UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTICAGIONES BIOMÉDICAS

BIOLOGÍA EVOLUTIVA

EFECTOS DEL DESTETE TEMPRANO SOBRE LA MORFOLOGÍA, FISIOLOGÍA Y CONDUCTA EN EL GATO DOMÉSTICO (*FELIS SILVESTRIS CATUS*)

TESIS

(POR ARTÍCULO CIENTÍFICO)

EFFECTS OF EARLY SOCIAL SEPARATION ON THE BEHAVIOUR OF KITTENS OF THE DOMESTIC CAT

QUE PARA OPTAR POR EL GRADO DE:

MAESTRA EN CIENCIAS BIOLÓGICAS

PRESENTA:

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MÉXICO, CD. MX. OCTUBRE, 2023

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P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **16 de junio de 2023** se aprobó el siguiente jurado para el examen de grado de **MAESTRA EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Biología Evolutiva** de la alumna **MARTÍNEZ BYER SANDRA NAITZÉ** con número de cuenta **311264528** por la modalidad de graduación de **tesis por artículo científico** titulado: **"Effects of early social separation on the behaviour of kittens of the domestic cat",** que es producto del proyecto realizado en la maestría que lleva por título: **""Efectos del destete temprano sobre la morfología, fisiología y conducta en el gato doméstico (*felis silvestris catus*)"**, ambos realizados bajo la dirección del **DR. PÉTER MARK SZENCZI,** quedando integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 12 de septiembre de 2023

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

c. c. p. Expediente del alumno

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Thank you Mom, I love you

ÍNDICE

RESUMEN

Para muchos mamíferos la presencia de la madre y/o hermanos es un componente importante en el entorno temprano de los individuos, y la falta de estas influencias sociales puede tener efectos duraderos sobre el desarrollo morfológico, fisiológico y conductual. Se sabe sorpresivamente poco sobre los efectos de la separación prematura de la madre y hermanos en el gato doméstico a pesar de que la crianza por humanos es bastante común (dado el gran número de crías huérfanas y rescatadas). En el presente estudio evaluamos 62 crías de gato doméstico de nueve semanas de edad (32 criados por la madre, 14 huérfanos criados con hermanos y 16 huérfanos criados en solitario) en tres pruebas conductuales: una prueba de manejo (forcejeo), una prueba de defensa del alimento (carne), y una prueba de separación/confinamiento. Adicionalmente, utilizamos termografía infrarroja para evaluar cambios en la temperatura de ojos y nariz inducidos por estrés en la prueba de separación/confinamiento. No encontramos diferencias entre el peso corporal de los grupos a la edad del destete (nueve semanas), pero encontramos diferencias conductuales entre el grupo control y los dos grupos de crías huérfanas en las tres pruebas. Comparadas a los gatos control criados por su madre, tanto los gatos huérfanos criados con sus hermanos como los huérfanos que crecieron como crías únicas forcejearon antes en la prueba del forcejeo, además de vocalizar más y mostrar mayor actividad locomotriz en la prueba de separación/confinamiento. En la prueba de la carne, sólo los huérfanos criados con hermanos comenzaron a consumir el estímulo de carne cruda antes que los individuos control. La termografía facial mostró una tendencia general donde la temperatura de ojos y nariz disminuyó en respuesta a la separación/confinamiento, sin ninguna diferencia entre las condiciones de crianza. Nuestros resultados muestran que el entorno social temprano influye en la formación de la conducta y fisiología de los gatos. Las diferencias conductuales entre individuos criados por su madre e

individuos criados por humanos deberían ser consideradas para el bienestar de estos animales cuando crecen en hogares temporales y durante los procesos de adopción. Nuestros resultados sugieren que, como era esperado, ser criado por humanos y desarrollarse sin la presencia de la madre (con o sin hermanos) tiene un efecto sobre la conducta de las crías de gato a la edad del destete y posiblemente incluso se adentre en la etapa adulta.

ABSTRACT

For many mammals the presence of the mother and/or siblings is an important component of an individual's early developmental environment, and lack of these social influences can have long-lasting effects on morphological, physiological, and behavioural development. Surprisingly, little is known about the effects of premature maternal and sibling separation in the domestic cat, even though hand-raising is rather common (due to the large number of orphaned and rescued kittens). In this study we assessed 62 nine-week-old kittens (32 mother-reared, 14 orphans hand-raised with siblings and 16 hand-raised orphan singletons) in three behavioural tests: a handling (struggle) test, a food defence (meat) test, and a separation/confinement test. Additionally, we used infrared thermography to evaluate stress-induced changes in eye and nose temperatures in the separation/ confinement test. We found no differences in body weight between groups at weaning at 9 weeks of age, but we found behavioural differences between the control and the two orphan groups in all three tests. Compared to control, mother-reared kittens, orphans raised with siblings and orphan singletons struggled sooner in the struggle test, plus vocalised more and exhibited greater locomotor activity in the separation/confinement test. In the meat test, only the orphans raised with siblings, but not the singletons, started consuming the raw meat stimulus sooner than control individuals. Facial thermography showed a general trend for eye and nose temperatures to decrease in response to the separation/confinement test, with no differences between rearing conditions. Our findings show that the early social environment is influential in shaping the long-term behaviour and physiology of kittens. The behavioural differences between mother-reared and hand-raised individuals should be considered for the well-being of the animals when fostering and during the adoption process. Our results suggest that, as expected, developing without the presence of the mother and raised by humans (with or without siblings) has an effect on the behaviour of kittens to weaning age and possibly even into adult life.

INTRODUCCIÓN

La influencia del entorno social temprano

En mamíferos, la relación madre-cría tiene consecuencias fundamentales no solo en desarrollo físico sino también en el conductual, endócrino y neuroquímico (Harlow, 1958; Keverne, 2004; Lauby et al., 2021). Es así que los efectos maternos influyen en las diferencias fenotípicas entre los individuos de muchas maneras más allá de la transmisión de genes. Moore y colaboradores describen que, en general, el cuidado materno influye sobre los rasgos juveniles más que los rasgos adultos, particularmente los caracteres morfológicos. Sin embargo, también muestran que el cuidado materno tiene efectos profundos sobre características de la conducta y fisiología que permanecen a lo largo plazo (Moore et al., 2019).

Los hermanos (y medios hermanos) también forman una parte prominente del entorno social temprano de muchos mamíferos (revisiones en: Mendl, 1988; Hudson and Trillmich, 2008). Por ejemplo, crías únicas o crías de camadas pequeñas típicamente son más pesadas al nacer, obtienen más leche, y consecuentemente son más grandes. Se puede esperar que tales diferencias tengan influencia sobre el desarrollo de las crías, incluyendo su conducta. En conjunto, los estímulos sensoriales y sociales de la madre y los compañeros de camada influyen sobre los fenotipos fisiológicos y conductuales de los individuos durante periodos sensibles postnatales, contribuyendo significativamente a las diferencias individuales estables a lo largo de la vida (Hall, 1998; Braun et al., 1999; Rödel et al., 2009; Groothuis & Trillmich, 2011; Hudson et al., 2011; Nicolás et al., 2011).

No es sorpresivo, entonces, que el aislamiento social abrupto pueda tener serias consecuencias sobre el desarrollo conductual y físico de las crías. Las diferencias permanentes en el desarrollo debido a separación de la madre o pérdida de los padres durante etapas tempranas han

sido estudiadas desde hace varias décadas en niños criados en orfanatos e instituciones afines (Spitz, 1945; Brown, 1966; Carlson & Earls, 1997). Anécdotas iniciales de guarderías residenciales durante la Segunda Guerra Mundial indican que la falta de relaciones emocionales sostenidas puede afectar marcadamente el desarrollo psicosocial de un niño (Burlingham & Freud, 1944). Por ejemplo, la falta de cuidados maternales incrementa el riesgo de ser diagnosticado con ansiedad, depresión, y psicosis en la adultez (Vetulani, 2013; Kaur et al., 2018). Más recientemente ha habido un esfuerzo por entender los cambios neurobiológicos que subyacen los efectos de las experiencias tempranas adversas. Crecer en una institución ha sido ligado a retrasos en el crecimiento físico y el desarrollo cognitivo, así como a una reducción de materia blanca y gris, reducción del volumen del cerebelo, y alteraciones en la producción de cortisol (revisiones en: van IJzendoorn et al., 2011; Bick & Nelson, 2016).

