $z = -3.82$, $p < 0.0001$; SCI: $z = -3.92$, $p < 0.0001$; mFCD: $z = -3.83$, $p < 0.0001$; WC: $z = -3.92$, $p < 0.0001$). In the same line, one animal always won more encounters than the other in the Tube Test (Fig. 4F, Wilcoxon signed-ranks for TT Day1: $z = -4.06$, $p < 0.0001$ and for TT Day2: $z = -4.12$, $p < 0.0001$), with the exception of one pair of animals in Day 1 and another in Day 2, where both animals of the pair won the same number of trials, thus no categorization as dominant or submissive was possible in these cases.

We decided to investigate how body weight would relate to dominance in established hierarchies of rats. Intriguingly, we did not observe differences in the body weight between dominant and submissive animals in any of the behavioral tests where animals would compete for food, sucrose or water (Fig. 4A–F; Paired Sample T-test for weights in mFC: $t = 1.208$, $p = 0.242$; SC: $t = -0.309$, $p = 0.761$; SCI: $t = -0.843$, $p = 0.410$; mFCD: $t = 0.522$, $p = 0.608$; WC: $t = 0.067$, $p = 0.947$). In contrast when they had to compete for territory for the first time in the Tube Test, a significant relationship between dominance and body weight was observed (Fig. 4F; Paired Sample T-test for weights in TTDay1: $t = 2.529$, $p = 0.021$). In conclusion, although no relation was observed between social hierarchy and body weight in the rest of the tasks, this was not the case in the Tube Test, where bigger rats had a higher probability of winning in the first encounters.

We then asked whether hierarchy following this criterion, amount of resources consumed, would also translate to differences in other behaviours within each test. General exploration of the resource location during the whole session did not differ between dominant or submissive animals when consumption in the same test was taken as criterion. However, we did observe that submissive animals would spend more time self-grooming in the modified Food Competition test ($z = -2.016$, $p = 0.044$) and that time spent pushing was modulated by dominance in some tests. Dominant animals tended to display more pushing in the Sucrose Competition with intermittent access ($z = -1.867$, $p = 0.062$) and surprisingly, submissive animals were the ones that pushed more in the Sucrose Competition with continued access to the bottle and the Food Competition under deprivation (SC: $z = -2.722$, $p = 0.006$ and FD $z = -3.472$, $p = 0.0005$). As this observation was unexpected, we next explored this further.

**Dominant rats are more efficient displacing their partners to gain access to resources.** Although the time animals spent pushing their partner should be a good measure of amount of conflict between the interacting animals, qualitative differences might be more informative of dominance status. One possibility is that even if a dominant rat pushes less often, its thrusts may be more successful in removing the partner from the resource. Thus, we calculated for each animal the percentage of successful pushing from the total number of pushing bouts, i.e. the fraction of pushing epochs that actually displaced the partner and allowed access to the resource.

Strikingly, dominant animals were more successful in displacing their partners in the modified Food Competition under deprivation, while submissive animals would push often but failed to displace their partner (Fig. 5, Wilcoxon signed rank test $z = -1.979$ $p = 0.048$). However, this was not observed in the Water Competition test (WC: $z = -0.821$ $p = 0.411$) nor in the tasks not involving deprivation (mFCD: $z = -0.933$ $p = 0.351$; SC: $z = -1.014$ $p = 0.310$; SCI: $z = -0.563$ $p = 0.573$). The lack of differences in successful pushing between dominant and subordinate rats in the water competition test, could result from a limited window within the test where asymmetric interactions are apparent. To investigate this possibility, we identified the epochs with highest conflict in the Water Competition test, i.e., those where the most drinking and pushing behavior was observed for each pair of animals (Supplemental Figure 2A). We then classified the animals as dominant and submissive according to the duration of drinking in that epoch and quantified pushing displayed by either dominant or subordinates. This new categorization led to a change in the hierarchy in 55% of the pairs (11 out of 20). Here, although submissive

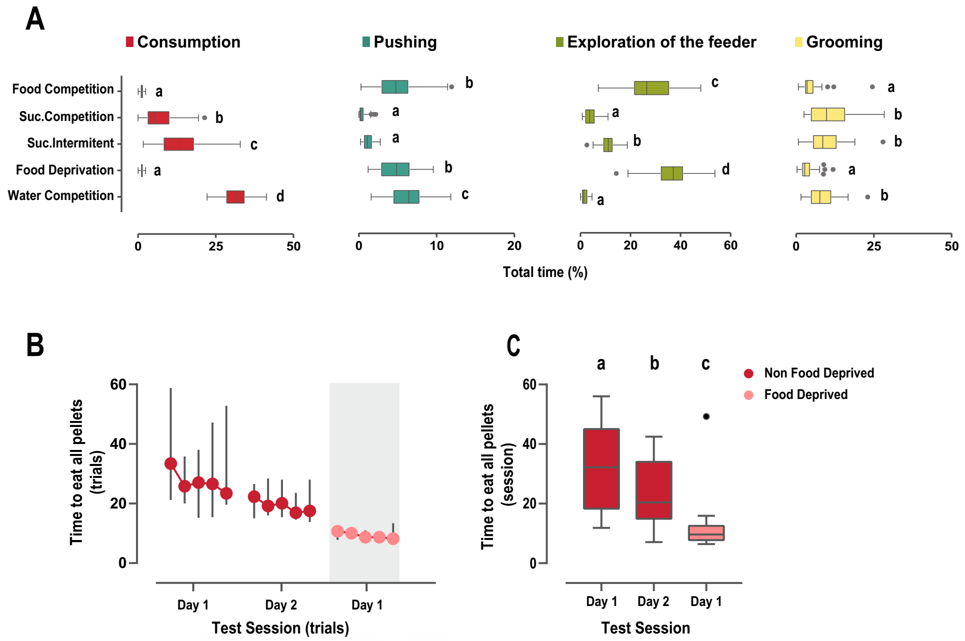


**Figure 3.** Descriptives of the behavioral analysis for all social tasks involving competition for resources. (**A**) Comparison of the behaviors of interest displayed by each animal across all behavioral tests. Data is presented as percentage of time performing a specific behavior related to the total duration of the task, enabling comparison between tests differing on duration. For those tasks with a trial structure, consumption corresponds to the first 80 s of reward availability for each trial, while pushing, exploration of the feeder and grooming durations correspond to the displays performed during the 40 s before and 80 s after reward availability. When more than one day of testing was performed, data represents average of the two days. Each competition test provided very different behavioral profiles, where the duration of consumption and exploration of the feeder or pushing behaviors to access the resources differed clearly over the tests. Note that pellets in the modified Food Competition test were consumed during the first seconds, but still high levels of pushing and exploration of the feeder where observed. However, the time spent consuming sucrose and the levels of pushing for accessing the bottle were low. Boxplots depict the median and quartile 1 and 3, whiskers indicate minimum and maximum values and extreme values are signaled as a dot. Consumption in red, Pushing in dark green, Exploration of the Feeder in light green, Grooming in yellow. Letters denote statistically significant differences between behavioral tests after one-way ANOVA with Tukey posthoc comparisons. Time spent grooming was not normally distributed, thus non-parametric analysis was performed. (**B**) Latency to eat all pellets for each trial is shown in the Food Competition tests, with or without deprivation where median and 95% CI for each trial are represented. Animals only took around 20 s to eat all pellets available, decreasing this latency when food deprived. Shaded background indicates session performed under food-deprivation. (**C**) Boxplots representing the average time to eat all pellets over testing days. Wilcoxon rank test revealed that latency to eat the pellets decreased on the second day of testing of the modified Food Competition test, and did further under deprivation state. $^{**}p < 0.01$, $^{***}p < 0.001$.

