The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK

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Murid stress odours: a review and a 'low tech' method of collection

GJ Mason*†, J Dallaire† and N Ware‡

† Department of Animal and Poultry Science, University of Guelph, Ontario N1G 2W1, Canada
‡ St. Peter's College, Oxford University, New Inn Hall Street, Oxford OX1 2DL, UK
* Contact for correspondence and requests for reprints: gmason@uoguelph.ca

Abstract

Stress cues can affect the welfare of animals in close proximity and are possibly useful non-invasive indicators of the emitters' welfare. To facilitate their study in murids, we tested whether rats' stress odours could be collected and stored using an enfleurage-type technique. 'Donor' rats were individually exposed to a compound stressor (carried circa *75 m inside a novel container, then euthanised with rising carbon dioxide) while on blotting paper dotted with melted vegetable lard. These sheets were sealed, left at room temperature for 2–5 h, and then 'bioassayed' by a blind observer for their effects on conspecifics. Compared with control sheets (exposed to unstressed rats, to CO_2 alone, or untreated), stress-exposed sheets significantly affected the unconditioned behaviour of 16 pairs of detector rats trained to enter an arena from their home cage to obtain sucrose. When used to line this arena, the stress-exposed sheets significantly increased: i) rats' latencies to eat, to place front feet into, and to completely step into the arena and ii) shuttling movements between arena and home cage. These pilot data thus suggest that odours produced by stressed rats can be simply and successfully collected and stored for several hours, though certain potential confounds (eg urine volume) may conceivably be alternative explanations for the observed effects. Future work should control for urine volume, and assess whether fat is needed for optimal odour absorption by paper and for how long sheets can be stored at various temperatures. Much fundamental work is also still needed on the nature, functions, and sources of stress odours.*

Keywords: *alarm, animal welfare, odour, rat, signal, stress*

Introduction

Animals' responses to pain, fear or stress often elicit strong physiological or behavioural reactions in nearby conspecifics. This is because they may inadvertently give off incidental cues (eg through their startle or flight responses) which are innately salient or become so through conditioning or, alternatively, they may emit signals — communicative cues evolved to influence other animals in a way that benefits the signaller (eg Maynard-Smith & Harper 2003). Vocal alarm or distress signals, for instance, may attract conspecifics and encourage predator-mobbing, or instead act as a warning for them to hide or flee; a response potentially benefiting the signaller's inclusive fitness (eg Russ *et al* 1998; Krams *et al* 2006; Blumstein 2007). Such signals may even be graded in magnitude or quality, to convey information about the proximity or nature of a threat (eg Manser *et al* 2001; Randall & Rogovin 2002; Kiriazis & Slobodchikoff 2006). Not all such cues are auditory: they can be visual, like the fin-flicking of glowlight tetras (*Hemigrammus erthrozonus*) (Brown *et al* 1999), or chemical. For example, water-borne chemical cues from injured fish, salamanders that have autotomised a tail, and many adult or larval amphibians aware of a predator's presence, cause conspecifics to avoid the area and to show enhanced vigilance, freezing and other anti-predator behaviours (eg Hucko & Cupp 2001; Brown *et al* 2004; Dupuch *et al* 2004; Rajchard 2006), while air-borne chemical cues are produced by many species when hurt or threatened, eg the pheromones released by social insects (Wilson 1965; Hughes *et al* 2001) and the odours produced by many mustelids (Dunstone 1993). In this paper, we refer to these various responses as 'stress cues', a broad term covering all cues produced in response to injury, restraint or perceived threat, which influence conspecifics either as a by-product of their emission or as specifically-designed signals.

Stress cues are of potential welfare significance for three reasons. First, they can be used as a relatively humane deterrent for pest species (Bishop *et al* 2003; Cook *et al* 2008). For instance, the playback of night heron (*Nycticorax nycticorax*) distress cries has been used to drive herons from trout ponds (Spanier 1980). Such approaches may be preferable on welfare grounds to many traditional means of pest control (Mason & Littin 2003). Second, stress cues may reduce the welfare of animals exposed to them. As we have seen, they are often avoided (see also Vieuille-Thomas & Signoret 1992 and Boissy *et al* 1998 on the aversiveness of urine-borne stress cues to farmed ungulates), and they can even act as negative reinforcers: for example,

jackdaws (*Corvus monedula*) will learn to peck a key that prevents recorded jackdaw distress calls from being played (Morgan & Howse 1973), while rats offered two concurrent levers that deliver, respectively, food or food plus a sound, are indifferent between these if that sound is a neutral tone, but prefer the 'food only' lever if the sound is a rat distress scream (Aoyama & Okaichi 1994, cited by Burn 2008). Thus, unsurprisingly, animals exposed to conspecific stress cues that they cannot avoid often show signs of stress. For instance, rats held in the same room as stressed conspecifics show elevated circulating corticosterone (Pitman *et al* 1988), and even simply the chemical cues from stressed rats or mice (eg via water they have been immersed in or air that has been blown over them) can, similarly, provoke corticosteroid responses in exposed conspecifics (eg Moynihan *et al* 1994) — effects we discuss in greater detail below. The third welfare implication of stress cues is that, especially if their biological meaning is understood, they can help us assess the fear, stress or anxiety of the emitter (Weary & Fraser 1995; Watts & Stookey 2000; Manteuffel *et al* 2004). For instance, piglet vocalisations have been investigated as a pain indicator (Weary *et al* 1998) and used to identify the most painful components of castration (Taylor & Weary 2000). Calf distress calls have been used to compare the effects of different weaning methods on farms (Haley *et al* 2005), while cattle vocalisations in slaughter plants have been used to pinpoint handling and equipment that are suboptimal for welfare (Grandin 1998, 2001). Rat vocalisations have been used as an index of distress during carbon dioxide euthanasia (Niel & Weary 2006a); while in rodent-based psychopharmacology research, the calls made by rats and mice during agonistic encounters, social separation, exposure to aversive stimuli or drug withdrawal, have been advocated as useful measures for screening potential anxiolytics (Miczek *et al* 1995; Covington & Miczek 2003).