Efectos de las separaciones tempranas

Los primeros relatos científicos sobre los efectos duraderos de la deprivación materna en animales provienen de los estudios de Harlow y colaboradores que documentaron los efectos de diferentes grados de deprivación maternal en macacos Rhesus (*Macaca mulatta*). Aquellos macacos criados en aislamiento desarrollaron más conductas estereotípicas, tales como mecerse y auto-abrazarse, tenían dificultad para adaptarse a nuevos entornos y socializar con otros individuos, y presentaban mayores niveles de ansiedad aún en la adultez (Harlow, 1958; Mitchell et al., 1966; Suomi et al., 1971).

Observaciones en otras especies han encontrado una amplia gama de consecuencias en individuos que pasan por separación/deprivación materna prematura. Por ejemplo, estudios sobre el destete prematuro y abrupto en ovejas reportan crecimiento inhibido y afecciones a la función inmune, junto con menor actividad en pruebas de campo abierto (Napolitano et al., 2008). En ratas,

periodos de separación materna durante las primeras semanas de vida resultan en una mayor incidencia de desórdenes físicos como úlceras (Ackerman et al., 1978), mayor presencia de estereotipias y conductas defensivas (Lippmann et al., 2007; Wang et al., 2020) al igual que disminución de la fertilidad y las conductas maternales en las siguientes generaciones de hembras (Kikusui et al., 2005; Miyaso et al., 2021). También se han observado aumentos en la incidencia de ansiedad, conductas tipo depresivas, agresión y respuestas pronunciadas a estresores tanto en ratas como ratones (Meaney et al., 1994; Aisa et al., 2007; Kikusui et al., 2008; Tractenberg et al., 2016), y se han reportado patrones de conducta similares en cerdos (Poletto et al., 2006).

Estudios con roedores han mostrado que estas alteraciones en la conducta como las que han sido descritas corresponden a alteraciones en varios sistemas neuroendocrinos debido a la influencia del cuidado materno postnatal (revisión en Lauby et al., 2021). La mayoría de los estudios se han enfocado en los mecanismos que subyacen a la reactividad al estrés y las alteraciones al eje Hipotálamo-Hipófisis-Adrenales (Meaney, 2001, Weaver et al., 2004), aunque también se ha sugerido una relación entre el cuidado materno recibido y cambios conductuales que involucran a los sistemas oxitocinérgico (Champagne et al., 2001) y dopaminérgico (Peña et al., 2014).

La influencia de los hermanos

La separación temprana puede significar que el animal no solo ha sido separado de su madre sino también de sus compañeros de camada. A pesar de que la mayoría de los estudios se han enfocado únicamente en la separación materna, los hermanos tienen gran importancia en el desarrollo de las crías y pueden contribuir significativamente a la variabilidad conductual entre individuos. (Li et al., 2008; Hudson et al., 2011; Martínez-Álvarez et al., 2022).

Los compañeros de camada son una fuente de estimulación sensorial y competencia durante el desarrollo temprano. Un factor importante es el tamaño de camada. Conforme aumenta el tamaño de camada las crías reciben más estímulos de sus hermanos, pero también se ven en mayor competencia por la leche y cuidados maternales (Drummond et al., 2000; Rödel et al., 2008). Las diferencias en la masa corporal y tasa de crecimiento entre compañeros de camada se han relacionado a diferencias conductuales. Por ejemplo, poco después del destete las ratas más pequeñas de la camada parecen ser menos osadas que sus hermanos de mayor peso (Rödel & Meyer, 2011). Se ha reportado un patrón similar en el cuis común (*Cavia aperea*), donde los cachorros más pesados exploran más y son más propensos a tomar riesgos que sus hermanos de menor tamaño (Guenther & Trillmich, 2015).

Pero la presencia de hermanos afecta la conducta de otras formas más allá de la competencia. Las crías reciben estimulación sensorial constante por parte de sus hermanos, primero a través de agruparse para ayudar en la termorregulación (Bautista et al., 2003; Zepeda et al., 2018), y después a través del juego (Nunes et al., 2004; Nicolás et al., 2011). Sin estos estímulos también hay efectos sobre el comportamiento. Por ejemplo, los gazapos de conejo que crecen sin compañeros de camada muestran un retraso en el desarrollo de habilidades motrices comparados con sus hermanos criados en camada (Nicolás et al., 2011). Y en ratas, la separación temprana de los hermanos ha sido asociada con el desarrollo de conductas adictivas y de ansiedad (Li et al., 2008).

Separación temprana en el gato doméstico

Sin embargo, a pesar de los posibles efectos desfavorables de una separación prematura, la "crianza a mano" de animales domesticados o de granja por cuidadores humanos a veces es necesaria, por ejemplo debido al rechazo la madre u orfandad. Esta es una situación común para

el gato doméstico, con crías siendo rescatadas frecuentemente y albergadas junto con su madre, como camadas huérfanas, o como crías únicas, presentando la oportunidad de estudiar su desarrollo conductual en estos diferentes contextos. Datos provenientes de 2,386 albergues en Estados Unidos reportan que en 2020 el 45.2% de gatos recibidos fueron cachorros/juveniles (aproximadamente 598,700) (Castelazo et al., 2020), aunque no hay información disponible sobre la proporción de cachorros que requieren ser criados por humanos o por madres adoptivas.

En animales de compañía, tales como el gato doméstico (*Felis silvestris catus*), el destete temprano ha sido ligado a respuestas elevadas al estrés, así como estereotipias y conductas agresivas (O'Farrell et al., 1994; Ahola et al., 2017). En gatos, el destete usualmente inicia a alrededor de las ocho semanas post-natales (Turner & Bateson, 2013; Bánszegi et al., 2017). Las semanas de lactancia también son un periodo de socialización con compañeros de camada y cuidadores humanos. Se ha reportado que la experiencia de camada es necesaria para el desarrollo de patrones de juego adecuados, y que la falta de esta experiencia puede resultar en niveles más elevados de agresión hacia la adultez, incluso si la madre estuvo presente en la crianza (Guyot et al., 1980; Mendl, 1988). Se ha mostrado que la presencia de la madre o compañeros de camada afecta la conducta de una cría al enfrentarse a un entorno no-familiar; donde las crías muestran menos señales de estrés en compañía de un miembro de su unidad familiar (Rheingold and Eckerman, 1971). Sin embargo, se sabe poco sobre cómo la separación social prematura afecta las respuestas de los gatos a eventos potencialmente estresantes. En su estudio comparando gatos criados por su madre y gatos huérfanos, Lowell et al. (2020) encontraron que las crías huérfanas emiten más llamados de alarma y muestran un incremento de actividad motriz durante una separación social breve. Sus resultados sugieren que la separación materna puede llevar a consecuencias de largo plazo en las respuestas al estrés, y que tanto las vocalizaciones como la

actividad motriz son buenos marcadores conductuales para estudiar tales efectos (ver también Martínez-Byer et al., 2020; Urrutia et al., 2022a; b).

En el presente estudio buscamos evaluar los efectos la separación temprana de la madre y los hermanos sobre la conducta de crías de gato en desarrollo al comparar individuos criados en diferentes entornos sociales. Para poner a prueba sus respuestas, utilizamos tres pruebas conductuales a las nueve semanas de edad, mismas que se han usado en estudios previos de personalidad, junto con marcadores fisiológicos y de crecimiento corporal. A nuestro conocimiento, este es el primer estudio en comparar la conducta de gatos criados por su madre con la conducta de gatos huérfanos criados con hermanos y huérfanos criados en solitario.

OBJETIVO GENERAL

Evaluar los efectos de la separación temprana de la madre y compañeros de camada sobre la conducta, fisiología y morfología de crías de gato doméstico en desarrollo, al comparar individuos criados en entornos sociales diferentes.