animals spent longer time pushing their cage mate (Fig. 5E middle plot, $z = -3.920$, $p < 0.0001$), again dominant animals displayed higher efficiency in displacing their subordinates, being successful practically 100% of the times (Fig. 5E plot on the right, $z = -3.627$ $p = 0.0002$). This fine grained behavioral analysis, where pushing is categorized into successful or unsuccessful, thus revealed that although in some tasks submissive animals displayed higher duration of pushing, they rarely managed to get the access to the resource, being dominant rats more successful to displace their cage mates.

**Social hierarchy as a stable trait between tests.** To examine whether social hierarchy is a stable trait in familiar animals, we analyzed reliability across the performed tests. To this end, we computed the *Dominance Index* (DI) for each test where the difference in resource consumption across partner animals was normalized by the summed resource consumption of the pair. A DI close to 0 means that animals did not have a strong hierarchy. Positive values indicate that animal A consumed more, while negative values indicate that animal B was the one having priority access to resources or won more trials in the Tube Test. Provided that behavioral measures were stable across testing days (Supplemental Figure 3) data was averaged for this analysis.

DI for positive reinforcers led to a highly variable distribution across dyads, where in some dyads animals would strongly differ in their consumption and in others differences were subtle (Fig. 6A). This was not the case in the Water Competition, where DI was mostly around 0 for all animals, indicating that both animals drank very similar amounts of water during the test. In contrast, the Tube Test gave very polarized DIs, where most of the pairs had one animal winning almost 100% of the trials. To refine our understanding of the hierarchy in the Tube Test, we computed a *Conflict Resolution Index (CRI)*, which would take into account not only who won a trial, but also how long it took for the conflict to be resolved (i.e., the latency for one of the animals to be pushed out of the tube). The conflict resolution index revealed a more continuous and fine-grained measure of hierarchy strength in each pair (Fig. 6B).

If social hierarchy was a stable trait, then dominance indexes should be correlated across tests. We found DIs from tests with competition for positive reinforcers were positively correlated (Fig. 6C). Moreover, the modified Food Competition test correlated with the DI index in the Tube Test, when conflict resolution time was taken into account. Water competition DI was not correlated with any of the other tests. Water deprivation could have challenged the homeostasis of the interacting animals bringing them to a very different internal state that disrupted the stable hierarchies revealed by the other tests. Alternatively, computing the DI for the water competition test using the whole test duration, may have diluted differences in water consumption across animals within the pair. Hence we re-calculated DI for different time windows, just as we observed for the pushing behavior (see above). No correlation was found between water competition test and the other test when other time windows were used to compute DI (Supplemental Figure 2B). Still, we have shown that during this test, the dominant animals accessed the water bottle in a qualitatively different manner, by successfully displacing their subordinates (Fig. 5E). This highlights the importance of considering multiple behaviors simultaneously and suggests that social status in the Water Competition task could be better assessed by finer behaviors rather than water consumption.

### Modified Food Competition tests as a tool to measure stable hierarchies.

Last, we calculated for each task a *Conflict Index* as a measure of the degree of conflict that our manipulations introduced in the home-cage, by dividing the time animals spent pushing in one test by either the time spent consuming in case of the Sucrose and Water Competitions, or latency to eat all rewards in the case of the modified Food Competitions. Food competition with and without deprivation were the tasks with higher conflict, as animals displayed high amounts of pushing and the time available to eat the resources in each trial was very short (Supplemental Figure 4A). Since the modified Food Competition test yielded significant levels of conflict but did not involve food deprivation, we next evaluated whether attributing dominance within dyads using this test, would allow correct identification of the dominant rat in the other tests. Specifically, animals were classified as Dominant or Submissive according to their DI in the second day of testing in this task, as the conflict index was higher in this day (Supplemental Figure 4B). Those pairs where the difference of the number of pellets eaten was small (less than 5% difference compared to equal consumption between the animals) were considered to have an unstable or unclear hierarchy ($n = 4$) and were not included in this last analysis. As expected, dominant animals consumed more pellets in the Food Competition test (Fig. 7A, Wilcoxon signed-ranks of average Consumption in mFC of both days $z = -3.362$, $p = 0.001$). Interestingly, they also successfully pushed their partner away from the feeder more ($z = -2.275$, $p = 0.023$), explored the feeder more during inter the trial interval, where the pellets were present but not accessible ($z = -2.844$, $p = 0.004$), and groomed less than their submissive pairs during the trial period ($z = -2.275$, $p = 0.023$). Moreover, dominant animals according to the modified Food Competition did consumed more sucrose, both when access was continuous (Fig. 7B, SC $z = -2.499$, $p = 0.012$) or intermittent (Fig. 7C, SCI $z = -2.275$, $p = 0.023$). Attributing dominance found in the modified Food Competition, to the same test run under food deprivation, revealed similar dominance interactions (Fig. 7D, Consumption in mFCD $z = -2.619$, $p = 0.009$; Successful Pushing in mFCD $z = -2.534$, $p = 0.011$; Anticipatory exploration of the feeder in mFCD $z = -2.902$, $p = 0.034$, Anticipatory grooming in mFCD $z = -1.992$, $p = 0.046$). Moreover, dominant animals also successfully pushed away their partner more in the Water Competition test (Fig. 7E, $z = -2.379$, $p = 0.017$). In the case of the Tube Test, the amount of wins did not differ between dominant and submissive animals, as determined by the modified food competition test (Supplemental Figure 4C). In Fig. 3 we show that differences in body weight affects the probability of winning in the Tube Test, especially on the first day. This was however not the case for the other tests. Thus, weight may dominate the outcome of the tube test, overshadowing the dynamics of social interactions within stable pairs. Therefore, we decided to examine the relationship between the hierarchy in the modified Food Competition and the Tube Test while controlling for the effect of the body weight. To this end, we first regressed the number of pellets eaten against the animals' body weight and calculated the residuals. Next, we calculated, in the same manner, the residuals when regressing out body weight from the conflict resolution index (see above) of the first day of the Tube Test. Interestingly, the linear regression of these residuals was statistically significant ($p = 0.010$) indicating that indeed, when correcting for the effect of body weight, consumption in the modified Food Competition predicts who will win in the first interactions of the Tube Test (Fig. 7F). Thus, dominant animal in the modified Food Competition test also won more trials in the Tube Test when the influence of the body weight was controlled for.

## Discussion

Here, we developed and validated the modified Food Competition task, a new tool designed to provide, for the first time, the means to evaluate established hierarchies in pairs of rats, and with the added value of doing so in the home cage, without inducing aggressive behaviors nor requiring food-deprivation.