The stress cues of laboratory murids — chemical as opposed to vocal — are the focus of this paper. Our aim was to increase the ease with which they might be investigated by seeing whether they could be collected simply using an enfleurage-type technique, stored, and then moved to another site for detection. Before introducing our methods, we first briefly review what is known about murid stress odours, in terms of their source, properties and effects on conspecifics. Previously we have discussed the effects of these odours on recipient welfare whereas now we focus largely on the use of recipients' responses in order to make inferences about stress odour presence and, potentially, donor welfare.

Initially investigated by Müller-Velten (1966), rodent stress odours are yet to be identified or characterised at the molecular level. Thus, their precise nature remains unknown, and their detection relies solely on their effects on conspecifics. Indeed, it is even unknown whether they are inadvertent cues of stress, or true chemical signals — evolved to communicate information of benefit to the sender. 'Donor' animals are typically stressed via footshock or forced-swim sessions, and recipient or 'detector' animals then exposed to the flooring the donors were shocked on, the water they were immersed in, or streams of air passing over the stressed animals and directed into another apparatus. Where recipients are able, they typically avoid the chemical cues from the stressed odour donors, for instance, avoiding areas where these are present (eg Carr *et al* 1970; Rottman & Snowdon 1972; Mackay-Sim & Laing 1980; 1981a,b; Zalaquett & Thiessen 1991). Activity levels often increase, eg Kikusui *et al* (2001) report increased 'restlessness', while Mackay-Sim and Laing (1980) recorded increased movement between areas containing stress odour and areas that did not, and in forced swim baths containing stress odours, immobility in the water greatly decreases (Abel & Bilitzke 1990; Abel 1991, 1992). In the presence of stress odours, behavioural signs of anxiety also increase, such as increased vigilance, freezing and sniffing (Kikusui *et al* 2001; see also Zalaquett & Thiessen 1991 for similar trends in mice), and increased acoustic startle responses (Inagaki *et al* 2008). Other effects on recipients include hyperthermia (Kikusui *et al* 2001), opioid-mediated analgesia (Fanselow 1985; Moynihan *et al* 2000) and, in individuals treated with imipramine (a tricyclic antidepressant), increased risks of convulsions (Abel *et al* 1992). Physiological stress may also be induced, as reviewed earlier; and immune functioning can alter: for example, in mice, stress odour exposure reduces natural killer cell activity while increasing humeral antibody responses (Moynihan *et al* 1994, 2000; see also Cocke *et al* 1993).

These various behavioural and physiological responses in recipients have been utilised experimentally to explore the properties of rodent stress odours. It has been shown that stress odours seem only to affect conspecifics (eg Müller-Velten 1966, cited by Carr *et al* 1970; Stevens & Gerzog-Thomas 1977). The odours are also graded, increasing in their impact the greater the stress experienced by the donor. For instance, the degree of preference for an odour donor shown by odour-exposed rats correlates negatively with the degree of footshock experienced by the donor (Mackay-Sim & Laing 1980). The immobility of rats in the forced swimming test held in water previously soiled by a conspecific also decreases more severely if the donor was held in the bath for a longer period (Abel & Bilitzke 1990). Finally, when rats were placed in a Y-maze, one arm containing the odour of a non-stressed rat and, the other, the odour of a stressed rat, levels of exploratory activity increased if the latter donor had been mildly stressed, but decreased if it had been more severely treated (Mackay-Sim & Laing 1981a; see also Dua & Dobson 1975). It is not yet known whether such graded or otherwise varying responses reflect changes in odour amount or in odour quality. However, it does seem clear from the effects on recipients that rodent stress odours are multifactorial, and that they have multiple sources (eg Kiyokawa *et al* 2004). Thus, the urine from stressed animals is aversive, compared with that from unstressed conspecifics (Müller-Velten 1966, cited by Rottman & Snowdon 1972; Mackay-Sim & Laing 1981b). However, so too is the blood (Mackay-Sim & Laing 1981a), or even just

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their homogenised muscle (Stevens & Gerzog-Thomas 1977). Air that has passed over a stressed animal is also effective, regardless of whether or not the odour donor has defaecated or urinated (Rottman & Snowdon 1972; Mackay-Sim & Laing 1980; Zalaquett & Thiessen 1991). Furthermore, it has been suggested that odours from the anal glands and sebaceous glands of the whisker provoke autonomic and behavioural stress responses, respectively (Kiyokawa *et al* 2004); while the Harderian glands could also be important, since these produce chromodacryorrhoea when rats are stressed and have been hypothesised to release pheromones (eg Mason *et al* 2004).