SOBRETIRO DEL ARTÍCULO

Effects of early social separation on the behaviour of kittens of the domestic cat

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Abstract

For many mammals the presence of the mother and/or siblings is an important component of an individuals' early developmental environment, and lack of these social influences can have long-lasting effects on morphological, physiological, and behavioural development. Surprisingly, little is known about the effects of premature maternal and sibling separation in the domestic cat, even though hand-raising is rather common due to the large number of orphaned and rescued kittens. In this study we assessed 62 nine-week-old kittens (32 mother-reared, 14 orphans hand-raised with siblings and 16 hand-raised orphan singletons) in three behavioural tests: a handling (struggle) test, a food defence (meat) test, and a separation/confinement test. Additionally, we used infrared thermography to evaluate stress-induced changes in eye and nose temperatures in the separation/confinement test. We found no differences in body weight between groups at weaning at 9 weeks of age, but we found behavioural differences between the control and the two orphan groups in all three tests. Compared to control, mother-reared kittens, orphans raised with siblings and orphan singletons struggled sooner in the struggle test, plus vocalised more and exhibited greater locomotor activity in the separation/confinement test. In the meat test, only the orphans raised with siblings, but not the singletons, started consuming the raw meat stimulus sooner than control individuals. Facial thermography showed a general trend for eye and nose temperatures to decrease in response to the separation/confinement test, with no differences between rearing conditions. Our findings show that the early social environment is influential in shaping the long-term behaviour and physiology of kittens. The behavioural differences between mother-reared and hand-raised individuals should be considered for the well-being of the animals when fostering and during the adoption process. Our results suggest that, as expected, developing without the presence of the mother and raised by humans (with or without siblings) has an effect on the behaviour of kittens to weaning age and possibly even into adult life.

Keywords: Early weaning, Social separation, Early development, Orphans, Stress responses, Facial thermography

1. Introduction

In mammals, the mother–infant relationship has fundamental and long-term consequences not only in physical but also in behavioural, endocrine, and neurochemical development (Harlow, 1958; Keverne, 2004; Lauby et al., 2021). Siblings (or half siblings) also form a prominent part of the early social environment of many mammals (reviews in: Mendl, 1988; Hudson and Trillmich, 2008). Singletons or young from small litters are typically heavier at birth, obtain more milk, and are consequently larger. Such differences can be expected to influence the development of the young, including their behaviour. Furthermore, sensory and social stimuli from the mother and littermates shapes physiological and behavioural phenotypes of individuals during sensitive postnatal periods, contributing significantly to stable individual differences in later life (Hall, 1998; Braun et al., 1999; Rödel et al., 2009; Groothuis and Trillmich, 2011; Hudson et al., 2011; Nicolás et al., 2011).

Not surprisingly, sudden early social isolation can have serious consequences for the behavioural and physical development of the offspring. Permanent developmental differences due to maternal separation or parental loss during critical early life stages have long been studied in children raised in institutions (Spitz, 1945; Brown, 1966; Carlson and Earls, 1997). Early accounts from residential nurseries during wartime indicated that a lack of sustained emotional relationships can markedly affect children's psychosocial development (Burlingham and Freud, 1944). For example, lack of maternal care increases the risk of anxiety, depression, and psychoses into adulthood (Vetulani, 2013; Kaur et al., 2018). More recently there has been an effort to understand

the neurobiological changes that underlie the effects of early adverse experiences. Institutionalized rearing has been linked to delays in physical growth and cognitive development, as well as to a reduction in white and grey matter, reduction of cerebellar volume, and alterations in cortisol production (reviews in: van IJzendoorn et al., 2011; Bick and Nelson, 2016).

The first scientific accounts of the lasting effects of maternal deprivation in animals stem from the research of Harlow and colleagues documenting the effects of varying degrees of maternal deprivation on rhesus macaques (*Macaca mulatta*). Isolate-reared animals developed more stereotypic behaviours, such as rocking and self-clasping, had difficulty adapting to novel environments and socializing with other individuals, and presented higher levels of anxiety well into adulthood (Harlow, 1958; Mitchell et al., 1966; Suomi et al., 1971). Research in other species has found widespread consequences for individuals that go through early maternal separation/deprivation. For example, studies on early and abrupt weaning in sheep report inhibited growth and impaired immune function coupled with lower activity in an open field (Napolitano et al., 2008). Maternal separation during the first weeks of a rat's life results in higher incidence of physical disorders (Ackerman et al., 1978), more stereotypic and defensive behaviours (Lippmann et al., 2007; Wang et al., 2020) as well as decreased fertility and decreased maternal behaviour of the daughters of these females (Kikusui et al., 2005; Miyaso et al., 2021). Increased anxiety, depressive-like behaviour, aggression and pronounced responses to stressors have been found for both rats and mice (Meaney et al., 1994; Aisa et al., 2007; Kikusui et al., 2008; Tractenberg et al., 2016) and similar findings have been reported in pigs (Poletto et al., 2006).

Early separation can mean that the animal is not only apart from the mother but littermates as well. Although most studies have focused only on maternal separation, there is considerable evidence for the importance of siblings on the physiological and behavioural development of the young (Li et al., 2008; Hudson et al., 2011; Martínez-Álvarez et al., 2022).

However, despite the possible unfavourable effects of premature separation, the hand-rearing of young domesticated or farm animals by humans is sometimes necessary, for instance due to maternal rejection or being orphaned. This is a common situation for the domestic cat, with kittens frequently being rescued and fostered together with their mother, as orphan litters, or as singletons, presenting the opportunity to study their behavioural development in these three different contexts. Data from 2,386 USA shelters showed that in 2020 45.2% of cat intakes were kittens (approximately 598,700) (Castelazo et al., 2020), although no information is available on the proportion of kittens that require hand raising or foster mothers.

In companion animals, such as the domestic cat (*Felis silvestris catus*), early weaning has been linked to elevated stress responses, and aggressive and stereotypic behaviour (O'Farrell et al., 1994; Ahola et al., 2017). In cats, weaning is usually initiated at around 8 weeks after birth (Turner and Bateson, 2013; Bánszegi et al., 2017). This is also a period of socialization with littermates and human caregivers. It has been reported that littermate experience is necessary for the development of appropriate social play patterns, the lack of which can result in higher levels of aggression into adulthood, even when mother-reared (Guyot et al., 1980; Mendl, 1988). Studies show that the behaviour of pre-weaning cats in a novel environment is affected by the presence or absence of their mother and littermates (Rheingold and Eckerman, 1971). Still, little is known about how premature social separation affects kittens' responses to potentially stressful events. In their study comparing mother-reared and orphan kittens, Lowell et al. (2020) found that orphans emitted more distress calls and showed increased activity during brief social separation. Their results imply that maternal separation might lead to long-term differences in stress responses, and that vocalisations and locomotor activity are good behavioural markers to study such effects (see also Martínez-Byer et al., 2020; Urrutia et al., 2022a; b).

With the present study we aimed to evaluate the effects of early separation from the mother and siblings on the behaviour of developing kittens by comparing individuals raised in different social environments. To test their responses, we used three behavioural tests at 9 weeks of age which we had used earlier in studies of personality assessment, together with physiological and growth markers. To our knowledge, this is the first study to compare the behaviour of mother-reared kittens to the behaviour of litter-reared orphans and singleton orphans.

2. Material and methods

2.1. Subjects

We collected data from 62 mixed-breed kittens belonging to 19 litters (7 mothered, 12 orphaned) divided into three groups according to their rearing context (Table 1). Detailed information regarding the experimental animals can be found in Supplementary Material 1.

Table 1. Main characteristics of the treatment groups

Group	n	Sex	Number of litters	Mean litter size	Mean age at separation
Mother-reared	32	10F 22M	7	4.57	------
Orphans raised with siblings	14	8F 6M	4	3.75	3.5 weeks
Orphan singletons	16	13F 3M	8	2.37*	2.87 weeks

*Three kittens were found alone, thus mean litter size is not precise

- **Mother-reared** (control, n=32 kittens from 7 litters, 10 female, 22 male): Kittens raised by their mother together with at least two littermates. All litters in this group were born from four multiparous, mixed-breed mothers belonging to an established free ranging breeding colony kept in a private home under seminatural conditions. Kittens were kept

exclusively indoors for the duration of the study. Individual litters were kept in rooms equipped with half doors so that mothers could jump freely in and out, but the kittens were unable to leave. Mothers and kittens were cared for as described in previous reports (Bánszegi et al., 2017; Bánszegi et al., 2021; Urrutia et al., 2022a). Before leaving the nest, around week 4, contact with human handlers was limited to short daily check-ups; afterwards they were handled during feeding times and at least twice weekly play/socializing sessions.