In our task, cage mates with a stable hierarchy competed for access to positive reinforcers. The introduction of a small conflict in the home cage, where consumption of appetitive food is only possible for one animal at a

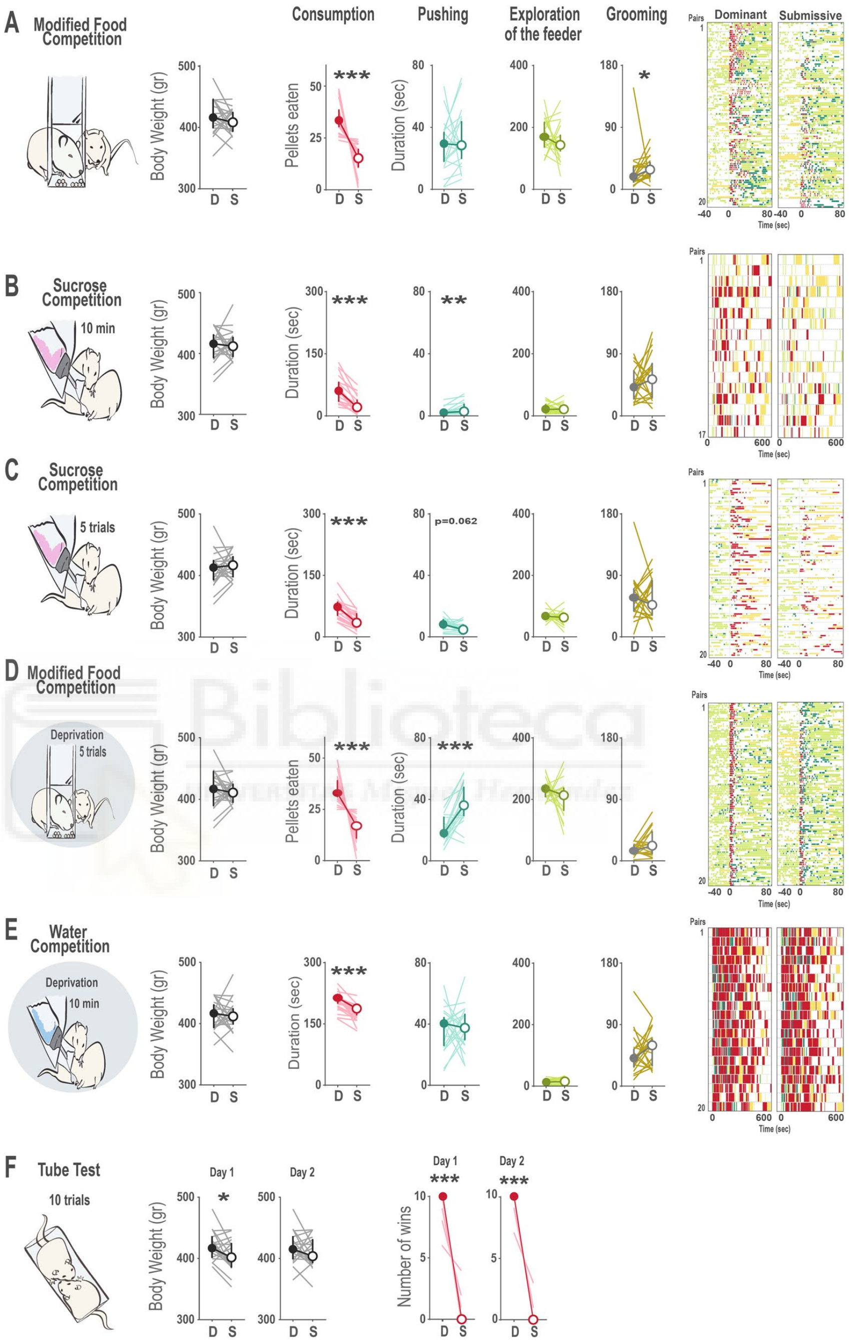
**Figure 4.** Categorization of animals as dominant and submissive according to behavior within each task. (**A**–**E**) Behavioral profiles when consumption of the resources within each test is used to define dominant and submissive animals. For all panels, a schematic cartoon with experimental design is provided. Cartoons with shaded background indicate that tests were performed under deprivation. For each test, differences between dominant and submissive animals are represented regarding body weight, time spent consuming the resource (or number of pellets eaten in the case of the food competition tasks), duration of pushing, exploration of the feeder and grooming is provided, where median and 95% interval confidence are displayed and individual values are showed with light lines. Color coded raster plots of behaviors of interests display raw data in a testing session, where pairs of animals are sorted according to the stronger differences in hierarchy for each test. For those tests with a trial structure, the five trials of each pair are plotted in separate lines, all aligned to time 0 (when access to reward was possible) and reflecting the behavioral data of the 40 s before and 80 s after that moment. When more than one day of testing was performed, data represents average of the two days and rasters display data of day 2. In those cases where the animals of one pair had identical values in the categorizing criteria (same amount of pellets eaten, duration of consumption or trials won in the Tube Test), no hierarchy was assumed for this specific task, and these pairs were removed from this analysis (one pair for the Sucrose Test, one for the Tube Test and one for the Food Competition under deprivation). For all graphs and rasters, Consumption is represented in red, Pushing in dark green, Exploration of the Feeder in light green and Grooming in yellow. Time of consumption in each test was significantly different between animals defined as dominant or submissive, and in some cases, these differences also were translated to a differential amount of grooming or pushing behavior. Interestingly, no differences in body weight were found in these tests, indicating that priority access to resources in established hierarchies is not influenced by the size of the animals. (**F**) In the Tube Test, the amount of winnings was clearly different between dominant and submissive rats, and was related to differences in body weight, which reached significance in the first days of testing, being bigger animals those more likely to win. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

time, led to subtle competition which translated into increased consumption by the dominant rat. This measure reliably predicted differences in other behaviors observed during the competition test. These differences held across the different social tasks evaluated, such as competition for sucrose solution or food and water competition under deprivation. Importantly, testing was performed in the home cage of the animals, thus minimizing the influence of the experimenter in the social interactions displayed, and interference by other factors such as anxiety or exploratory behaviors usually displayed in novel environments. Conflict in the modified Food Competition increased over days, providing the second day a clearer picture of the established hierarchy, most probably because during the first of competition in the home cage, differences in attention to the appearance of the conflict situation might be modulating the interactions. In future experiments, it might be desirable to include a third day of testing to confirm whether conflict continues increasing or is stable after the second day.

Interestingly, although competition was observed and hierarchy could be identified, no agonistic behaviors (biting, boxing, keep down, lateral threat) were observed between the cage mates in our task. This is in accordance with previous reports indicating that once social hierarchies are established, the number of agonistic encounters decreases[6]. Moreover, the fact that no food deprivation was required, nor aggressive behaviors were observed, can be considered as an added value of our task. Aggressive behaviors induce robust stress reactions in rodents[20], and food deprivation, although widely used in neuroscience to increase motivational salience during behavioral testing, modifies internal state[21] and social behavior[22]. Minimizing the possible long term effects of these manipulations on the internal state of tested animals is particularly important for studies regarding the impact of social hierarchy on other behavioral, physiological and brain functions.

