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Assessment of intradimensional/extradimensional attentional set-shifting in rats

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ABSTRACT

The rat intradimensional/extradimensional (ID/ED) task, first described by Birrell and Brown 18 years ago, has become the predominant means by which attentional set-shifting is investigated in rodents: the use of rats in the task has been described in over 135 publications by researchers from nearly 90 universities and pharmaceutical companies. There is variation in the protocols used by different groups, including differences in apparatus, stimuli (both stimulus dimensions and exemplars within), and also the methodology. Nevertheless, most of these variations seem to be of little consequence: there is remarkable similarity in the profile of published data, with consistency of learning rates and in the size and reliability of the set-shifting and reversal 'costs'. However, we suspect that there may be inconsistent data that is unpublished or perhaps 'failed experiments' that may have been caused by unintended deviations from effective protocols. The purpose of this review is to describe our approach and the rationale behind certain aspects of the protocol, including common pitfalls that are encountered when establishing an effective local protocol.

1. Introduction

Rats and mice account for more than 70% of animals used in the UK under the Animals (Scientific Procedures) Act 1986, with a third of these being in the translational research category of 'Applied – human medicine' (UK Home Office, 2017). Although there has been a recent retreat from translational neuroscience in psychiatry, in part due to a lack of understanding the neurobiology of psychiatric disorders (Insel et al., 2012), research with non-human animals is providing important insight into the nature of cognitive impairments in conditions such as depression, dementia and psychosis. All of these conditions have impairments of so-called 'executive functions' of the frontal lobes, the severity of which are associated with poor functional outcome. Cognitive flexibility – "the ability to switch thought and/or response patterns" (Powell and Ragozzino, 2017) – is one such function: how the brain solves the problem of being, simultaneously, consistent and efficient (able to learn and generalise that learning to new situations) and yet also flexible (able to know that 'things change' and that 'rules have exceptions').

The early psychology literature is replete with a great variety of demonstrations of cognitive flexibility in many different contexts and in many species, ranging from fish to rodents and humans. Reversal learning has been called a "pre-eminent test of cognitive flexibility" (Izquierdo et al., 2017), not least because it is observed ubiquitously and is also easily quantified in different species. Other demonstrations of cognitive flexibility include task switching (Jersild, 1927), when response strategies need to change, and the shifting of attention as the relevance of perceptual features changes (demonstrated, for example, in the Wisconsin Card Sorting Test (Berg, 1948) and the intra/extradimensional (ID/ED) attentional set-shifting task (Lawrence, 1949)).

We have previously argued that task switching, attentional shifting and reversal learning are unlikely to reflect a unitary function called 'cognitive flexibility' (Brown and Tait, 2015). Shifting and switching tasks have in common the idea that prior experience causes the cognitive system to be dynamically set, or prepared, to perform particular mental operations or process particular information. This cognitive preparedness – also known as 'mental set' – confers a processing advantage (either stimulus processing in the case of a perceptual attentional set, or response selection in the case of a task or learning set) for as long as the preparation is appropriate. When the set of the system is not appropriate, the model-based processing will be disadvantageous, thus the system must be flexible and able to reset. The ID/ED task (Lawrence, 1949) enables this to be demonstrated by comparing new learning in two different states of mental set. At the ID stage, novel

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stimuli are presented but prior experience of particular perceptual features being relevant (*e.g.*, colour) ensures that the processing of those features are prioritised, which confers an advantage for learning. At the ED stage, different perceptual features of novel stimuli (*e.g.*, shape) are now relevant to solve the task, but as they are not the features prioritised, this results in a learning decrement. A comparison of learning rates in these two different states thus provides inference of the state of the mental set.