Whatever the sources of murid stress odours, their potential use in practical welfare assessment would be more feasible if they could somehow be collected and stored, in order that they could be 'bioassayed' (via conspecific response) at a later time and perhaps even at another locale. Piloting such a method was the aim of this experiment.

Materials and methods

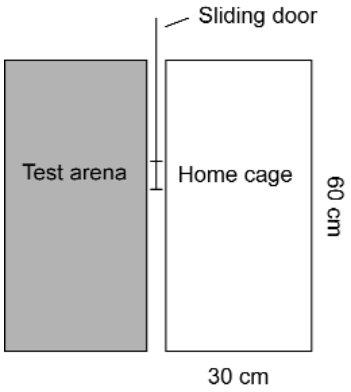
The subjects to be used as odour-detectors were 16 same-sex pairs of young adults (approximately four months of age; 450–550 g bodyweight) Lister Hooded (Long Evans) rats (*Rattus norvegicus*) (Harlan, Bicester, UK) — eight male and eight female. They were housed in a unit with a semi-reversed light cycle (0100–1300h and 1300–0100h; light and dark), each pair living in a medium-sized polypropylene cage (60 × 30 × 28 cm; length × width × height [North Kent Plastic Cages, Kent, UK]) with a mesh lid and front, aspen wood chip bedding, and enriched with cardboard tubes (Lillico, Surrey, UK), nesting material, hanging parrot toys and a hammock made out of a facecloth to sleep in (Figure 1). Subjects were maintained on RM3 pelleted diet (Special Diet Services, Essex, UK) from a food-hopper and *ad libitum* tap water from a bottle. Each cage was attached via a sliding door on one side to a second cage — the test arena — of similar size and structure, but empty except for a lining of plain paper (45 × 25 cm; length × width). Once a day for five days, the sliding door was withdrawn during the early part of the dark phase, and the rats habituated to exploring the attached arena for ten minutes. Exploration was rewarded by means of a level teaspoon of sugar that had been sprinkled evenly onto the paper prior to the start of each training trial. Between training trials, each test arena was cleared of paper, droppings and uneaten sugar, and lined with new paper. After five days, the rats appeared highly motivated to explore, and the experiment proper was commenced.

During the experiment, instead of the plain paper, the test arena of each pair of rats was lined with specially-treated blotting paper (45 × 25 cm, 90 gsm [Southfield Stationers, Midlothian, UK]), some sheets of which had been in contact with stressed conspecifics. Solid, low-scent fats are extremely effective at absorbing certain odours, and this underlies the enfleurage scent collection techniques used in the perfume industry (eg Rimmel 1865; Süskind 1986). We therefore used blotting paper treated with melted vegetable lard. However, since at least part of murid stress odour is water-soluble (eg Abel & Bilitzke 1990; Kiyokawa *et al* 2005), the whole paper was not impregnated, but instead the fat was placed in dots of approximately 1 cm across, approximately every 3 cm. This paper was always handled using clean gloves, and stored in individual polythene bags when not in use. Sixteen such sheets were prepared the evening prior to each experimental day. The morning before each experimental trial, four of these were left unmanipulated, four were exposed to rats stressed and euthanised with carbon dioxide, four were exposed to carbon dioxide alone, and four were exposed to unstressed rats (Figure 2). This was repeated for four successive days.

Each afternoon, starting at 1200h, the four fat-treated sheets to be exposed to unstressed rats were taken to the test subjects' room and used to line four test arenas (two male, two female). The remaining 12 arenas were lined with plain paper. All arenas were then sprinkled with sugar as before, and all rats allowed to explore for 15 min (a period chosen from prior pilot runs, see below). The four treated sheets were then removed, dusted free of sugar and any droppings, individually bagged, and sealed and numbered. The time was also noted. Thus all test (detector) rats had identical experiences each day, although only four of the 16 pairs were acting as odour donors for that afternoon's experimental trial.

Simultaneously, the eight sheets allocated for exposure to stressed rats and to carbon dioxide alone were taken to a separate rat room, where animals of the same age and strain as the detector rats were to be euthanised. Four sheets were removed from their storage bags and used to line four separate, lidded cardboard carrying boxes. Two males and two females were individually placed in each box, and a stopwatch started. Each box was carried by hand (for

Figure 1



Housing configuration. Subjects were kept in the home cage, containing food, water, and enrichments, with the sliding door shut. Daily, for 15 min, the sliding door was removed to allow subjects access to the test arena, empty save for paper lining on the floor and sugar that had been sprinkled over the paper. Photographs of the home cages, including the doorway, can be found in Burn and Mason's recent (2008) publication (their Figure 1).