Moreover, the fine behavioral characterization across the different social tasks used, allowed us to identify very interesting social patterns, that to our knowledge have not been reported before. In this direction, we describe that social hierarchy tasks with a trial structure (modified Food Competition tasks and Sucrose Competition with Intermittent access), where access to resources was presented in a repeated and intermittent manner, promoted competition. Moreover, we describe that when measuring conflict, the time spent pushing by the animals is not indicative of dominance, but rather how efficient an animal is to displace its partner while pushing. Although the dominance index in tasks that involved competition for positive reinforcers reliably revealed the strength of social hierarchy within a pair, this was not the case in the Water Competition test under deprivation, where the animals drank around the same amount of water (dominance index around 0), nor in the case of the Tube Test, where very polarized results were observed. In the Water Competition task, animals tended to perform long bouts of drinking, alternating consumption until both animals were satiated, which resulted in very similar final consumption levels in both animals. We asked whether an analysis with finer temporal resolution could unveil structured dynamics of water consumption in this task, such that asymmetries across rats in the dyad would emerge during bouts of conflict, and these asymmetries would correlate with other social competition tests. However, to the extent that we could quantify, we did not observe such a pattern. Strikingly, we found that although consumption between animals was very similar, other behaviors displayed while approaching the water bottle were clearly different. Although submissive animals in this task spent more time pushing their cage mate in moments of high conflict, they were rarely successful in accessing the water if the dominant was already drinking. Indeed, the ability of dominant rats to successfully displace their partners from the resource location was not limited to the Water Competition, being reliable in those tests where more conflict (pushing) was observed.

In the case of the tube test, we showed that computing conflict resolution index which takes into account multidimensional behavioral measures, such as the winner of a trial and conflict duration (latency for one of the animals to be pushed out of the tube) revealed a more granular view of the strength of social hierarchy in this



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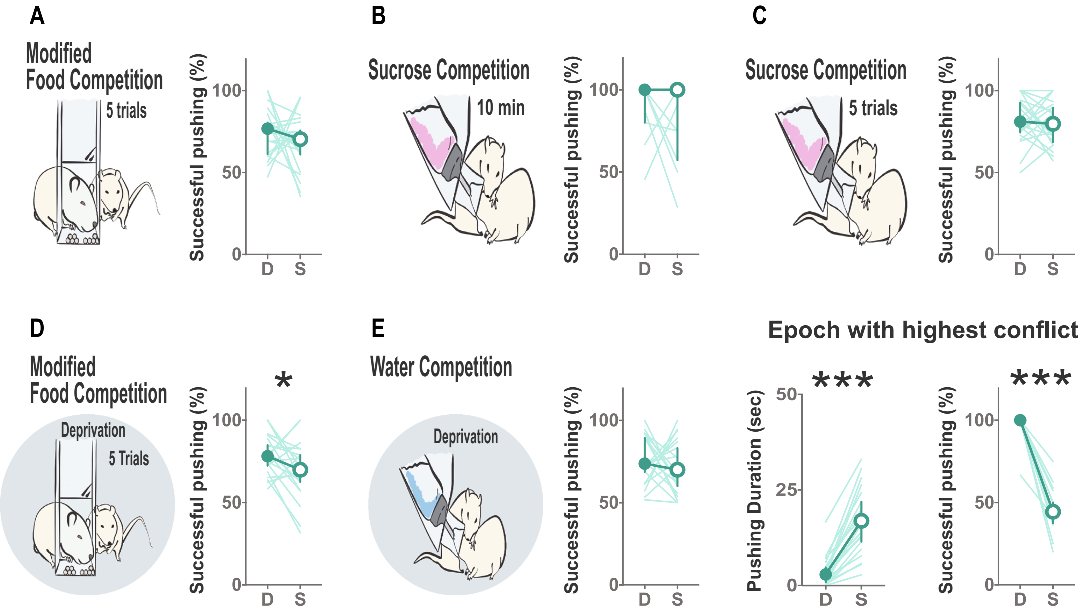
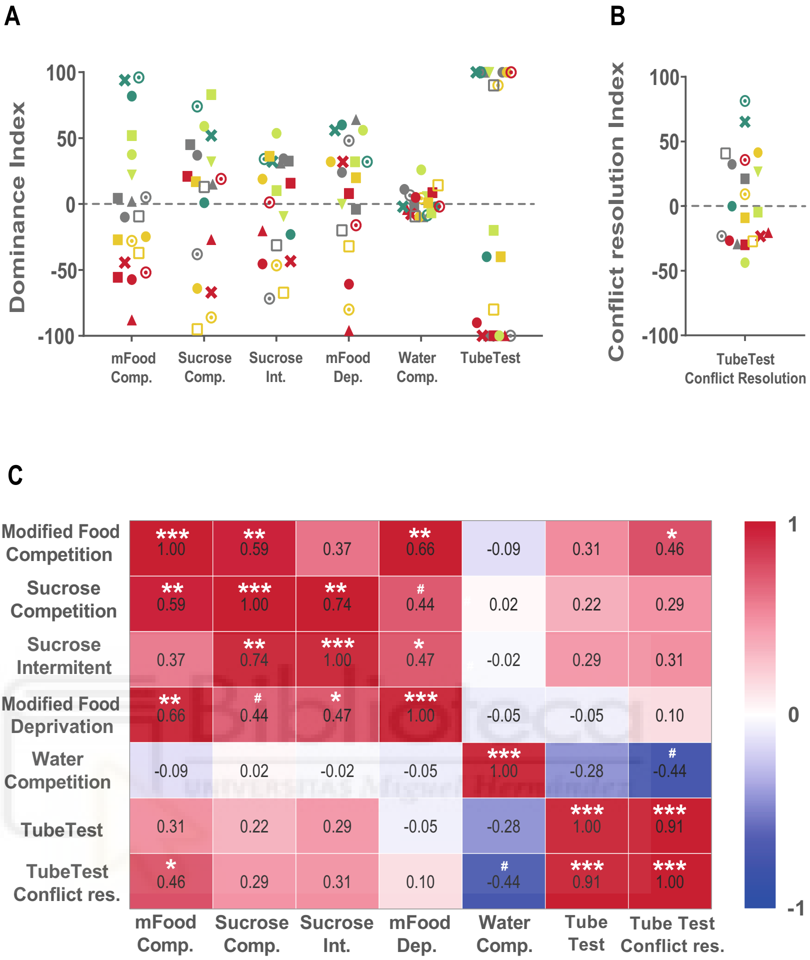


**Figure 5.** Dominant animals are more successful in displacing their subordinate partners to gain access to resources when in deprivation states. Dominant animals showed a higher percentage of successfully pushing away their subordinates while gaining access to the resources in moments with high conflict. Percentage of successful pushing did not reach significance in the modified Food Competition (**A**), Sucrose competition with continuous (**B**) or Intermittent access to the bottle (**C**) but was significantly different in the Food Competition under deprivation (**D**). No differences were observed in the efficiency of pushing behavior in the Water Competition task (**E**). However, when assessing these differences in the moments with highest conflict in this test, defined as the bout where intense drinking was displayed and high levels of pushing behavior were observed, submissive animals spent more time pushing, but dominant animals were almost always successful to displace their partners in every bout of pushing. D: dominant, S: submissive according to the consumption in each test. Median, 95% CI and individual values for all animals are represented. $*p < 0.05$, $***p < 0.001$.