It is possible that the process or mechanisms that enable reversal learning may have been repurposed to support cognitive flexibility. In other words, cognitive (covert) flexibility could be a special case of behavioural (overt) flexibility. On the other hand, it seems more likely that reversal learning – like any learning – can occur in the context of various states of cognitive preparedness, ranging from model-free (no prior set) to entirely model-based, and this will probably be determined by the context or task variant (Izquierdo et al., 2017). 'Learning set' (Harlow, 1949) describes an increase in the rate of reversal learning as a function of experience of learning reversals, and it indicates that mental set (and its corollary, cognitive flexibility) is not an intrinsic, let alone necessary, aspect of adaptive behaviour resulting from learning processes (which includes reversal learning), but rather is additional to it. In other words, having a mental set (a model) can influence the rate of any learning, including reversal learning, but the nature of the mental set cannot be known by observing an isolated instance of learning. The mental set is only revealed by assessing the relative advantage or disadvantage that the model confers. This is one of the reasons we suggested that it is important that a task does not conflate reversal learning with either switching or shifting (Brown and Tait, 2015). This is particularly problematic in rule- or strategy-switching tasks for rats that employ mazes or operant chambers (see Floresco and Jentsch, 2011) because the responses to the different rules are not unique. On 50% of trials, the response to a new rule (*e.g.*, "turn left") will be the same as when an old rule (*e.g.*, "approach the light") is applied. This partial reinforcement effect, which is the result of a learning process, cannot be distinguished from the effects of cognitive flexibility. In shifting tasks, this problem can be overcome by having a sufficiently large number of stimulus exemplars so that it is possible to have a 'total change design': previously rewarded stimuli are no longer present and therefore not partially reinforced (Slamecka, 1968).

In summary, although we acknowledge that aspects of cognitive flexibility are undoubtedly relevant to, and can be assessed in the context of, reversal learning (see also Dhawan et al., in press), we do not think that all examples of reversal learning are relevant or that it is a simple way to measure cognitively flexibility. We think it is yet to be determined whether shifting and switching represent a unitary executive function, although the involvement of prefrontal cortex in both seems a compelling reason to suggest that these behaviours have aspects in common. The purpose of this paper is to describe our methods and protocol for assessing cognitive flexibility and our rationale for these. We do not intend to imply that we think this is the only, or even the best, way to assess these psychological constructs. Rather, we hope to provide helpful information for other researchers' who might consider adopting or adapting the ID/ED attentional set-shifting task (ASST) for the rat.

1.1. The ID/ED ASST

The ID/ED ASST is a well-established behavioural assay which is used in humans, primates and rodents (for review see Brown and Tait, 2016). Performance in this task specifically is impaired in neurodegenerative diseases (*e.g.*, Parkinson's disease; Downes et al., 1989) and neurological disorders (*e.g.*, schizophrenia; Elliott et al., 1995) with frontocortical neuropathology, and in rodent models of these disorders (*e.g.*, subchronic phencyclidine as a model of schizophrenia; Rodefer et al., 2005). We believe that the particular value of the task is that, regardless of species, the ID/ED ASST is formally the same: it requires the participant/subject to learn a series of two-choice compound discriminations with (typically) two systematically varied, uncorrelated stimulus dimensions – one is relevant to solving the discrimination (*i.e.*, predicts reward), the other is irrelevant. Over multiple ASST stages, an attentional set is formed to the persistently relevant dimension, and then the participant/subjects' ability to flexibly shift attention from that dimension to the previously irrelevant dimension is tested. The trials required to learn the discrimination at the ED stage is compared to learning at the ID stage and the difference is assumed to reflect the strength of the set and the cognitive cost ('shift-cost') of flexibility. Manipulations that increase shift-cost relative to control performance (which is generally expressed as additional trials at the ED stage, because there is often little room for improvement in ID acquisition) are typically interpreted as reflecting an impairment in cognitive flexibility, although the specific latent mechanisms can only be inferred. A reduced shift-cost is more difficult to interpret, as it could result from performance change at either ID (increased trials) or ED (decreased trials) or both (changes to shift-cost are discussed in more detail in Section 4.2.2).