- **Orphans raised with siblings** (n=14 kittens from 4 litters, 8 female, 6 male): Kittens raised without the mother but together with at least two littermates. We define orphans as kittens that were hand-reared at most from 4 weeks of age or before (mean 3.5 weeks), meaning that they were exclusively cared for by humans for at least a month prior to testing. Individuals in both this and the singleton group were either found or given up to rescuers without their mother and were fostered by human caretakers in private homes. No kittens were deliberately orphaned for the purpose of this study. Age at the time of rescue was estimated with the help of veterinarians. Orphan kittens were bottle-fed with a commercial kitten formula (Royal Canin® Babycat Milk or Super-Cria® Kittens). Kittens were fed according to their estimated age specific needs: 0–1-week-olds were fed 2-6 ml every 2 hours, 1–2-week-olds were fed 6-10 ml every 2-3 hours, 2–3-week-olds were fed 10-14 ml every 3-4 hours, 3–4-week-olds were fed up to 20 ml every 4-5 hours. At the 3–4-week stage wet food was slowly introduced along with the formula until the kittens completely transitioned to eating solid food, after which they ate both wet and dry food similarly to the mother-reared group. Kittens up to 4 weeks old were anogenitally stimulated to induce elimination before each bottle-feeding. Warmth was provided by an electric heating pad under the bedding until the kittens could regulate their own temperature. Foster homes

without the presence of adult cats were preferred but not always available. In the cases where there were other cats in the home, the owners were asked to keep the kittens separate to limit the contact with non-littermates and avoid the kittens receiving maternal-like care from the other cats, such as grooming. In all foster homes, kittens had at least one room where they were free to roam, with water and sandboxes always available.

- **Orphan singletons** (n=16 kittens from 8 litters, 13 female, 3 male): Kittens raised without the presence of their mother or littermates. Some individuals in this group were siblings, however most foster homes offered care for only one kitten rather than the entire litter thus they were reared alone, and hence they were considered singletons for the purposes of this study. Kittens in this group were cared for in the same way as described for the orphans raised with siblings.

2.2. Procedures

Kittens were tested when they were between 9-10 weeks old. All kittens were weighed regularly to monitor growth; their weight at the time of testing was recorded as well as their length (tip of the nose to base of the tail). Tests were conducted at the kittens' foster homes across two days, except on eight occasions (four mother-reared and four orphan singletons) where it was only possible to test on one day. They were given a rest period of at least 20 minutes between tests. The order of the tests was fixed (struggle, separation/confinement, meat); however, kitten testing order was randomized for mother-reared and litter-reared individuals. All tests were video recorded (Sony Handycam HDR-CX405) for subsequent behavioural analysis. At the end of testing all kittens were adopted out into private homes.

2.3. Behavioural testing

2.3.1. Struggle test

The struggle against being restrained can be a useful measure of the ease of handling (Grandin, 1997) and has been used to evaluate temperament in different mammalian (Steru et al., 1985; Erhard et al., 1999; Bautista et al., 2015; Martínez-Byer et al., 2020; Urrutia et al., 2022a) and bird species (Fucikova et al., 2009; Wang et al., 2013). A high latency to struggle is thought to represent a calm temperament and greater tolerance of human handling. It may be noted that this test under the name "dangle test" recently gained attention in social media as a way for owners to test their cat`s temperament.

The test was performed in the room where the kitten normally lived, in the presence of littermates and/or one of their human caretakers, since taking them to another room could have interfered with the struggle response due to the additional handling. An experimenter (S.M-B.) approached the kitten and petted it three times from the head to the base of the tail, then picked it up, holding it with both hands around the thoracic area, under its forelimbs and facing away from the experimenter (Supplementary Material 2; Martínez-Byer et al., 2020; Urrutia et al., 2022a). The test lasted until the kitten began to struggle (see Table 2 for behavioural definition) or until 30 seconds had passed after picking the kitten up. When this happened, it was immediately set down.

Table 2. Behavioural variables recorded in each test

Behaviour measured	Definition
Struggle test	
Latency to struggle	Lifting one of the hind paws and touching or kicking the experimenter's forearm
Meat test	
Latency to chew	Starting to consume the meat by chewing with the molars. Maximum latency 60s
Active defence/aggression (frequency)	Swiping at the tongs or stomping on the floor with forepaw as the tongs approach
Hissing or growling	Vocalised when defending the meat from the tongs
Separation/confinement test	
Vocalisation (latency and number)	Meow-type vocalisations
Motor activity (duration)	Displacement of any of the limbs on the floor or on the sides of the carrier for at least 1s

2.3.2. Meat test

Aggressive interactions around certain foods have been observed from a young age in kittens. For example, they display agonistic behaviours in defence of raw beef from siblings and humans (González et al., 2018; Urrutia et al., 2022b). Motivation to eat the stimulus is also a factor and rearing context might influence an individual's motivational state.

The test was performed in the room where the kitten normally lived without the presence of the mother or littermates. Before the test began, the kitten was given a bite-sized piece of raw beef shank as a taste, to test its motivation since it was a novel food. For the test, a piece of the meat was placed on the center of a plastic mat in the middle of the room. The piece of meat was cut to approximately 3.5 x 2.5 cm (8-10 g), such that the kittens could easily chew on it and carry

it but not consume it too quickly. Once the stimulus was placed, the experimenter called the kitten's attention by tapping their fingers next to the meat and the test began. When the kitten started consuming the meat, marked by a sideways chew using the molars, a pair of long bamboo tongs were introduced by snapping them twice and slowly sliding them towards the kitten (see Supplementary Material 3). The experimenter then used the tongs to attempt to take the meat from the kitten four times. Each attempt consisted of grasping at the meat and pulling it away, unless the kitten impeded it actively by swiping, stomping and/or growling (see Table 2 for behavioural definitions). The test ended after the fourth attempt, whether the experimenter succeeded in taking the meat away or not, or if the kittens did not approach the meat for 1 minute.

2.3.3. Separation/confinement test

In previous studies, this type of test has been used for evaluating individual differences in kittens of the domestic cat (Hudson et al., 2017; Urrutia et al., 2022a) and adult shelter cats from varied backgrounds (Martínez-Byer et al., 2020). Social separation and confinement are stressful even when brief, eliciting distress calls and behaviours such as scratching, circling, and pacing (coded as motor activity in this paper). Previous studies suggest that stress can also be reflected in skin temperature changes, such as a decrease in the nose area (macaques: Kuraoka and Nakamura, 2011; cows: Proctor and Carder, 2015; chimpanzees: Kano et al., 2016), and depending on the species, eye temperature has been reported to increase (horses: Bartolomé et al., 2013; dogs: Travain et al., 2015; sheep: Cannas et al., 2018) or to decrease (cows: Stewart et al., 2007; cats: Foster and Ijichi, 2017; chickens: Herborn et al., 2018). Since responses to separation have been found to be different between hand-raised and mother-raised kittens at postnatal weeks 1 and 3 (Lowell et al., 2020), we thus expected a similar results at a later age.

The test was performed in a small, closed room, within their home but unfamiliar to the animal. The room was cleared of any objects that could be distracting to the cat. If there were other animals in residence, they were not allowed in or immediately outside the room during the test. Thus, kittens could not see, hear, or smell their usual companions or known surroundings.

Immediately before the test, three baseline infrared thermal pictures Fluke (Ti300+, Fluke Corp., Washington, USA) were taken of the kitten's face with both eyes and nose in focus. The kitten was placed on a flat surface and gently scruffed to face the camera which was ~30cm away. Then, the kitten was brought into the testing room by an experimenter and was placed in a commercial transport carrier (42 x 61 x 38 cm), which was a closed plastic box with a wire grill door at one end, and holes along the sides which the kitten could look out of. The carrier, with the kitten, was placed at a marked position, and the experimenter left the room. All tests were recorded with a video camera set up in front of the carrier 1 meter away (see Supplementary Material 4). To facilitate observation, a light was mounted above a small hole in the roof of the carrier. The test lasted five minutes. Once this time had elapsed the kitten was taken out of the carrier and three more thermal pictures were taken. The carrier was cleaned between tests with alcohol. The behaviours measured are listed and defined in Table 2.

2.4. Thermal imaging analysis

Pictures were analysed using SmartView software version 4.3.329.0 (Fluke Corp., Everett, Washington, USA). We registered the maximum temperature of the medial canthus of each eye as well as the minimum temperature of the naked area of the nose. We obtained the mean maximum temperature of both eyes and the mean minimum temperature of the nose for both before and after the separation/confinement test to assess the changes in temperature.