test. In addition, this conflict resolution index correlated to the social hierarchy observed in the modified Food competition test, especially when taking into account the body weight of the animals. It is important to note that the final output of the tube test (i.e. who was the winner) did not correlate with other measures of social hierarchy. Although this test is widely used in mice (see[3] for review), our data indicates that in rats this test might not be an appropriate tool to measure established hierarchies, as who manages to push out of the tube its partner is largely affected by the body weight of the interacting animals. However, the conflict displayed inside the tube in the first encounters (incorporating the latency to win in the conflict resolution index), might be a better measurement of established hierarchies for rats. These results thus underscore the necessity of including multidimensional analysis of behavior and the importance of taking into account qualitative measurements when describing social interactions. Furthermore, although it might be surprising that the tube test is not a reliable measure of established hierarchy in rats, important differences in social behavior between mice and rats are starting to be reported[22,23]. Rats are more socially tolerant, and less hierarchical compared to mice. This might be related to their natural behavior in the wild, where rats are often observed in larger groups[12]. These ecological differences should be carefully taken into account when borrowing tools from one species to the other[24].

Finally, body weight has been largely assumed to be a good indicator of social hierarchy in rats[25], although this has not been replicated in mice[19,26]. This view was inspired by classic works[27] and the seminal contributions in the field upon the development of the visible burrow system[13]. However, our data, obtained in animals maintained with ad libitum access to food and water, did not support this observation, as dominant and submissive animals showed no differences in body weight, with the notable exception of the Tube Test. It is important to take into account that in our experiments the difference in body weight between the animals of a pair was lower than 10%, as they were age-matched. It is possible that larger body mass differences would indeed influence social rank in these tests, as previously shown in mice[28,29] and rats[25]. However, when using animals with marked body weight asymmetries, differences in age, and thus social experience, should be then taken into consideration as possible modulators of hierarchy. In our conditions, only the Tube Test was affected by body weight, where bigger animals had indeed more chances to win in the first encounters in the tube, probably indicating that in rats, as opposed to mice[8], body mass does affect the pushing behavior and the output in this task.



| | mFood Comp. | Sucrose Comp. | Sucrose Int. | mFood Dep. | Water Comp. | Tube Test | Tube Test Conflict res. |
|---|---|---|---|---|---|---|---|
| Modified Food Competition | *** 1.00 | ** 0.59 | 0.37 | ** 0.66 | -0.09 | 0.31 | * 0.46 |
| Sucrose Competition | ** 0.59 | *** 1.00 | ** 0.74 | # 0.44 | 0.02 | 0.22 | 0.29 |
| Sucrose Intermitent | 0.37 | ** 0.74 | *** 1.00 | * 0.47 | -0.02 | 0.29 | 0.31 |
| Modified Food Deprivation | ** 0.66 | # 0.44 | * 0.47 | *** 1.00 | -0.05 | -0.05 | 0.10 |
| Water Competition | -0.09 | 0.02 | -0.02 | -0.05 | *** 1.00 | -0.28 | # -0.44 |
| TubeTest | 0.31 | 0.22 | 0.29 | -0.05 | -0.28 | *** 1.00 | *** 0.91 |
| TubeTest Conflict res. | * 0.46 | 0.29 | 0.31 | 0.10 | # -0.44 | *** 0.91 | *** 1.00 |

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**Figure 6.** Reliability between dominance measurements between tests. (**A**) Dominance index (DI) based in consumption of resources for each behavioral test is represented being each pair of animals identified with a specific color/symbol. In those cases where two days of testing was performed, values plotted correspond to the average DI of the two days. Competition for positive reinforcers produced highly variable DI indicating detectable differences in the strength of social hierarchy between the pairs. Water Competition, however produced DI close to 0 indicating that the animals of most of the dyads drank very similar amounts during this test. The Tube Test produced a very polarized distribution of DI, where one animal of each pair would win most of the trials. (**B**) These differences in hierarchy became less polarized when taking into account the time the animals took to solve the conflict in the tube (DI in the Tube Test /latency to finish the trial). (**C**) Correlation matrix between DI from all tests. Competition for positive reinforcers DIs were correlated across tests, and DI from the modified Food Competition test correlated with the Tube Test, when conflict resolution time was taken into account. However, no significant correlations were observed with the Water Competition tests. $^{\#}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

In summary, here we provide and validate a novel trial-based dominancy assay to be performed in the home cage of familiar non-deprived rats, the modified Food Competition test, which is easy to adapt and implement in any behavioral laboratory. The ability to assess dominancy in stable social hierarchies opens the possibility to study how social status affects different aspects of an individual cognitive, behavioral and physiological functions in the context of various social interactions, regarding which very little is known. Intense efforts in the last years have highlighted the Norway rat as a very interesting animal model to identify the proximate mechanisms and neural circuits of complex social functions[30–42]. It is conceivable that each of these behaviors is modulated in some way by hierarchy.