An ID/ED ASST that is suitable for testing humans or monkeys typically uses compound (multidimensional) visual stimuli presented on a computerised touchscreen (Roberts et al., 1988). For example, the ID/ED ASST in the Cambridge Neuropsychological Test Automated Battery (CANTAB) (Cambridge Cognition, Ltd) uses stimuli which are opaque shapes with superimposed line-configurations. An ID/ED ASST employing a total-change design suitable for testing rodents was described by Birrell and Brown (2000). This approach relies on the natural propensity of rats and mice to forage for food, with subjects digging for food bait in small bowls which are discriminable by the digging media, or the scent, or the bowl itself may even have a different appearance or texture. This adaptation of the ASST for rodents, allows researchers to understand the same mechanisms governing attentional set-shifting in mammals, but using species-appropriate stimuli and responses. There is a common standard in the stages of the rat ASST: the majority of published designs use seven stages (Tait et al., 2014) – a simple discrimination (SD); a compound discrimination (CD); a reversal of the CD (REV1); the ID; a reversal of the ID (REV2); the ED; and finally a reversal of the ED (REV3) – which we refer to as the standard 7-stage task (Chase et al., 2012; Tait et al., 2014).

It does not seem to be important that the apparatus and materials are standardised for the rat ID/ED ASST: research groups typically construct their own testing chamber or arena, and the various elements of the stimuli (odours and digging media) are largely determined by local availability. On the one hand, this variability indicates the robustness of the task, nevertheless there are aspects that are important to consider when selecting materials. Here we will, therefore, discuss some of the reasoning behind choices made during the development of the rat ID/ED task, including changes made since the original Birrell and Brown publication so that researchers wishing to adopt or adapt the task in the future are informed by our experience of what worked or did not work. We will focus on designs for use with rats, as mouse ASSTs, although often similar in design to the rat ASST, have their own requirements (see Tait et al., 2014 for review). In this methods paper, we will discuss rat strain; the apparatus; the choice of stimulus exemplars; stage and trial order; counterbalancing; and data analysis.

2. Rats

ASST data have been collected in many different rat strains – including Lister Hooded (Birrell and Brown, 2000); Long Evans (Rodefer et al., 2008); Sprague Dawley (Tunbridge et al., 2004); Wistar Kyoto (Cao et al., 2012); Fischer 344/Brown Norway cross (McCoy et al., 2007) – and although the majority of the published work has used male rats, there are also data from female rats (Lovic and Fleming, 2004; McLean et al., 2012). Whereas there may be some strain or sex differences in willingness to perform the task, the pattern of data in terms of

trials to criterion is fairly consistent in the different strains/sexes. Indeed, those studies that have directly compared the sexes find no effect of sex on control performance regardless of strain: Lister Hooded (Snigdha et al., 2011); Sprague Dawley (Mohamed et al., 2011); or Long Evans (Murphy et al., 2017). There does appear to be a difference between male and female performance at the ED shift after phencyclidine administration, although the exact effect varies between studies (Broberg et al., 2008; Snigdha et al., 2011). There are fewer studies comparing across strains, although Cao et al. (2012) report no difference between male Sprague Dawley and Wistar-Kyoto rats. The majority of our experience is with male Lister Hooded rats (Birrell and Brown, 2000; Chase et al., 2012; McAlonan and Brown, 2003; Tait et al., 2007), although we have also used female Lister Hooded (Lindgren et al., 2013) and male Sprague Dawley rats (Whyte and Brown, unpublished observations). We find male Lister Hooded rats are highly motivated to engage with the task and seek out the reward (we currently use half a Nestlé® Honey Cheerio, as Kellogg's® Honey Loops are no longer available in the UK). We use minimal food control: we maintain the colony on 15–20 g per day of standard laboratory chow, which is within the range of what a healthy active rat typically eats (Siegal, 1961) and which results in a steady weight increase as they grow. We test rats during the light cycle (colony lights are on 07:00–19:00) and before they are fed. Thus rats are fed in their cages, typically between the late afternoon and before the lights turn off at 19:00. All of the food is usually consumed during the dark period, such that they are without food for several hours before being tested in the light period. Female Lister Hooded rats are as equally motivated as males but, as they are smaller, we feed them slightly less (closer to 15 g a day) and use a smaller reward (*e.g.*, quarter to a third of a Honey Cheerio) to avoid satiety. We have found that Sprague Dawley rats are less willing to engage (and stay engaged) in the ASST, so we also offer them closer to 15 g than 20 g of 'free access' food each day, whilst monitoring their weight to ensure that they are still gaining at a healthy rate. A number of other published studies report using food restriction regimes. These regimes actively aim to reduce the weight of the rat to ~85% of their starting weight or an assumed 'free-feeding' body weight (Deschenes et al., 2006; Gastambide et al., 2012). For example, Lovic and Fleming (2004) use adult female Sprague Dawley rats, raised on *ad libitum* food, which is then reduced to 10 g/day for 2–3 weeks to achieve a reduction to 85–90% of their starting body weight. The aim of controlling access to food is for the rats to be motivated to forage for an appetising reward without making them so hungry or calorically-deprived that it will compromise cognitive function. Our data show that motivation to engage with the task is typically sufficient without the need to restrict food to the extent that weight loss occurs – although our data also show that strain and sex differences may affect motivation.