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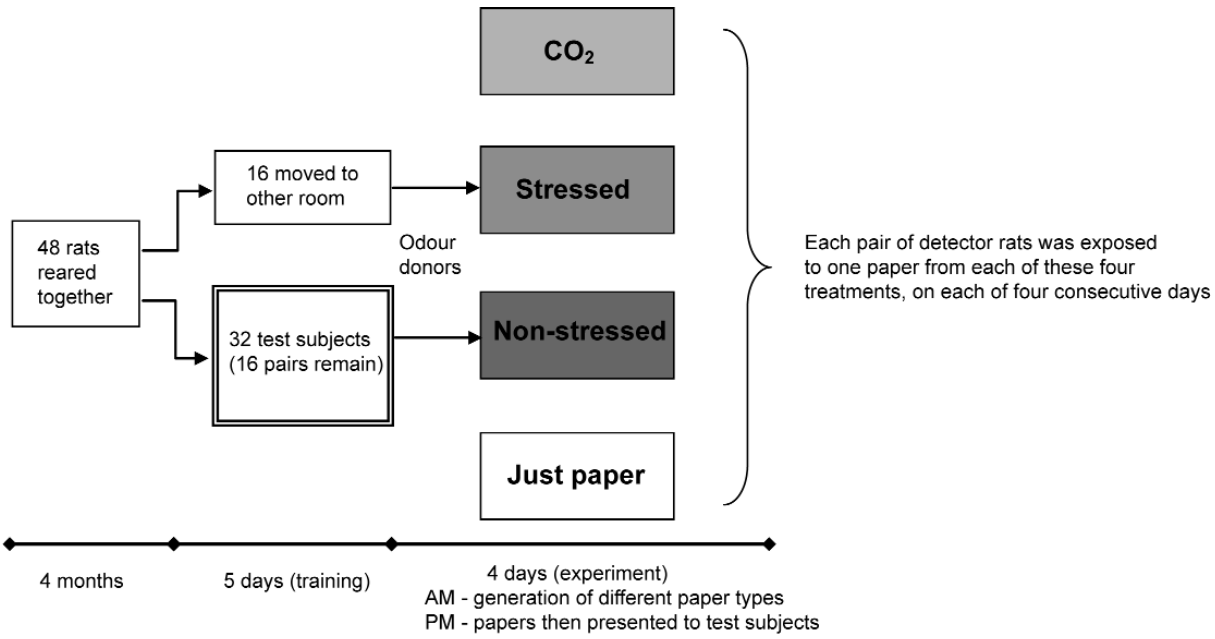
Figure 2



Schematic representation of the experimental procedure. At approximately four months of age, rats were separated into Stressed donors and test subjects (unstressed donors/detectors). Housed in pairs, these were first trained to explore the arena for a sugar reward. They were then used independently as Non-stressed odour donors, and as odour detectors in the experimental phase. Each pair of detectors was exposed to one of the four types of sheets, on four separate days; note that when exposed to the Non-stressed paper, they were never exposed to one produced by themselves, but always to one generated by other non-stressed donor rats that morning.

2–3 min) for approximately 75 m to a 'procedures room' containing the euthanasia chamber. Here, still within its box, each animal was placed in the chamber, the lid sealed, the time noted, and the rat exposed to rising levels of carbon dioxide at a rate of 10 l min^{-1}. Rats usually became unconscious after 2–3 min and died within 4–6 min. This was deemed the 'Stressed' treatment, on the grounds that (i) handling and being placed in a new cage may be stressful (Balcombe *et al* 2004; cf Burn *et al* 2006), and (ii) carbon dioxide levels above 25% are aversive (eg Leach *et al* 2002a,b; Niel & Weary 2006b), cause distress vocalisations (Hawkins *et al* 2006; Niel & Weary 2006a), and exposure to the blood or muscle of rats euthanised under rising carbon dioxide provokes fright responses in conspecifics (Stevens & Gerzog-Thomas 1977). At death, each sheet was removed and brushed clean of droppings, and the time again noted. It was then individually bagged, sealed and labelled, a teaspoon of sugar also being added for 15 min and then shaken out (so that sucrose odours were not specific to the Non-stressed sheets). The period of contact between paper and stressed donor rats meaned approximately 15 min, because rats remained in their cages for several extra minutes while others were being carried or euthanised. This was similar to that judged from previous pilot runs and used to inform the exposure period for the Non-stressed paper (see above). The timed periods of exposure to carbon dioxide during this treatment were used similarly to expose four further blotting papers each day to the gas alone (by placement in the euthanasia chamber). Again, once bagged, these papers were each sprinkled with sugar, which was left for 15 min before being shaken out. Bags were then resealed and labelled.

The procedure in practice meant that Non-stressed papers were exposed to donor rats for 15 min, starting at 1200h each day; while Stressed sheets were exposed to donor rats for a mean of 16 min 20 s, starting on average at 1220h each day, during which carbon dioxide exposure occurred for 10 min. The Carbon dioxide control papers were exposed to the gas alone for a mean of 10 min 10 s, with a mean start time of 1240h each day. Behavioural data from the detector rats were collected between 1400 and 1700h. Thus, the scents on the Stressed and Non-stressed sheets were between two and five hours old when tested, while those on the Carbon dioxide sheets were between approximately 1.5 and 4.5 h old. Overall, the Stressed sheets and two exposed controls were thus similar if not identical in exposure time, plus broadly similar if not identical in freshness at test (exposure time for the different treatments varied due simply to human error).