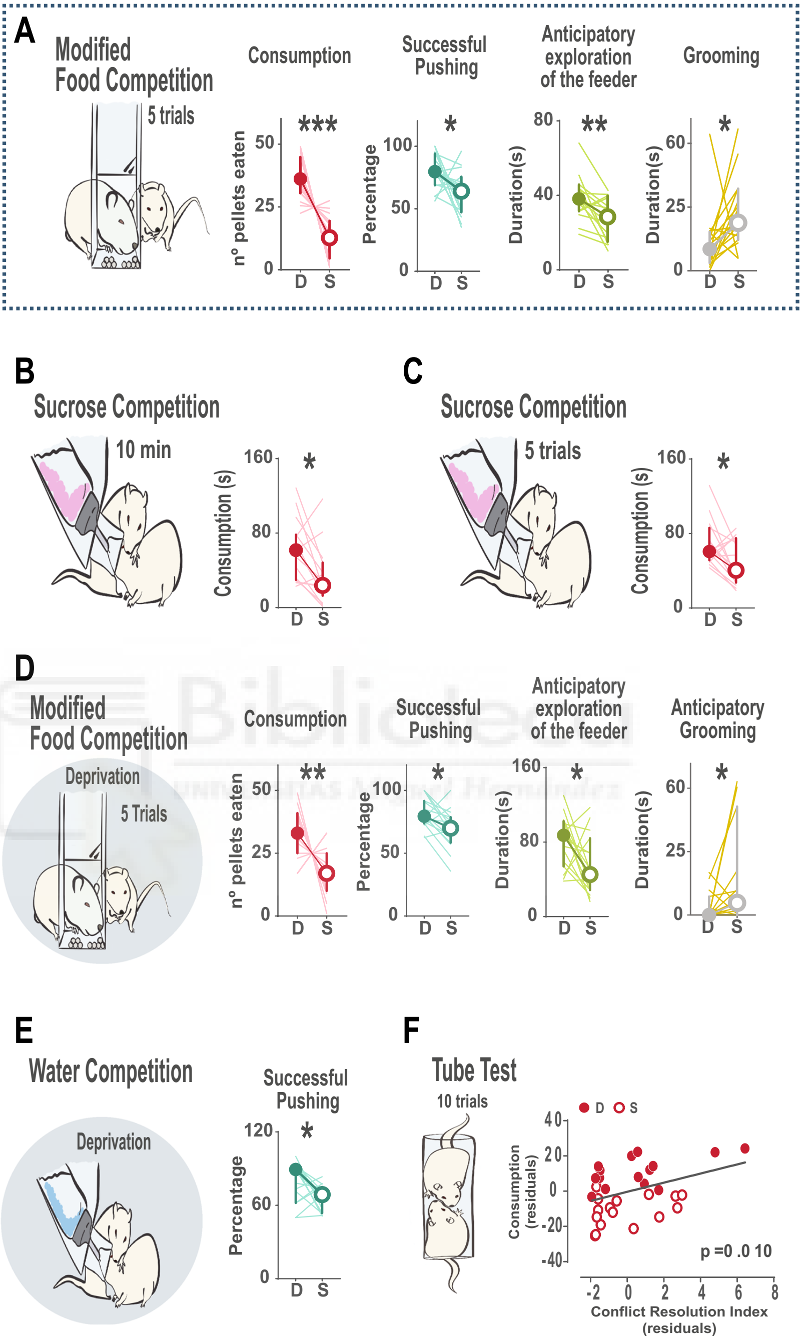
**Figure 7.** Food competition in the home cage is a simple and reliable measure of established social hierarchies in rats. Animals displaying differences in the amount of pellets eaten in the modified Food Competition test were classified as dominant or submissive animals, and their behavioral profiles studied in the rest of the tests. (**A**) When considering average behaviors of both testing days in the modified Food Competition test, dominant animals significantly ate more pellets, were more successful to displace their partners from the food magasin during the competition and explored more the location of the resource when access was still prevented, and groomed less time throughout the session (considering the last 40 s of intertrial interval and first 80 s of reward exposure of each trial). Moreover, they (**B**) drank more in the Sucrose Competition test with continuous, and (**C**) with intermittent access to the bottle. (**D**) Similar behavioral profiles were displayed in the Food Competition under food-deprivation, where differences in consumption, successful pushing, anticipatory exploration of the feeder and grooming were also observed. We defined the anticipatory window as the last 40 s of the intertrial interval, just before the bottle was placed in the lid thus consumption was still not possible (**E**) Dominant animals according to the modified Food Competition test, were also more successful to displace their submissive in the Water Competition test, indicating that although no differences in the amount of water drank were observed, the quality of the interaction was significantly different. (**F**) The amount of pellets eaten in the modified Food Competition test significantly predicted the probability of wining in the first interactions in the Tube Test, when latency to win was taken into account, and values were regressed out of the influence of body weight. Dashed line around panel A indicates that consumption in second day of testing in the modified Food Competition test was the criteria to evaluate differences in all the tasks. Median, 95% CI and individual values for all animals are represented. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

While most of the available tests for the evaluation of social hierarchy in rats are based on the identification of dominant animals during the establishment of a new hierarchy[3,11,15,16,26] it is uncertain whether becoming the dominant in a first encounter will translate into keeping the same rank when the hierarchy becomes stable. Indeed, previous reports indicate that repeated encounters are needed for two unfamiliar animals to establish a stable social rank[14–16]. By studying social status only during the early phases of its development, we are losing a huge aspect on the richness of social behavior and how it might be impacting brain function, in health and disease. The differences between the establishment of a hierarchy and its maintenance are largely unexplored, and the field would vastly benefit from new behavioral tools to address this fascinating question. Our new behavioral task opens the possibility for the study of such differences in rats.

Although we acknowledge that the general tendency in the field is to favor the use of mice as a model species, due to the large advantages related to its genetic tool box, cross-species validation is of utmost necessity, and validation of tools in different species an urgent need in Neuroscience. On the other hand, rats display much more sophisticated social and non-social behaviors compared to mice, and the development of tools such as CRISPR/Cas9 and state-of-the-art viral approaches, are making more accessible the precise monitoring and manipulation of neural circuits in other species. Taking all together, we foresee a drift towards a diversification of the species used in Neuroscience in the following years, and validation of ethologically relevant behavioral tools within the ecology of each species is needed.

Our modified Food Competition task provides a simple, robust, and unintrusive means of assessing established social hierarchy that can be readily incorporated into future studies, with the notable advantage of not inducing aggressive behaviors between the interacting individuals, nor having to manipulate internal state (deprivation), and being performed in the home-cage. One limitation of our work was the use of a very specific population for our study: male adult Sprague Dawley rats. Although we do not anticipate major problems in using the modified Food Competition test in other rat strains, behavioral differences have been already reported in several social and nonsocial behaviors between Long-Evans, Wistar and Sprague–Dawley rats[23]. It could be possible that different rat strains would react differently to the subtle conflict we induce in the cage during the modified Food Competition test, and that acute aggressive encounters could be observed depending on the strain. Future studies could also expand our task to other developmental ages (such as peripuberty or late adolescence), where play-fight behavior and aggressive profiles are being acquired[20]. Importantly, future studies should investigate whether social hierarchy could be assessed in female rats using the modified Food Competition Task. The neural mechanisms of female dominance and aggression have been surprisingly overlooked, and most of the knowledge in this direction has been obtained in the context of maternal aggression[43–45], with some notable exceptions[46,47], moment where females display robust, clear and strong aggressive episodes. However, established social rank, contrary to de novo establishment of social hierarchies, is not based in aggressive encounters. We therefore believe our task is well suited for the study of social hierarchy in female rats, as dominance is evaluated not according to the quantification of agonistic behaviors, which female rats do not typically or strongly display, but on the behavioral response towards a subtle conflict to gain access to appetitive reinforcers. Moreover, although not the aim of the present work, our task can be easily scalable to larger groups of animals living in standard home-cages or more naturalistic environments. A collective evaluation of established hierarchies by introducing subtle conflicts during discrete periods in the home cage might provide very interesting information of complex social structures, and would suppose a clear advantage when evaluating groups of animals, as compared to repeated testing across multiple pairs, as the round-robin design currently used in the tube test in mice.

In conclusion, here we present a tool that allows, for the first time, to identify established social hierarchies in rats. After the precise description and validation of the modified Food Competition test presented here, this task is very easy and cheap to implement in any behavioral laboratory, which we expect will substantially help accelerate discovery on the effects of established social hierarchies on brain function. Our work adds to recent efforts towards the development of ethologically relevant paradigms[48], where rich information of the social



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interactions of rodents can be obtained with minimal intervention of the experimenters but in controlled and highly quantifiable laboratory settings.