3. Apparatus

3.1. The testing arena

The ASST arena used by Birrell and Brown (2000), and in all our subsequent studies, is made from a modified opaque white plastic homecage, and has overall outer dimensions of 70 × 40 × 18 cm (Fig. 1). It is divided into three sections: roughly two thirds of the length of the arena serves as the holding area, separated from the remaining third by removable semi-transparent acrylic barriers, which is further subdivided by an opaque white plastic wall, creating two choice chambers. Access to either of the choice chambers is controlled by the acrylic barriers: a full barrier to block both of the choice chambers; and a small barrier to block only one of the choice chambers. Other designs either do not specify barrier transparency (Egerton et al., 2005b), or use opaque barriers (Izquierdo et al., 2010), and we have not explicitly tested whether rats use visual cues through a semi-transparent barrier to determine responding. However when challenging rats with a visual discrimination in our arena (Tait et al., 2009) we observed that rats did not appear to be able to discriminate between a black versus a white bowl until they had approached the bowls and they were inside the choice chambers and 'nose-to-bowl' – suggesting that it is very unlikely that rats can use visual cues through the (semi-)transparent barriers.

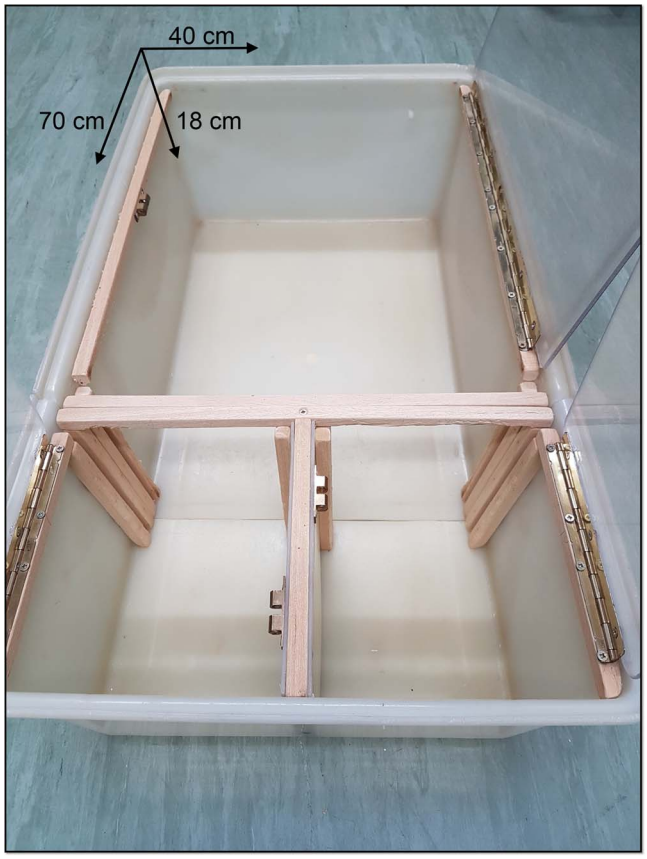

Fig. 1. Our attentional set-shifting arena. Roughly two thirds serves as a holding area, with the remaining third subdivided into two bowl-containing choice chambers – access to which can be blocked (either individually, or both simultaneously) by semi-transparent barriers. The arena's dimensions are 70 (length) × 40 (width) × 18 (height) cm.

A semi-transparent acrylic hinged lid seals the top of the holding area, with two individual hinged lids of the same material sealing the choice chambers. This reduces the ambient lighting in the arena to ~500 lx, when the ambient light in the room is typically ~800 lx. The floor of the holding area is covered with clean sawdust, although the choice chambers are left bare to allow efficient recovery of spilled digging media between trials and to diminish the risk of cross-contamination. Because a testing session could last many hours (particularly if a rat is impaired following a manipulation), water is provided in the holding area. Our early studies (Birrell and Brown, 2000; McAlonan and Brown, 2003) were conducted