At the start of each afternoon's experimental session, the four types of paper sheet ('Stressed', plus the three controls: 'Non-stressed', 'Carbon dioxide' and 'Just paper') were pseudo-randomly allocated by GJM to each pair, such that: each treatment was tested on four pairs per day; Stressed and Non-stressed papers were presented to detector animals of the same sex as the odour donor and Non-stressed papers were

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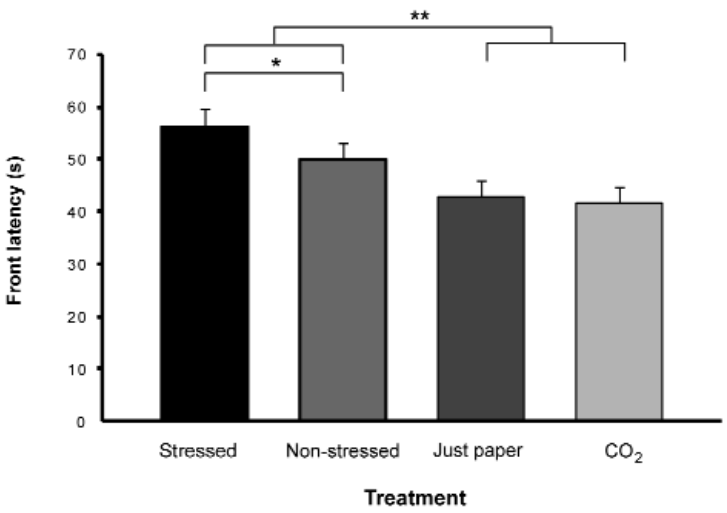
Figure 3



Mean (± SEM) Front latency. Treatment affected the latency to place the forepaws in the test arena ($F_{3,40}$ = 26.27, $P < 0.0001$), and this effect held when the non-significant sex × treatment term was removed ($F_{3,43}$ = 26.87, $P < 0.0001$) to run Tukey's *post-hoc* comparisons. These *t*-tests showed the Stressed treatment to have greater effects than all controls ($t > 3.54$, $P < 0.005$ for all three comparisons). The Non-stressed value also was significantly greater than the other two controls ($t > 3.90$, $P < 0.001$). * $P \leq 0.01$; ** $P \leq 0.001$.

never given to the rats that had actually generated them. Importantly, the ensuing procedure was conducted by NW, blind to the nature of each sheet. The allocated sheet was removed from its bag and used to line a test arena. One teaspoon of sugar was sprinkled evenly across it, the sliding door opened, and a stopwatch started. That pair of detector rats' behaviour was then observed for 10 min, the following data being collected based on previous studies of stress odours' behavioural effects (see *Introduction*): the time taken for each rat to place its front feet in the newly-opened compartment ('Front latency'), to completely step into the opened compartment ('Body latency') and to eat the sugar ('Latency to eat'); plus the number of times rats moved between the test chamber and the home cage ('Shuttling'). This procedure was repeated for each of the remaining 15 pairs and again over the following three afternoons. The order in which pairs were observed and tested was the same every day to ensure that test order or time of day did not cause extraneous within-pair variation; thus, the four treatments were also evenly distributed in time and order over the course of the experiment. The collected data were used to calculate two additional measures: i) 'Body latency' was subtracted from 'Front latency' to quantify how long each rat took between starting to enter the chamber and completing the movement (approximating to the 'Stretch attend' posture rats show when investigating potential threat, eg Blanchard *et al* 1990) and ii) 'Body latency' was subtracted from the full trial length (600 s) to give an approximate index of the total time spent in the chamber, and 'Shuttling' then divided by this number to give an index termed 'Shuttling rate'. For the two rats per pair, each of the six behavioural measures was meaned (since non-independent), and data analysed using a general linear model (GLM; Minitab 14) of the form: y = pair (sex) + sex + treatment + sex × treatment', with 'pair' specified as a random effect. Data were suitably transformed if their residuals did not meet the assumptions underlying parametric tests. The significance level used for treatment effects was 99% ($P = 0.01$), to avoid Type I errors arising through multiple testing ($P = 0.05$ was treated as a trend). Where there were significant treatment effects and the term sex × treatment was found to be insignificant ($P > 0.05$), this interaction term was excluded and the model re-run to allow *post-hoc* Tukey's *t*-tests to compare the four treatments.

As additional investigations to see if pairs were consistent in their responses, regressions (of the form: y = sex + x, where x is a covariate) were run to compare each behavioural measure obtained in the Stressed treatment with the equivalent as assessed during all three controls (meaned); and also to investigate whether the different behaviours co-varied, both within the Stressed and control conditions. These analyses were merely exploratory, and so the conventional level of alpha ($P = 0.05$) was used.

In terms of the ethical implications, the 'stress donors' were surplus rats already scheduled to be euthanised. They were treated no differently for this experiment than they would have been had no data been collected from them. The work did not require a Home Office licence, but was approved by Oxford University's ethical review process.

Results

In the detector rats, all four measured behaviours were affected by treatment, ie the nature of the paper to which they were exposed (see Figures 3–6). Values were always higher for the Stressed treatment than for all three controls. These effects were particularly marked for Shuttling between the test chamber and the home cage, and for Latency to eat, which doubled during the Stressed treatments compared with the controls (Shuttling was also affected by sex, males returning to the home cage more often than females).