2.5. Video and statistical analysis

All behavioural variables were coded using the Solomon Coder software for video analysis (Péter, 2019). To assess inter-rater reliability, we randomly selected 15 videos from each of the tests (24% of all videos), and a naïve second observer coded them independently. Inter-rater reliability was assessed by calculating intra-class correlation coefficients using the R package irr (Gamer et al., 2012). Agreement between the two independent observers was high for all variables. Struggle test: latency to struggle ($R_{ICC} = 0.99$, $P < 0.001$). Separation/confinement test: latency to vocalize in ($R_{ICC} = 0.98$, $P < 0.001$), number of vocalizations ($R_{ICC} = 0.99$, $P < 0.001$), duration of motor activity ($R_{ICC} = 0.84$, $P = 0.005$). Meat test: latency to chew ($R_{ICC} = 0.99$, $P < 0.001$) number of aggressive displays (stomping, swiping, hissing; $R_{ICC} = 0.96$, $P < 0.001$).

Statistical analysis of the data was carried out using the program R, version 2022.02.1 (R Core Team, 2022) with the package~~s~~ *lme4* (Bates et al., 2015). We tested for significant effects of rearing condition (group) by fitting linear mixed models (LMMs) for normally distributed data or generalized linear mixed models (GLMMs) for count data. Plots of residuals versus fitted values, and Q-Q plots were used to visually inspect model residuals for homogeneity of variance and normal distribution. For the morphological development model, we analysed the weight with length as a covariate and litter ID as a random factor. Models of behavioural variables included litter ID as random factors, and sex and weight as covariates. Models were reduced using backward stepwise selection, starting with all candidate variables in the model, and removing the nonsignificant variable with the highest p-value in each step, until no nonsignificant variables remained. P values were extracted by Wald Chi-square tests.

Paired t-tests were used to evaluate if nose and eye temperature had significantly increased or decreased. We then calculated the change in ºC of the eye and nose measures to analyse the differences in face thermography between groups by fitting an LMM as previously described.

3. Results

We found no difference in the morphological development of the kittens across the study. For the weight at the time of testing we found that males (mean = 1127 g ± 187 SD) tended to weigh more than females (mean = 1019.5 g ± 161.13 SD), but we found no difference when comparing treatment groups (LMM sex: χ^2 = 11.24, $P < 0.001$, treatment: χ^2 = 0.86, $P = 0.652$). Results were similar for the length (from the tip of the nose till the base of the tail, LMM sex: χ^2 = 5.51, $P = 0.019$, treatment: χ^2 = 0.30, $P = 0.862$), which suggests normal morphological development of the orphans. All kittens were considered to be in good condition by the experienced handlers and were within a healthy weight range (see Berliner et al., 2022); they continued to grow favourably after adoption.

3.1. Behavioural tests

We found that orphan kittens overall, whether singletons or raised with siblings, struggle significantly sooner than mother-reared kittens (χ^2 = 14.28, $P < 0.001$; Figure 1).

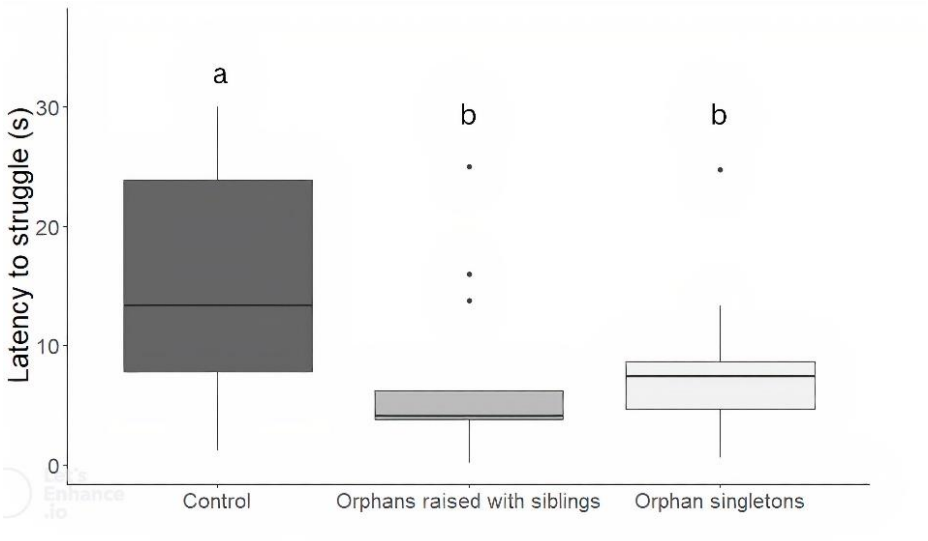

Figure 1. Latency (s) to struggle by control individuals (n = 32), orphans raised with siblings (n = 14) and orphan singletons (n = 16). Box plots give the median latency to struggle (horizontal lines within boxes) and interquartile ranges (upper and lower limits to the boxes). Dots represent outliers. Different letters indicate significant differences as reported, followed by Tukey post hoc tests.

In the meat test we initially found no differences among groups in the latency to begin consuming the meat (latency to chew; $\chi^2 = 3.39$, $P < 0.18$). However, there were seven individuals that took longer than a minute to begin eating or even approach the stimuli. Differences emerged when omitting these individuals (four control and three orphan singletons), where the orphans raised with siblings begin consuming the meat sooner than the control kittens ($\chi^2 = 6.98$, $P < 0.03$; Figure 2). Once the kittens were interacting with the resource, we found no differences between the groups regarding their displays of active defence/aggressive behaviours, such as growling, hissing, swiping and/or stomping ($\chi^2 = 0.67$, $P = 0.71$).

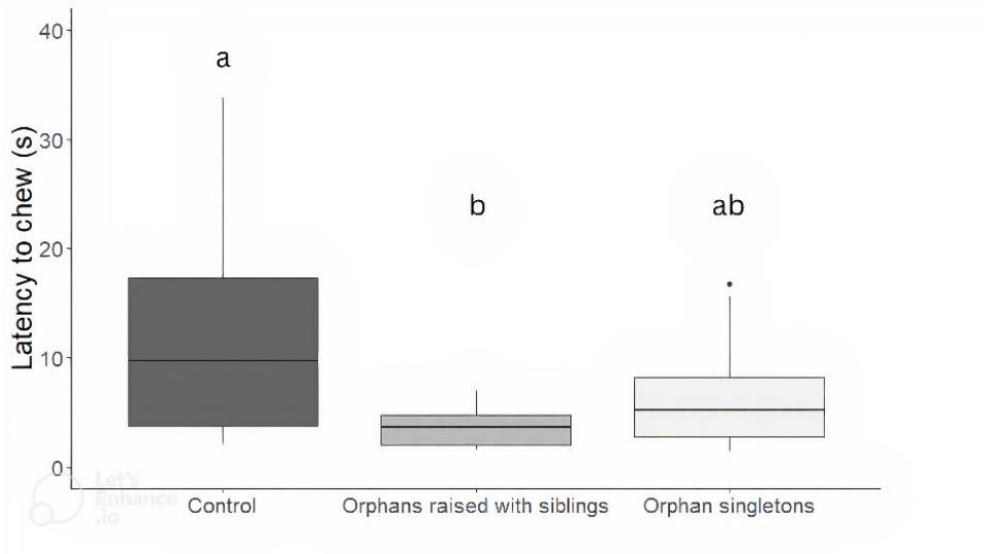

Figure 2. Latency (s) to begin consuming the meat stimulus by control individuals (n=28), orphans raised with siblings (n=14) and orphan singletons (n=13). Box plots give the median latency to begin chewing (horizontal lines within boxes) and interquartile ranges (upper and lower limits to the boxes). Dots represent outliers. Different letters indicate significant differences as reported, followed by Tukey post hoc tests.

In the separation/confinement test we found that orphans raised with siblings vocalise sooner than control individuals (χ^2 = 89.45, P < 0.001). We also found significant differences in the number of vocalisations between control kittens and orphans raised with siblings, and between control kittens and orphan singletons (χ^2 = 89.45, P < 0.001), with the orphan groups emitting more vocalisations when left alone in the carrier (Figure 3). A similar pattern can be seen for locomotion, where orphans overall exhibited more motor activity (walking, scratching, turning) during the test (χ^2 = 26.66, P < 0.001; Figure 4).