## Materials and methods

**Animals.** 40 three-months old male Sprague–Dawley rats (OFA, Charles-River, France) weighing 325–410 g at the beginning of the experiment were used. Upon arrival from the commercial vendor (Charles River, France), rats were pair-housed and maintained with ad libitum access to food and water under a reversed light cycle (12 h dark/light cycle; lights off at 10 AM) in controlled temperature conditions, and with a transparent red tunnel as environmental enrichment (8 cm diameter, Bio-Serv, # K3325). Animals were left undisturbed in their home-cages for approximately three weeks, allowing rats to habituate to our Vivarium Facility and routines, and to reverse their circadian rhythm. After this period, animals were handled four times every other day during one week. Body weight was controlled weekly and prior to each testing session. Experiments were performed during the dark cycle, waiting at least 2 h after the lights were off to start with behavioral procedures. Animals were provided by a commercial company, thus previous social experience, social status and degree of relatedness between the animals was not known. Animal husbandry and all experimental procedures were approved by the Animal Care and Users Committee of the Champalimaud Neuroscience Program and the Portuguese National Authority for Animal Health (Direcçao Geral de Veterinaria), which is in strict compliance with the European Directive 86/6097EEC of the European Council. We confirm that the study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org/).

**Experimental procedures.** Twenty pairs of animals were tested in the different behavioral paradigms (Sucrose Competition, Food Competition with and without food deprivation, Tube test and Water Competition, see below for description of each task) in order to identify social rank and study reliability of social status between them. Each pair of animals consisted of cage mates living in the same cage for 4 weeks before starting the behavioral procedures. The interacting animals were thus familiar and the same pairs were maintained throughout the entire duration of the experiment. In order to control for possible influences of the order of behavioral testing on the evaluation of social status, we divided the animals in two independent groups (n = 10 pairs each) where the order of the tests that involved competition for positive reinforcers was counterbalanced (Supplemental Figure 1). Those tests that required food or water deprivation, and thus were more stressful and/or could a priori induce strong aggressive behaviors, were performed towards the end of the experiment. All pairs were tested in all the tasks with a 2 to 6 days of interval between testing sessions.

At the beginning of the experiment, we randomly identified each rat of a cage as 'Animal A' and 'Animal B' and quantified their behavior and consumption of the resources for each of the behavioral tests. Before each habituation or test session the fur of the animals was marked using a black pen in order to enable clear identification of each animal for post hoc video-annotation analysis. All the tasks, except for the Tube Test, were performed in the animal's home cage with small modifications to the lid to accommodate a customized feeder/water bottle. During testing, standard chow and water bottles were removed from the home-cage, to accommodate the modified lids, being replaced immediately after behavioural testing.

*Modified Food Competition test (mFC).* Food competition for palatable pellets (Dustless Precision Pellets, 45 mg, Rodent Purified Diet, Bio-Serv) was performed in the home-cage of non-food restricted pairs of animals. For this test, the home-cage lid was replaced by a modified laser-cut acrylic one that accommodated a fully transparent feeder (Fig. 1A). The feeder was designed so only one animal could access the food pellets at a time, promoting conflict and competition for the reward. Moreover, the feeder accommodated a sliding door that prevented access to food pellets during the inter-trial interval, and an opening on the top to facilitate delivery of food pellets in each trial with minor interference from the experimenter. This customized lid was used during habituation and test sessions.

Animals were exposed to palatable pellets in their home-cages during the handling period for four days in order to reduce neophobic responses to the food. Then, during three consecutive days, all the animals went through a habituation period to the modified lid, where they were allowed to explore and consume the pellets individually without competition, while the partner would be kept in a separate cage. Specifically, during habituation days, the new lid holding the feeder was placed on the home cage containing 10 palatable pellets. The sliding door was closed, preventing access to the pellets. Two minutes after, the door was opened, allowing the rat to access the pellets for 2 min, after which the door was closed again and 10 new pellets were placed. In total, the animal was given 4 min' access to 20 pellets in a total session of 10 min. Next, food competition in a social context was performed for two consecutive days. Pairs of animals were re-marked, and the home-cage lid was replaced by the modified one with the feeder and 10 palatable pellets. 1 min after, the sliding door was opened allowing the rats to have access to the pellets for 2 min, after which the door was closed again for a 1-min inter-trial interval and 10 new pellets were delivered. We repeated this procedure for 5 trials and a total session of 15 min and 50 pellets. After the session, the customized lid was replaced by their home-cage lid.

*Modified Food Competition with food deprivation (mFCD).* The modified Food Competition test was performed in the home cage of familiar animals as described above, but in this case animals underwent only one session of social competition after a 24 h period of food deprivation. After the test, the modified lid was replaced by the standard one and rats were allowed to eat and drink ad libitum for the rest of the cycle.

*Sucrose competition (SC).* Pairs of non-water-restricted cage mates competed for access to a bottle containing 1% sucrose solution placed in a modified lid on their home-cages. The lid was designed so the bottle holders were prolonged with a transparent acrylic tube, in a manner that the tip of the bottle was surrounded by an extension that would allow the head of only one animal to drink at a time (Fig. 1B). We performed three habituation sessions. In the first habituation day, animals were exposed to the new lid for 20 min where no bottle was available. In the two following days, the new lid was holding two bottles of 1% sucrose solution and animals were given free access to the sucrose solution for 20 min. Then, animals were re-marked and tested for sucrose competition in two consecutive days for 10 min, where the modified lid presented only one bottle of sucrose this time.

*Sucrose competition with intermittent access to the resources (SCI).* In this test, access to the 1% sucrose solution followed an intermittent schedule. One minute after the beginning of the session, a bottle with sucrose solution was placed in the dispenser allowing the animals to access the solution for 2 min. After this time, the bottle was removed for 1 min and put back again for 2 more minutes, performing a total of 5 trials. The SCI was performed over two consecutive days. After each session, the customized lid was replaced by their home-cage lid.

*Water competition (WC).* Animals were water deprived for 24 h and tested for competition for water in their home cage. At the moment of the test, the cage lid was replaced by a modified one where access to the bottle was only possible for one animal at a time. The duration of the test was 10 min, after which the standard lid was replaced and both rats had ad libitum water access.

*Tube test (TT).* We used a transparent Plexiglas tube with 60 cm length and 8 cm diameter, a size that allows an adult rat to pass through without reversing its direction, and, when two rats are placed in the tube, prevents one rat from crossing the tube by passing the other. We performed one habituation session where animals individually explored the apparatus, allowing spontaneous entering and crossing of the tube for 5 min. During this habituation session, animals were placed initially in front of one of the ends of the tube, and freely allowed to enter the tube and explore the behavioural table. In our hands, rats immediately entered and crossed the tube, spontaneously performing 5 to 6 crossings during the habituation period, without the need to force exploration nor to push them, as they did not display extended periods of immobility. After the 5 min' habituation, animals were returned to their home-cages. During test days, each pair of cage mates rats was simultaneously placed into opposite ends of the tube and met in the middle. At this time, a partition placed at the center of the tube was removed. The rat that first retreated from the tube was designated as the 'loser' and the other as the 'winner'. After each trial, both rats were placed back into their home-cages until the beginning of the next trial. From trial to trial, animals were released at either end of the tube alternately. We performed one testing session of the Tube Test with 10 trials at the beginning of the experiment and assessed stability of social rank within this test with another testing session at the end of the experiment. We quantified the amount of time that one of the animals took to push the other out of the tube and annotated the winner and loser of each interaction.