without water in the testing cage and we have not observed any obvious change in the rats' performance of the task or their level of motivation when water is provided. However, as they do sometimes drink, we think it is good practice to make water available. Perhaps because our ASST arena is constructed from homecage materials that are familiar to the rats, the rats do not appear to require habituation to the arena itself in order for them to begin to explore and start to forage. However, rats might require a period of habitation in an ASST arena that is made from unfamiliar materials.

Some ASST arena designs use only one-third of it as a holding area, with one-third subdivided into the choice chambers and the middle third being an open space that the rat must cross to get to the choice chambers (Lapiz and Morilak, 2006). In such a design, if the choice chambers cannot be individually blocked (Izquierdo et al., 2010), rats must be manually moved from the choice chambers to the holding area after completion of a trial. The intent of having this 'open area' is

Table 1
The current exemplar pairings used in our attentional set-shifting task.

	Odours		Digging media	
Pairings				
Training	Mint	Oregano	Polystyrene	Shredded Paper
Pairing 1	O1 – Cinnamon	O2 – Ginger	M1 – Coarse tea	M2 – Fine tea
Pairing 2	O3 – Sage	O4 – Paprika	M3 – Sand	M4 – Grit
Pairing 3	O5 – Turmeric	O6 – Cloves	M5 – Coarse shavings	M6 – Fine shavings
Pairing 4	O7 – Dill	O8 – Coriander	M7 – Cotton pads	M8 – Cigarette filters
Pairing 5	O9 – Fenugreek	O10 – Tarragon	M9 – Coarse cork	M10 – Fine cork
Pairing 6	O11 – Cumin	O12 – Marjoram	M11 – Long wire coat	M12 – Short wire coat
Pairing 7	O13 – Thyme	O14 – Caraway seeds	M13 – Beads	M14 – Gravel
Pairing 8	O15 – Fennel seeds	O16 – Chives	M15 – String	M16 – String knots

We use the above as exemplar pairings, with pairings 1–3 used in the standard 7-stage task, and pairings 4–8 added as necessary in tasks with multiple ID stages.

reportedly to prevent the formation of a response side-bias, with the rat waiting outside one or the other choice chamber. Whilst we regularly observe an investigative side bias (*i.e.*, rats initially may show a systematic spatial bias in the bowl they approach first), we have not observed any tendency for a rat to develop a persistent response bias to one side. The side of the baited bowl is pseudo-randomly varied, therefore the probability of the baited bowl in either choice location is 0.5.