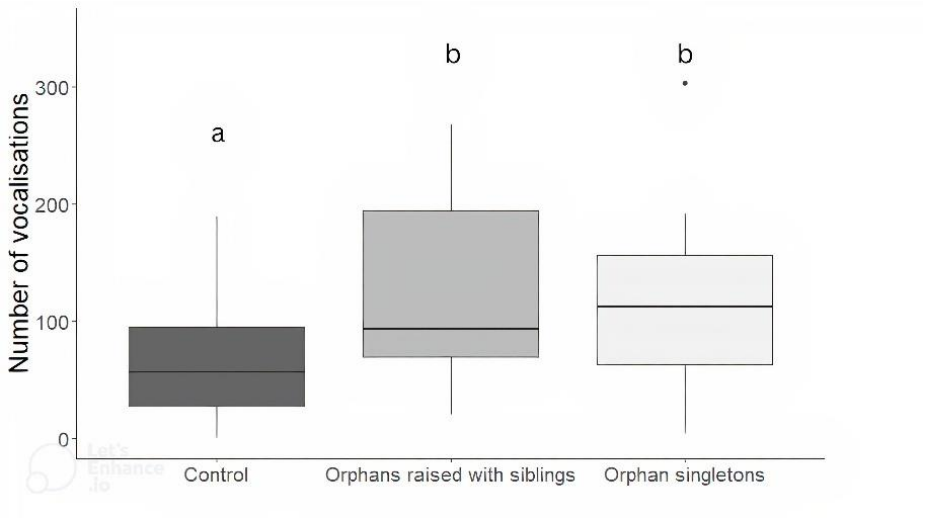

Figure 3. Number of vocalisations emitted by control individuals (n=32), orphans raised with siblings (n=14) and orphan singletons (n=16) during the carrier test. Box plots give the median latency to begin chewing (horizontal lines within boxes) and interquartile ranges (upper and lower limits to the boxes). Dots represent outliers. Different letters indicate significant differences as reported, followed by Tukey post hoc tests.

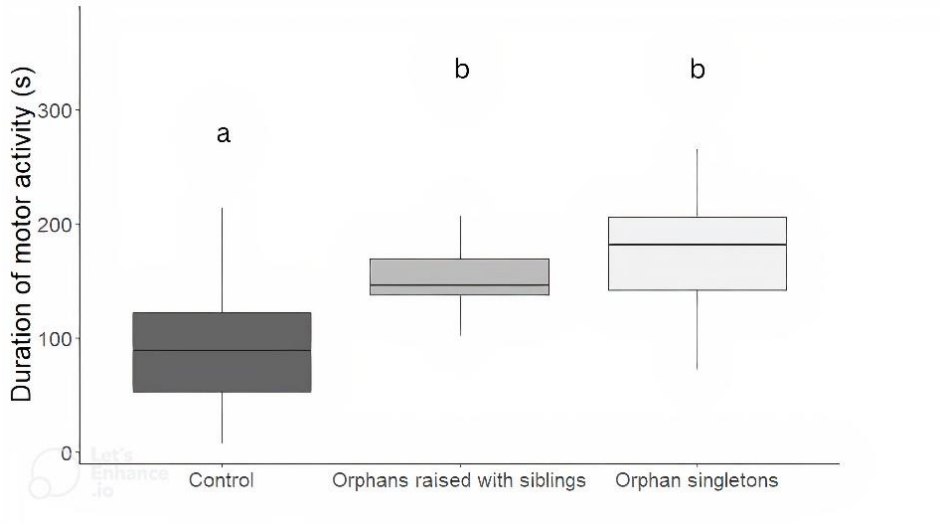

Figure 4. Time spent moving in the carrier (walking, scratching, turning) by control individuals (n=32), orphans raised with siblings (n=14) and orphan singletons (n=16). Box plots give the median latency to begin chewing (horizontal lines within boxes) and interquartile ranges (upper and lower limits to the boxes). Different letters indicate significant differences as reported, followed by Tukey post hoc tests.

3.2. Change in facial thermography due to the separation/confinement test

In all groups, the maximum recorded temperature of the eyes significantly decreased after the 5-minute separation (paired t test: $t_{61} = 5.99$, $P < 0.001$; mean temperature change = - 0.55 °C ± 0.723 *SD*). The minimum temperature of the nose also showed an overall significant decrease by the end of the test (paired t test: $t_{61} = 7.04$, $P < 0.001$; mean temperature change –2.66 °C ± 2.98 *SD*). However, we found no differences between groups, neither in the change in maximum eye temperature ($\chi^2 = 3.698$, $P = 0.157$), nor the change in minimum nose temperature ($\chi^2 = 0.443$, $P = 0.801$).

3. Discussion

In this study we aimed to evaluate the effects of early separation from the mother and siblings on the morphology and behaviour of kittens assessed with three behavioural tests at 9 weeks old. We first looked at the possible differences in body size between the groups as a way of assessing the kittens' physical condition. We found only that males weighed more than females at the time of testing, as has been reported in other studies of kitten development and is consistent with the strong sexual dimorphism in body size of adult cats. This is an indicator that the orphan kittens received adequate nutrition and care. Concerning the behavioural measures, we found differences between the control and orphan groups in all three tests. Overall, both orphans raised with siblings and orphan singletons struggled sooner, vocalised more often and showed higher levels of locomotor activity in the separation/confinement test. The orphans raised with siblings show further differences from the mother-reared individuals by having the shortest latency to vocalise and the shortest latency to begin consuming the meat (the only significant difference found in the meat test). Our findings are consistent with what has been reported by Lowell et al. (2020), where orphan kittens vocalised more and showed increased motor activity as compared to mother-reared

individuals during a brief social separation at one and three weeks of age. Our results show that such differences are still evident at the age of weaning. Put together, these observations suggest that, as expected, developing without the presence of the mother and raised by humans (with or without siblings) has an effect on the behaviour of kittens that persists at the age of weaning and possibly even into adult life, although this remains to be investigated. Contrary to our expectations, the presence of littermates had no visible effect on the behavioural development of the kittens, at least not in the tests we used.

The separation/confinement test presents two measures of stress, one behavioural and one physiological. Firstly, vocalisation has been increasingly recognized as a useful way to measure the emotional state of individuals quickly and reliably, both in experimental settings and in applied situations. For example, distress calls have seen applied use in welfare (Grandin, 1998; Manteuffel et al., 2004). Some vocalisations, such as separation calls, have been linked to negative emotions (Jürgens, 2002; Brudzynski, 2007; Newman, 2007), as vocal and physiological responses to stress are partially controlled by the same central neuroendocrine systems (Jürgens, 2009). Our results show that vocalisation in response to separation/confinement is affected by the early social environment of the kittens. Individuals raised in different early social environments might cope with everyday occurrences (such as being put in a carrier or confined for a short period of time) differently depending on their susceptibility to stress. The validation of vocalisation as a behavioural indicator of stress with additional qualitative vocal analysis, could enable a faster, easier and non-invasive assessment of stress reactivity in cats and potentially other animals in the future.

For our second measure of stress we used facial thermography. Thermal imaging has become increasingly popular to evaluate stress given that the activation of the HPA axis and the autonomic nervous system (ANS) affect peripheral vasodilatation changing skin temperature

(Palkovits, 2014). Studies have shown that changes in emotional state do reflect changes in skin temperature, usually measured in the eye and nose area in various mammals (Godyń et al., 2013; Travain et al., 2015; Lecorps et al., 2016; Kim and Cho, 2021). Given the differences in vocalisation behaviour between the groups of kittens, we expected to see similar differences in thermography. In general, we found a mean negative change in the eye as well as in the nose temperature. Even though stress-related hyperthermia is well documented in several species (e.g Bartolomé et al., 2013; Travain et al., 2015; Cannas et al., 2018), decreases in body temperature, or at least in certain parts, has also been reported. A similar decrease as in our findings has been previously found in cats (Foster and Ijichi, 2017), rabbits (Jaén-Téllez et al., 2020), cows (Stewart et al., 2007), and hens (Herborn et al., 2018). Contrary to what we expected, we found no differences between groups of kittens. Stewart et al. (2007) described that, in cows, HPA stimulation does not influence eye temperature but rather the heat emitted by capillaries around the eyes changes as blood flow is regulated by the ANS. It's possible that maternal separation affects the HPA axis and the ANS differentially. Behavioural responses during the separation/confinement test could be reflecting differences between groups in the activation of the HPA axis while face thermography reflects no differences in the activation of the ANS. However, we can't discount the possibility that the equipment used might not be sensitive enough to detect these small thermal differences between groups.