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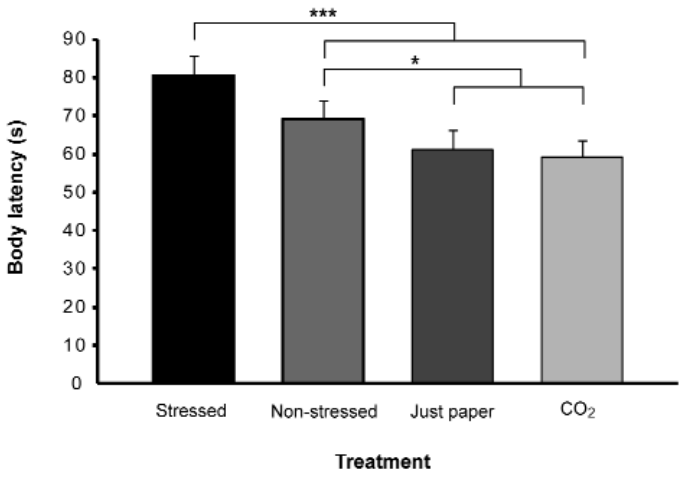
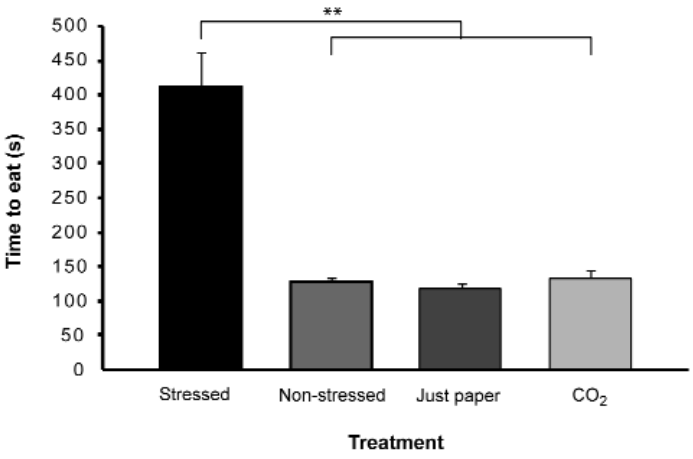
Figure 4



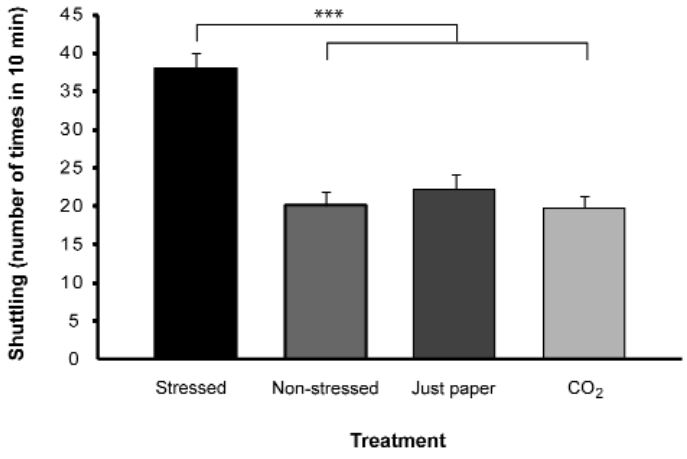
Mean (± SEM) Body latency. There was a significant effect of treatment on the latency to place all four paws in the arena ($F_{3,40}$ = 32.73, $P < 0.0001$) which held when the non-significant sex × treatment term was removed ($F_{3,43}$ = 34.12, $P < 0.0001$) to run Tukey's *post-hoc* comparisons. These showed that the Stressed treatment had a greater effect than the controls ($t > 5.1$, $P < 0.0001$ for all three comparisons). The Non-stressed treatment also significantly differed from the other two control treatments ($t > 3.40$ and $P < 0.01$ for both). * $P \leq 0.01$; *** $P \leq 0.0001$.

Figure 5



Mean (± SEM) Latency to eat. A square-root transformation was performed prior to analysis, since the data did not meet the necessary assumptions due to ceiling effects (eight pairs did not eat throughout the whole Stressed trial, cf two pairs in each of the other three treatments, and so were allocated the maximum latency, 600 s). Treatment affected the time taken to start eating the sugar ($F_{3,40}$ = 9.09, $P < 0.0001$; and with the non-significant sex × treatment term removed $F_{3,43}$ = 9.28, $P < 0.0001$). Tukey's *post-hoc* comparisons showed that the Stressed treatment had a greater effect than all three controls ($t > 4.28$, $P < 0.001$ for all comparisons), and that the three controls did not significantly differ from each other. ** $P \leq 0.001$.

Figure 6



Mean (± SEM) Shuttling between test chamber and home cage. Treatment affected rats' returns to the home cage and movements back into the test chamber ($F_{3,40}$ = 32.86, $P < 0.0001$), an effect which held once the non-significant sex × treatment term was removed ($F_{3,40}$ = 32.90, $P < 0.0001$) to allow Tukey's *post-hoc* comparisons. These *t*-tests showed that the Stressed treatment elicited significantly more Shuttling behaviour than all controls ($t > 7.42$, $P < 0.0001$ for all three comparisons), while the three controls did not significantly differ from each other. *** $P \leq 0.0001$.