The purpose of having a divider between the two bowls is to prevent the rat from rapidly moving between the bowls, particularly if it initially makes an incorrect response. Our protocol for ending a trial is the same regardless of whether a correct or incorrect choice has been made: the partition on the non-selected chamber is lowered immediately once the rat begins to dig, thereby blocking access to the non-selected bowl; the rat is then left to dig in the selected bowl with no time limit; when the rat obtains the reward (correct trial), or moves away from the bowl into the holding area showing no further interest in the bowls (incorrect trial), the second partition is lowered, blocking re-entry to the selected chamber. If the rat did not retrieve the reward, it can continue to investigate the bowl until it is no longer interested in digging in it anymore. This can involve the rat leaving the incorrect chamber, and encountering the barrier to the correct chamber, and then promptly returning to the bowl to dig further, which is a pattern frequently seen during reversal learning. If there is no barrier between the two bowls (Rodefer et al., 2005), or there is no way to block access to the individual choice chambers (Lapiz and Morilak, 2006), the rat would need to be manually prevented from digging in the other bowl after making an initial selection, with obvious implications for disruption of learning. This is particularly important after the incorrect bowl is selected and particularly during reversal learning: the trial should not be ended by the experimenter before the rat is convinced that there is no point in further digging in the bowl it had selected. In addition, manually moving rats may be stressful, and this may be more so for some strains or after some manipulations, which may affect performance.

Arenas may be self-constructed (Featherstone et al., 2007; Lapiz and Morilak, 2006; McGaughy et al., 2008), or adapted from other boxes (Gregg et al., 2009; McCoy et al., 2007), as the size and dimensions of the ASST apparatus is less important than features such as sufficient space for bowl placement in the choice chambers, strategically-positioned dividers and a low-stress holding area.

3.2. Bowls

The glazed ceramic bowls used in Birrell and Brown (2000), and all our subsequent studies, have vertical sides and an internal diameter of 7 cm, a depth of 4 cm, and weight of 200–230 g (Cane 8 cm small pet bowl; Mason Cash, Liverpool, UK). We have found this size of bowl suitable for all rats we have tested – from 200 g to 750 g animals The dimensions of bowls varies between research groups based on their local availability: some groups use bowls of the same/similar dimensions as Birrell and Brown (Egerton et al., 2005a; Featherstone et al., 2007; Gastambide et al., 2012); whilst other groups use small terracotta flower pots (Lapiz and Morilak, 2006; McCoy et al., 2007; McGaughy et al., 2008). Deep flower pots can be partially filled with material (*e.g.*, paraffin wax; McGaughy et al., 2008) to reduce the digging medium depth necessary to fill them and provide weight for stability; whilst in other cases they are placed in recesses in the floor of the arena to lower the edge (Goetghebeur and Dias, 2009). Practicality demands that the bowls be relatively stable so that the rats' natural exploratory behaviours such as investigating/digging/climbing are unlikely to cause the bowl to tip over; of a size that the reward can be placed deep enough in the media so that the rats cannot use the reward's scent as an olfactory cue; but not so deep that the reward is too difficult to find and the rat gives up searching.

3.3. Stimulus exemplars

Choice of stimulus exemplars is based on three factors – availability, suitability, and discriminability. We have previously discussed the needs of stimulus exemplar choice (see Tait et al., 2014), but many of those comments require repeating. The standard 7-stage rat ID/ED ASST requires four pairs of stimuli in each of the dimensions – a pair for training; a pair at the SD/CD/REV1; a pair at the ID/REV2; and a pair at the ED/REV3 (Table 1). In designs where there are multiple novel discriminations (Chase et al., 2012; Lindgren et al., 2013), additional pairs of stimuli are necessary for each novel stage. By always pairing stimuli, one from each dimension, the need for counterbalancing is reduced, and it also allows for consideration of how particular odours and media will interact, as a function of, for example, the density or absorbance.

Acquiring multiple olfactory stimuli is relatively simple, regardless of whether the method used is to add herbs/spices to the digging media (Birrell and Brown, 2000) or administer scented liquid on the rim of the bowl (Barense et al., 2002). Suitable herbs/spices are easily incorporated into the digging media, and preferences or aversions for particular olfactory stimuli are readily established.