Decades of maternal separation studies have established the importance of maternal care during the pre-weaning period for the adequate behavioural and physiological development of the young (reviews in: Wang et al., 2013; Nishi et al., 2014; Tractenberg et al., 2016). Thus, maternal separation is a commonly used model for the effect of early life stress on various aspects of development and has been found to affect the regulation of the hypothalamic-pituitary-adrenal axis (HPA) throughout life stages (Aisa et al., 2007; Lippmann et al., 2007; Nishi et al., 2014).

However, the effects of separation from siblings have generally been less often considered (but see Martínez-Álvarez et al., 2022; Hudson et al., 2011). The general observation of these studies is that separation during early life results in individuals that exhibit elevated reactivity to stress, which is what we expected to find for our orphan kittens.

Finally, we did not find behavioural differences due to the absence of siblings. Orphan singletons did not differ significantly from orphans raised with siblings in any of our measured variables. This does not mean that differences might not exist. Our study is limited by a small sample size and, additionally, there could be differences in behaviours not considered by our tests. For example, anecdotal accounts from rescue organizations imply that singleton kittens tend to play more aggressively with their owners. Further research is needed on the behaviour of kittens that developed with or without siblings in contexts other than the ones presented in this study, like their interactions with humans and non-related cats.

In conclusion, early maternal separation might cause long-lasting and possibly irreversible changes in the behaviour and physiology of kittens; even when they received adequate care and nutrition by human caregivers. Our findings show that the early social environment of an individual is an important factor in shaping their coping style in later life stages. The behavioural differences reported here between mother-raised and hand-raised kittens should be considered by adoption centres and rescue organizations for the future well-being of the animals. For example, adoptions of kittens of pre-weaning age should be discouraged, and having a lactating foster queen raise the kittens should be favoured over hand-raising whenever possible. Research is needed on the possible behavioural effects of raising by a foster queen, but it could still be preferable to being raised as an orphan in terms of nutrition and receiving maternal stimuli. Additionally, since orphan kittens appear to be more stress-reactive they could have a higher probability of developing behavioural problems when adopted out or faced with abrupt changes in their environment. These

considerations are important when matching to owners, as behavioural problems are one of the main reasons cats are rehomed or returned to shelters (Salman et al., 2000).

Ethics note

Throughout the study, animals were kept and treated according to the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences, as well as the relevant legislation for Mexico (National Guide for the Production, Care and Use of Laboratory Animals, Norma Oficial Mexicana NOM-062-200-1999). The project was approved by CEI (Comité de Ética en Investigación, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz), protocol number CONBIOETICA-09-CEI-010-20170316.

Declarations of Competing Interest

The authors declare no conflicts of interest.

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DISCUSIÓN

En el presente estudio nuestro objetivo fue evaluar el efecto de una separación temprana de la madre y hermanos sobre la morfología, fisiología y conducta de crías de gato doméstico mediante tres pruebas conductuales aplicadas a las nueve semanas de edad. Primero analizamos las posibles diferencias en tamaño corporal entre los grupos, como una manera de valorar la condición física de las crías. Encontramos únicamente que los machos pesaron más que las hembras a la edad del destete, como ha sido reportado en otros estudios del desarrollo del gato y es consistente con el dimorfismo sexual marcado en el tamaño corporal de los gatos adultos. Esto es un indicador de que las crías huérfanas recibieron una nutrición y cuidados adecuados.

En cuanto a las variables conductuales, encontramos diferencias entre el grupo control y los grupos huérfanos en las tres pruebas. En general, tanto los gatos huérfanos criados con hermanos como los huérfanos criados en solitario forcejearon antes, y vocalizaron más demostrando mayor actividad motriz en la prueba de separación/confinamiento. Este patrón es consistente con lo observado en gatos adultos donde individuos que tienden interactuar más con humanos tienen una latencia baja para forcejear y son más vocales en una prueba de separación/confinamiento (Martínez-Byer et al., 2020). Los huérfanos criados con hermanos muestran diferencias adicionales con respecto a los individuos criados por su madre al tener la menor latencia para vocalizar, así como la menor latencia para empezar a consumir la carne (la única diferencia significativa en la prueba de la carne). Nuestros resultados son consistentes con lo reportado por Lowell et al. (2020), donde crías huérfanas vocalizaron más y presentaron mayor actividad motriz en comparación a individuos criados por su madre durante una breve separación social a la semana y tres semanas de edad. Nuestros resultados muestran que tales diferencias aún son evidentes a la edad del destete. En conjunto, estas observaciones sugieren que, como era de esperar, desarrollarse

sin la presencia de la madre (con o sin hermanos) tiene un efecto sobre la conducta de las crías que persiste a la edad del destete y posiblemente incluso en la vida adulta, aunque esto queda por ser investigado. Contrario a nuestras expectativas, la presencia de compañeros de camada no tuvo ningún efecto aparente sobre el desarrollo conductual de las crías, al menos no en los contextos que evaluamos.

La prueba de separación/confinamiento presenta dos medidas de estrés, una conductual y una fisiológica. En primer lugar, las vocalizaciones han sido cada vez más reconocidas como una manera útil de evaluar el estado emocional de los individuos de forma rápida y confiable, tanto en entornos experimentales como situaciones aplicadas. Por ejemplo, los llamados de auxilio han sido útiles en evaluaciones de bienestar (Grandin, 1998; Manteuffel et al., 2004). Algunas vocalizaciones, como los llamados por separación, se han relacionado con emociones negativas (Jürgens, 2002; Brudzynski, 2007; Newman, 2007), dado que las respuestas vocales y fisiológicas al estrés son parcialmente controladas por los mismos sistemas centrales neuroendocrinos (Jürgens, 2009). Nuestros resultados muestran que la vocalización en respuesta a separación/confinamiento es afectada por el entorno social temprano de las crías. Los individuos criados en diferentes entornos sociales tempranos podrían afrontar situaciones cotidianas (tales como estar en una trasportadora o ser confinados por un breve periodo de tiempo) de manera diferente dependiendo de su susceptibilidad al estrés. La validación de la vocalización como un indicador conductual del estrés con un análisis vocal cualitativo adicional podría permitir una evaluación más rápida, fácil y no invasiva de la reactividad al estrés en gatos, y potencialmente otros animales en el futuro.

Para nuestra segunda medida del estrés utilizamos termografía facial. La termografía se ha vuelto cada vez más popular para evaluar estrés dado que la activación del eje Hipotálamo-Hipófisis-Adrenal (HHA) y el sistema nervioso autónomo (SNA) afecta la vasodilatación

periférica afectando la temperatura de la piel (Palkovits, 2014). Estudios han demostrado que cambios en el estado emocional se ven reflejados en cambios de la temperatura de la piel, usualmente medidos en el área ocular y la nariz de varios mamíferos (Godyń et al., 2013; Travain et al., 2015; Lecorps et al., 2016; Kim and Cho, 2021). Dadas las diferencias en la conducta de vocalización entre los grupos de crías, esperábamos ver diferencias similares en la termografía. En general, encontramos un cambio promedio negativo en la temperatura tanto de los ojos como la nariz. A pesar de que la hipertermia por estrés ha sido bien documentada en varias especies (e.g Bartolomé et al., 2013; Travain et al., 2015; Cannas et al., 2018), disminuciones de la temperatura corporal, o al menos en ciertas áreas del cuerpo, también han sido reportadas. Un descenso similar al de nuestros resultados se ha encontrado previamente en gatos (Foster and Ijichi, 2017), conejos (Jaén-Téllez et al., 2020), vacas (Stewart et al., 2007), y gallinas (Herborn et al., 2018). Contrario a lo esperado, no encontramos diferencias entre los grupos. Stewart et al. (2007) describe que, en vacas, la estimulación del eje HHA no tiene influencia sobre la temperatura ocular, sino que el calor emitido por los capilares alrededor de los ojos cambia a medida que el flujo sanguíneo es regulado por el SNA. Es posible que la separación maternal afecte al eje HHA y al SNA de manera diferencial. Las respuestas conductuales durante la prueba de separación/confinamiento podrían estar reflejando diferencias entre los grupos en la activación del eje HHA, mientras que la termografía facial refleja que no hay diferencias en la activación del SNA. Sin embargo, no podemos descartar la posibilidad que el equipo utilizado podría no ser lo suficientemente sensible para detectar estas pequeñas diferencias térmicas entre los grupos.