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Table 1 Rat pair response consistency.

Behavioural measure	Ranking of each rat pair consistent between Stressed and control treatments	Regression output
Front latency	Yes	$F_{1,13} = 26.54$, $P < 0.0001$
Body latency	Yes	$F_{1,13} = 64.74$, $P < 0.0001$
'Stretch attend' duration	Yes	$F_{1,13} = 34.54$, $P < 0.0001$
Latency to eat	Yes	$F_{1,13} = 8.71$, $P < 0.011$
Shuttling (number)	Yes	$F_{1,13} = 7.08$, $P < 0.020$
Shuttling rate	Yes	$F_{1,13} = 5.80$, $P < 0.032$

Table 2 Consistency between behaviour measures in control conditions.

	Body latency	'Stretch attend'	Latency to eat	Shuttling	Shuttling rate
Front latency	$F_{1,13} = 26.54$, $P < 0.0001$ (+ve)	$F_{1,13} = 10.66$, $P = 0.006$ (+ve)	ns	ns	ns
Body latency	–	$F_{1,13} = 42.20$, $P < 0.0001$ (+ve)	ns	ns	ns
'Stretch attend'	–	–	ns	ns	ns
Latency to eat	–	–	–	ns	ns
Shuttling	–	–	–	–	$F_{1,13} = 770.68$, $P < 0.0001$ (+ve)

Table 3 Consistency between behaviour measures in Stressed condition.

	Body latency	'Stretch attend'	Latency to eat	Shuttling	Shuttling rate
Front latency	$F_{1,13} = 98.53$, $P < 0.0001$ (+ve)	$F_{1,13} = 6.72$, $P = 0.02$ (+ve)	ns	ns	ns
Body latency	–	$F_{1,13} = 27.88$, $P < 0.0001$ (+ve)	ns	$F_{1,13} = 3.89$, $P = 0.070$ (-ve)	ns
'Stretch attend'	–	–	ns	$F_{1,13} = 4.21$, $P = 0.061$ (-ve)	ns
Latency to eat	–	–	–	ns	ns
Shuttling	–	–	–	–	$F_{1,13} = 466.99$, $P < 0.0001$ (+ve)

Furthermore, for these two measures, the Non-stressed paper (ie the scent of other rats *per se*) was treated just like the other controls, whereas for both Front latency and Body latency measures, it caused detector rats to enter the test chamber more slowly than in the two other control treatments. Our two additional calculated measures also showed strong treatment effects. Once their forepaws were in the chamber, rats stayed for longer in a Stretch attend-like posture in the Stressed treatment than for all other controls (treatment effect in full model: $F_{3,40} = 5.90$, $P = 0.002$; Tukey's *t*-test results: compared with Non-stressed, $t = 3.05$, a trend at $P = 0.019$, and for the other two controls, $t > 3.4$ and $P < 0.01$). Their Shuttling rates per unit of time were also higher (treatment effect in full model: $F_{3,40} = 36.62$, $P < 0.0001$; Tukey's *t*-test results: compared with all controls, $t > 7.70$ and $P < 0.0001$). For this measure, there was also a trend to a sex effect, males Shuttling at an almost significantly faster rate: $F_{1,40} = 5.10$, $P = 0.036$).

In every analysis, 'pair' was significant ($P < 0.001$). The results of the regressions performed to investigate these 'pair effects', to see whether pairs showed consistency between Stressed and control treatments, are shown in Table 1. Results of further regressions performed to see whether pairs were consistent across the different behavioural measures, first in control conditions, and second when exposed to Stressed sheets, are shown in Tables 2 and 3, respectively. Each of the six measures correlated between Stressed and control treatments: pairs were thus consistent in their ranked scores, with those pairs that entered the arena (etc) relatively rapidly in the control conditions also doing so in the Stressed treatment. However, although the three positional latencies all co-varied, they did not correlate with latencies to eat, nor with Shuttling behaviour (indeed in the Stressed condition there was a negative trend between Shuttling and Body latency, since animals which took a long time to enter simply had less time in which to then move between the two cages). Thus, different pairs seemed to have different styles of responding to the arena, including in the Stressed treatment.

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Discussion

Olfaction is a vital sensory modality for rodents. Murids have evolved both to produce many information-rich scents (eg Hurst *et al* 2001; reviewed by Burn 2008), and to detect them via a large family of genes, coding for over 1,000 different types of odour receptors (eg Axel & Dulac 1995; Ma 2007). Conspecific odours affect feeding and food-choice, mate-selection and mating behaviour, the care of young, and the establishment and maintenance of dominance hierarchies (eg reviewed by Latham & Mason 2004). Via scents, murids can even detect sickness and parasitism in conspecifics (Willis & Poulin 2000; Kavaliers *et al* 2003; Zala *et al* 2004), and perhaps even recent social defeat (eg Carr *et al* 1970; but see also Brown 1992) and frustration (Taylor & Ludvigson 1987). Small wonder, then, that rodents produce and can detect olfactory stress cues. Our *Introduction* reviewed the current level of understanding regarding these, and emphasised their potentially important role for assessing the welfare of animals producing them: after all, we would pay great attention to signals like piloerection or whimpering, so it seems wrong to ignore olfactory stress cues simply because they are not conspicuous to humans.