**Video acquisition and behavioral quantification.** Experiments were performed under the dark cycle of the animals and video recordings were obtained by a high resolution infra-red camera (PointGrey Flea3 -U3-13S2M CS, Canada) under infra-red illumination, capturing frames at 30 Hz at 1280 × 960 pixel resolution. Supervised offline frame by frame video annotation of behaviors of interest was performed by a trained blind experimenter (DFC) after confirmation of highly reliable quantifications. For each animal of the dyad we quantified frequency, latency, and duration of (1) consumption of resources (number of pellets eaten or time spent drinking water or sucrose); (2) exploration of the feeder (sniffing behavior inside and outside the feeders or bottle holders); (3) self-grooming; and (4) pushing the other animal to gain access to the resource. In those tests with a trial structure, behaviors were aligned to time 0, i.e. the moment where the sliding door that gave access to food pellets was opened for food competition tests, or when the bottle was placed in the modified lid in the case of the Sucrose Competition Intermittent. For these tests with trial structure, intertrial interval (the time where reward was not present) was set to 60 s and the duration of a trial (where animals could access to the reward) to 120 s. However, due to the manual control of the timings of the experiment, some trials of some pairs of animals resulted with shorter durations than aimed. In order to ensure comparable behavioral profiles across trials and dyads across these tests, we decided to narrow our behavioral quantification window and focus our statistical analysis to the 40 s before and 80 s after time 0 (the moment where access to rewards was possible). This resulted in a total of 10 min duration test for both tasks with and without trial structure. However, note that consumption in tasks with trial structure was only quantified for 400 s (80 s during 5 trials in a day), while in the other tasks this could be possible during 600 s. To take this difference into account, comparisons between consumption levels across tasks were performed with the percentage of time spent consuming relative to the duration of the session. Exploration of the feeder was measured during the whole session, and in those tests with a trial structure, we differentiated between exploration of feeder when reward was accessible and before that, when the sliding door was closed or there was no bottle there yet, as a proxy for anticipatory exploration. Pushing behavior was divided in two distinct categories depending on the outcome: Successful Pushing, if the animal managed to displace the partner from having access to the resource and Unsuccessful Pushing, when animals would attempt to get access to the resources but were unsuccessful to displace their partner from the reward area. In the Tube Test, we quantified the number of wins for each animal and the duration of each trial as a proxy for the time the animals used to solve the territorial conflict.

Bonsai[49] and Python Video Annotator (https://pypi.org/project/Python-video-annotator/), both open source computer vision software available online, were used to perform behavioral quantification. First, digitally assigned behaviors were quantified with Bonsai, which created timestamps for the beginning and ending of each behavioral event. Then, the start and end of each behavioral bout was curated with frame-by-frame investigation using Python Video Annotator, which allows fine modification of the timeframes with subsecond resolution.

Moreover, Python Video Annotator allowed easy post hoc categorisation of the two types of exploration of the feeder (anticipatory or during the presence of the resource) and pushing behavior (successful or unsuccessful) which can only be identified once the bouts of pushing behaviour are finished and is thus not possible to analyse with online video analysis.

**Data analysis.** Data was parsed and processed with Python (Python Software Foundation, v.2.7). In addition to comparing raw data obtained, we calculated several indexes to compare hierarchies across tests.

*Dominance Index.* The *Dominance Index* (DI) was calculated for each test, where the difference in resource consumption across partner animals was normalized by the summed resource consumption of the pair, following this formula:

$$DI = \frac{Consumption\ \ of\ Animal\, A - Consumption\, of\ Animal\, B}{Total\, Consumption} * 100$$

Consumption corresponded to the number of pellets in the modified Food Competition tests, the duration of drinking in the Sucrose and Water Competition tests, and the number of wins displayed by an animal in the case of the Tube Test. The sign of this index would indicate whether animal A or B would consume more, i.e. positive values would indicate that animal A consumed more, and negative values that animal B consumed more. DIs close to 0 would indicate no differences in consumption between the animals of a pair. Differences of 5% to equal consumption, i.e. DI ranging from − 10 and + 10, were considered noise and indicative of no reliable hierarchy.

*Conflict Resolution Index in the tube test.* The *Conflict Resolution Index (CRI)* was calculated taking into account not only who won a trial, but also how long it took for the conflict to be resolved (i.e., the latency for one of the animals to be pushed out of the tube):

$$CRI = \frac{DI}{Time\, to\, solve\, the\, conflict}$$

Conflict Index. The *Conflict Index* (CI) was calculated by dividing the time animals spent pushing in a specific test by either: (1) the time spent consuming, in case of the Sucrose and Water Competitions, or (2) the latency to eat all rewards in the case of the modified Food Competition with and without food deprivation.

Statistical analysis. The statistical analysis was performed using IBM SPSS Statistics version 24.0 for Windows. The normality of the data was tested using Kolmogorov–Smirnov normality test, and when normality was not observed non-parametric tests were applied and median and 95% confidence interval were chosen to represent data in figures. Wilcoxon signed-rank tests with Bonferroni correction were used to study differences between counterbalanced groups in each protocol, of each behavior across the tasks, and to study differences between dominant and submissive animals on behavior. Paired t-test were performed to assess differences in the weights between dominant and submissive animals. One-Way ANOVA followed by post-hoc test Tukey was used to compare behaviors of interest across tasks. Here, when normality was not observed, a Kruskal–Wallis test with post-hoc Dunn-Bonferroni correction was performed. Bivariate Pearson Correlation was performed to measure the strength and direction of association between the Dominance Index of all the tasks and linear regressions for assessing predictive value of Food Competition and Tube Tests controlling for body weight. Statistical significance was set at $p < 0.05$.

## Data availability

All data generated to support the findings of this study are available from the corresponding author upon reasonable request.

Received: 29 April 2021; Accepted: 24 June 2021
Published online: 16 July 2021

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## Acknowledgements

This work was supported by grants of the NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation under the grant number 26478 to C.M., the Spanish Agency of Research (grant RTI2018-097843-B-100 to C.M.), the "Severo Ochoa" Program for Centers of Excellence in R&D (SEV-2013-0317 and SEV-2017-0723) and the Champalimaud Foundation. D.F.C. was further supported by the Ministerio de Ciencia e Innovación (BES-2016-07674) and C.M. by a Ramon y Cajal contract (RYC-2014-16450). We thank the Marquez lab for fruitful discussions and specially, Kevin Caref for insightful comments on the manuscript. We also express our gratitude to Gonçalo Lopes for his help with Bonsai workflow for annotation of behavior, Antonio Dias for feedback on the python scripts and to Cristina Savin for discussion of the data analysis.

## Author contributions

DF Costa: Conceptualization, Investigation, Methodology, Formal analysis, Visualization, Writing original draft and Review and editing the manuscript; MA Moita: Methodology, Review and editing manuscript, Funding acquisition; C Márquez: Conceptualization, Supervision, Investigation, Methodology, Formal analysis, Visualization, Writing—original draft, Writing—review & editing, Funding acquisition.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-93818-0.

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