Décadas de estudios sobre separación materna han establecido la importancia del cuidado maternal durante el periodo de lactancia para el desarrollo fisiológico y conductual adecuado de las crías (reviews in: Wang et al., 2013; Nishi et al., 2014; Tractenberg et al., 2016). Por lo tanto, la separación maternal es un modelo común para estudiar el efecto del estrés temprano sobre varios

aspectos del desarrollo y se ha encontrado que afecta la regulación del eje HHA a través de las etapas de vida (Aisa et al., 2007; Lippmann et al., 2007; Nishi et al., 2014). Sin embargo, los efectos de la separación de los hermanos/compañeros de camada no han sido considerados con mucha frecuencia (pero ver Martínez-Álvarez et al., 2022; Hudson et al., 2011). La observación general de estos estudios es que la separación durante la etapa temprana de la vida resulta en individuos que muestran una reactividad al estrés elevada, que es lo que esperábamos encontrar en nuestros grupos de crías huérfanas.

Finalmente, no encontramos diferencias conductuales debidas a la ausencia de los hermanos. Los gatos huérfanos criados en solitario no difirieron significativamente de los huérfanos criados con hermanos en ninguna de las variables evaluadas. Esto no significa que no podrían existir diferencias. Nuestro estudio está limitado por un tamaño de muestra reducido y, adicionalmente, podría haber diferencias en conductas que no fueron consideradas en nuestras pruebas. Por ejemplo, relatos anecdóticos de organizaciones de rescate e individuos que han criado gatos sugieren que aquellos gatos criados en solitario tienden a jugar más agresivamente con sus dueños. Se requiere más investigación sobre la conducta de crías que se desarrollaron con o sin sus hermanos en contextos distintos a los presentados en este estudio, como su interacción con cuidadores humanos y gatos no familiares.

En conclusión, la separación maternal temprana podría causar cambios a largo plazo posiblemente irreversibles en la conducta y fisiología de las crías de gato doméstico; aún cuando recibieron nutrición y cuidados adecuados por parte de cuidadores humanos. Nuestros resultados muestran que el entorno social temprano de un individuo es un factor importante en moldear el estilo de afrontamiento (coping style) hacia las siguientes etapas de la vida. Las diferencias conductuales que reportamos entre gatos criados por su madre y gatos criados por humanos deberían ser consideradas por centros de adopción y organizaciones de rescate para el bienestar

futuro de los animales. Por ejemplo, se debería desaconsejar la adopción de crías en edad lactante, favoreciendo una madre lactante substituta sobre la crianza por humanos cuando sea posible. Se requieren estudiar los posibles efectos conductuales de ser criado por una madre adoptiva, pero podría ser preferible a ser criado como huérfano en términos de nutrición y de recibir estímulos maternales que como humanos no podemos replicar en su totalidad. Adicionalmente, dado que las crías huérfanas parecen ser más reactivas al estrés, podrían tener una mayor probabilidad de desarrollar problemas conductuales al ser adoptados como mascotas o al enfrentarse a cambios abruptos en su entorno. Estas consideraciones son importantes al empatar las expectativas y estilo de vida de dueños potenciales con gatos en adopción, ya que los problemas conductuales son una de las razones principales que los gatos son realojados o devueltos a refugios (Salman et al., 2000).

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MATERIAL SUPLEMENTARIO

id	grupo	camada	tamaño de camada	sexo	cuidador	edad de separación	peso al destete
enigma1_blue	control	enigma1	5	m	RH	no separado	771
enigma1_green	control	enigma1	5	m	RH	no separado	1270
enigma1_pink	control	enigma1	5	m	RH	no separado	1078
enigma1_purple	control	enigma1	5	m	RH	no separado	1156
enigma1_red	control	enigma1	5	f	RH	no separado	1028
enigma2_blue	control	enigma2	6	m	RH	no separado	1310
enigma2_green	control	enigma2	6	f	RH	no separado	1008
enigma2_pink	control	enigma2	6	f	RH	no separado	1067
enigma2_purple	control	enigma2	6	f	RH	no separado	1142
enigma2_stripy	control	enigma2	6	m	RH	no separado	1252
enigma2_yellow	control	enigma2	6	m	RH	no separado	1146
harley1_bw	control	harley1	5	f	RH	no separado	993
harley1_calico	control	harley1	5	f	RH	no separado	810
harley1_minibalam	control	harley1	5	m	RH	no separado	1095
harley1_slash	control	harley1	5	m	RH	no separado	1154
harley1_spot	control	harley1	5	m	RH	no separado	1083
nova2_green	control	nova2	4	m	RH	no separado	1137
nova2_pink	control	nova2	4	m	RH	no separado	1172
nova2_red	control	nova2	4	m	RH	no separado	1268
nova2_yellow	control	nova2	4	m	RH	no separado	1027
nova3_bigfluff	control	nova3	4	m	RH	no separado	1350
nova3_gw	control	nova3	4	f	RH	no separado	1130
nova3_littlefluff	control	nova3	4	m	RH	no separado	1250
nova3_minibalam	control	nova3	4	f	RH	no separado	1087
willow2_black	control	willow2	3	m	RH	no separado	1263
willow2_bw	control	willow2	3	m	RH	no separado	1256
willow2_tortie	control	willow2	3	f	RH	no separado	1329
willow3_blue	control	willow3	5	m	RH	no separado	986
willow3_green	control	willow3	5	m	RH	no separado	850
willow3_gw	control	willow3	5	f	RH	no separado	895
willow3_orange	control	willow3	5	m	RH	no separado	937
willow3_purple	control	willow3	5	m	RH	no separado	990
g1_green	camada huérfana	orphan_g1	4	m	JG y RH	4 semanas	1504

g1_pink	camada huérfana	orphan_g1	4	f	JG y RH	4 semanas	1304
g1_red	camada huérfana	orphan_g1	4	f	JG y RH	4 semanas	1369
g1_yellow	camada huérfana	orphan_g1	4	m	JG y RH	4 semanas	1422
g2_black	camada huérfana	orphan_g2	4	m	AL y SMB	2 semanas	1309
g2_orange	camada huérfana	orphan_g2	4	m	AL y SMB	2 semanas	1152
g2_pink	camada huérfana	orphan_g2	4	f	AL y SMB	2 semanas	919
g2_purple	camada huérfana	orphan_g2	4	m	AL y SMB	2 semanas	1053
g3_blue	camada huérfana	orphan_g3	3	f	PNA y SMB	4 semanas	768
g3_red	camada huérfana	orphan_g3	3	m	PNA y SMB	4 semanas	701
g3_teal	camada huérfana	orphan_g3	3	f	PNA y SMB	4 semanas	789
g4_blue	camada huérfana	orphan_g4	4	f	SMB	4 semanas	1064
g4_pink	camada huérfana	orphan_g4	4	f	SMB	4 semanas	1175
g4_yellow	camada huérfana	orphan_g4	4	f	SMB	4 semanas	1122
s1_blue	huérfano único	orphan_s1	4	f	AU	1 semana	1052
s1_pink	huérfano único	orphan_s1	4	f	SMB	1 semana	840
s1_teal	huérfano único	orphan_s1	4	f	RC	1 semana	952
s1_yellow	huérfano único	orphan_s1	4	f	DG	1 semana	1005
s2_luna	huérfano único	orphan_s2	1	f	LAF	2 semanas	1100
s3_indy	huérfano único	orphan_s3	1	f	LT	1 semana	850
s4_baguira	huérfano único	orphan_s4	3	f	VO	4 semanas	818
s4_gattini	huérfano único	orphan_s4	3	m	AU	4 semanas	1008
s4_kinoa	huérfano único	orphan_s4	3	f	RD	4 semanas	992
s5_dutchess	huérfano único	orphan_s5	3	f	SMB	4 semanas	891
s5_gala	huérfano único	orphan_s5	3	f	SO	4 semanas	
s5_vinnie	huérfano único	orphan_s5	3	m	ADR	4 semanas	814

s6_canoli	huérfano único	orphan_s6	1	f	CG	4 semanas	1235
s7_pearl	huérfano único	orphan_s7	2	f	AC	3 semanas	900
s7_ruby	huérfano único	orphan_s7	2	f	SMB	3 semanas	951
s8_red	huérfano único	orphan_g4	4	m	RM	4 semanas	1200