Here, our goal was to pilot techniques for making their assessment more practically feasible. Our results showed that rat stress odours could apparently be 'harvested', simply and cheaply, on specially-prepared blotting paper, and stored at room temperature for assessment up to five hours later. As we had predicted, the sheets from the stressed donor rats had a marked effect on the recipient animals, eliciting signs of increased caution (ie prolonged latencies to enter the chamber), vigilance (ie prolonged Stretch attend-like postures; less eating), and restlessness (ie increased Shuttling between the chamber and the home cage). These behavioural responses by the detector rats are typical of rodents exposed to stress odours (see *Introduction*), indicating that the blotting paper and enfleurage had almost certainly worked. These findings therefore suggest that in future work aimed at investigating the welfare of rats or mice exposed to different treatments, the surfaces on which the subjects in question stand or lie could be similarly lined with treated paper, and used to collect any stress odours for later bioassay via conspecific reaction. Thus, this potentially gives us a practical way of assessing whether stress odours are released during practices whose humaneness we are trying to assess

In terms of the best 'bioassay' for detecting stress odours, we suggest that Latency to eat and Shuttling rates of paper-exposed detector rats are potentially the most useful indices: these showed the greatest proportional increases from baseline and, unlike latencies to enter, seemed unaffected by cues from unfamiliar conspecifics *per se*. Future work should assess whether these measures show a graded response to odours from differentially-stressed donors. It might also be worthwhile to see whether responses during forced odour exposure are more easily measured, or show greater effects, or change more sensitively with graded odour cues. Non-behavioural responses by detector animals, like chromodacryorrhoea, might also prove useful (cf eg Mason *et al* 2004).

Having said this, how certain is it that the effects seen were really due to stress odours? All sheets were equally exposed to the vegetable fat, humans' gloved hands, polythene (when bagged) and sugar; all data were collected blind to treatment and, furthermore, the Non-stressed and Carbon dioxide controls demonstrated that unfamiliar conspecifics *per se*, or residual traces of the euthanising gas, did not underlie the effects. Thus, none of these could have acted as confounds. However, other potential confounds may have contributed. Stressed donors were in contact with paper sheets for slightly longer than Non-stressed donors, including a period of a few minutes following death. The observed differences could also be conceivably due to the amounts of urine and faeces found on Stressed sheets: stressed animals appeared to urinate more than the unstressed controls (unfortunately, amounts were not recorded) and, thus, it could perhaps be the volume of urinary odour, rather than any qualitative change, which was effective. Furthermore, Stressed animals appeared more likely to have diarrhoea, and although faeces were brushed off the sheets, these loose stools had a bilious odour, distinctive even to the human nose, that could perhaps have clung to the paper. It is reassuring that previous work has suggested that urine cues are not essential for stress odour detection, and that faeces are not involved at all (see *Introduction*). However, future work should clearly control for such effects to check for qualitative rather than quantitative differences in odour.

Next, we turn to the recipient animals' responses, and what they may reveal about their own welfare on being exposed to such odours. Stress odour exposure seems extremely effective at inducing anxiety or stress in laboratory rodents (see *Introduction*; also Cocke *et al* 1993 and Inagaki *et al* 2008). However, if we are concerned about the welfare of stress odour-exposed animals, which are the most reliable signs of stress or anxiety? Our exploratory findings in this 'voluntary exposure' set-up suggest that individual rats vary greatly in the style with which they respond to potential threat. Thus, pairs of rats that took a long time to enter the chamber did not take a long time to eat nor show the greatest degree of 'Shuttling' between the two cages. These distinct differences in style made it hard to assess which pairs found the stress odours most threatening. Future work using graded stress odour exposure could perhaps better assess which of these measures suggests the greatest level of anxiety. In the meantime, if responses to stress odour are used to make inferences about the welfare state of different recipients, we suggest measuring a variety of behavioural responses.

Finally, more fundamental future work is clearly needed into the nature and signal value of murid stress odours: much remains unknown about the true biochemical properties, sources and biological functions of these influential and important odours. Returning to more prosaic matters, more work is also needed on stress odour collection. Is fat necessary for odour absorption? If so, are some fats more effective than others? How long an exposure time is needed? Are some types of paper more effective than others? How long can the odour be stored for? And would refrigeration, or even freezing, allow odours to be stored for

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longer? Overall, we thus hope this paper inspires further research into these potentially important murid welfare indices — indices that we humans tend to be oblivious to.

Animal welfare implications

Murid stress odours could act as non-invasive welfare indicators, revealing donor anxiety, fear and/or stress, and thus be potentially useful for assessing the impact of husbandry and procedures on laboratory or wild murids. Stress odours could also affect the welfare of conspecifics living nearby; another important reason for detecting their production. Finally, if isolated and synthesised, murid stress odours could even help in pest control via their aversive, deterrent properties — important because much rodent pest control is currently extremely inhumane (Mason & Littin 2003). Despite this, and despite the crucial role of olfaction to many mammals, stress odours have been generally ignored in welfare research, perhaps because humans are largely oblivious to them. Our simple, cheap techniques for i) collecting stress odours from 'donors' and ii) using conspecific reactions to them as a detection method or bioassay, could thus potentially help advance the future uses of stress odours in welfare-relevant work on rats and mice.

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