Acquiring suitable digging media to pair together is more difficult, as most have an inherent odour. It is important that paired digging media share a similar background odour – ideally the same odour – so that the added odour is discernible as a discriminable feature, and does not become two distinct odours when combined with scents of two different media. If the odours of the media are too different, the rat might solve the task by learning which two of four distinct odours are baited. This would therefore not require selective attention to dimensions and an attentional set would not form. Thus, we pair coarse and fine wood shavings, creating the fine shavings from coarse shavings using a food blender/processor (Birrell and Brown, 2000): although

both media have an inherent odour, it is the same, and cannot be used to differentiate the bowls. We originally paired gravel and wooden beads (Birrell and Brown, 2000; McAlonan and Brown, 2003) as another digging medium pair, but replaced beads with gravel (Tait et al., 2009) to reduce the odour dissimilarities, have since replaced both of those with sand and grit (Tait et al., 2013). The purpose of this final change arose during investigations of test-retest reliability: prior to Tait et al. (2009), our rats were only ever tested once on the task. However, having established that retesting was an option, we found that learning the gravel/pebbles discrimination was particularly fast during retesting, which seemed to be due to the low particulate density enabling the rats to learn to detect the odour of the reward. To address this concern, some research groups (Barense et al., 2002) add some ground-up reward to the digging media, such that all bowls smell slightly of reward, and therefore the reward's odour is an unreliable cue to whether the bowl is baited. We have not, however, found this to be necessary: our current stimuli provide a regular and robust difference between novel learning and reversal learning over multiple tests in control rats, indicating that they do not learn to use the scent of the reward as a cue.

Acquiring textures for the outer surface of the bowls presents similar problems as the prior discussion regarding the digging media – in that they should differ only in the dimension that they are intended to be discriminated by. Thus, the odour and visual properties of any textures should be considered when pairing them. Our initial use of textures paired stimuli that could be 'reversed' (*e.g.*, velvet and its reverse side) such that they had the same odour and looked similar (Birrell and Brown, 2000; McAlonan and Brown, 2003). We have since moved away from using bowl texture, in part due to a concern that it requires the rat to attend to a different part of the bowl to solve the discrimination (*i.e.*, to solve a discrimination where the odours are embedded within the digging media, the rat needs to attend to 'what's *in* the bowl'; to solve a texture-based discrimination, the rat needs to attend to 'what's *around* the bowl'). Pragmatically, we also found that bowls with added texture on the surface are more difficult to maintain and clean. We have previously discussed the potential problem of presenting odours on the bowl-rim rather than embedded in the media (Tait et al., 2014), as separating the stimuli means that they are not sampled or perceived as a 'compound' stimulus. This same concern also applies to the use of textures applied to the bowls: separately configured stimuli might require a different sampling behaviour, so that what appears to be an attentional shift is actually a "shift in the strategy employed to discover the solution to the discrimination problem" (Baxter, 2009). This might be an issue for approaches to automating the task (for example, the "ID/ED Operon task" (Scheggia et al., 2014)). If exemplars from different dimensions (*e.g.*, visual, odour and texture) are presented in different locations rather than as a single compound stimulus, it might necessitate behavioural flexibility to effect a change in sampling strategy (*e.g.*, Floresco et al., 2008; Floresco et al., 2006) without requiring an attentional shift. However, whilst there are no published data directly comparing shifting between digging media and embedded odours *versus* digging media and rim odours, there are also no reports of differential shift-costs arising from task variants using three perceptual dimensions (although there is not a substantial body of literature for such; see Section 4.3.2).

4. The ID/ED ASST

4.1. Training

As foraging is a spontaneous behaviour, training is not a requirement. However, familiarising the rats with digging in the bowls for food does speed subsequent testing. Furthermore, rats show food neophobia, and therefore familiarisation with the cereal, which is a novel food, is necessary (Modlinska et al., 2015). Typically, rats are given a small quantity of the cereal to be used as reward the day before they are exposed to the reward-baited bowls. Although this form of habituation

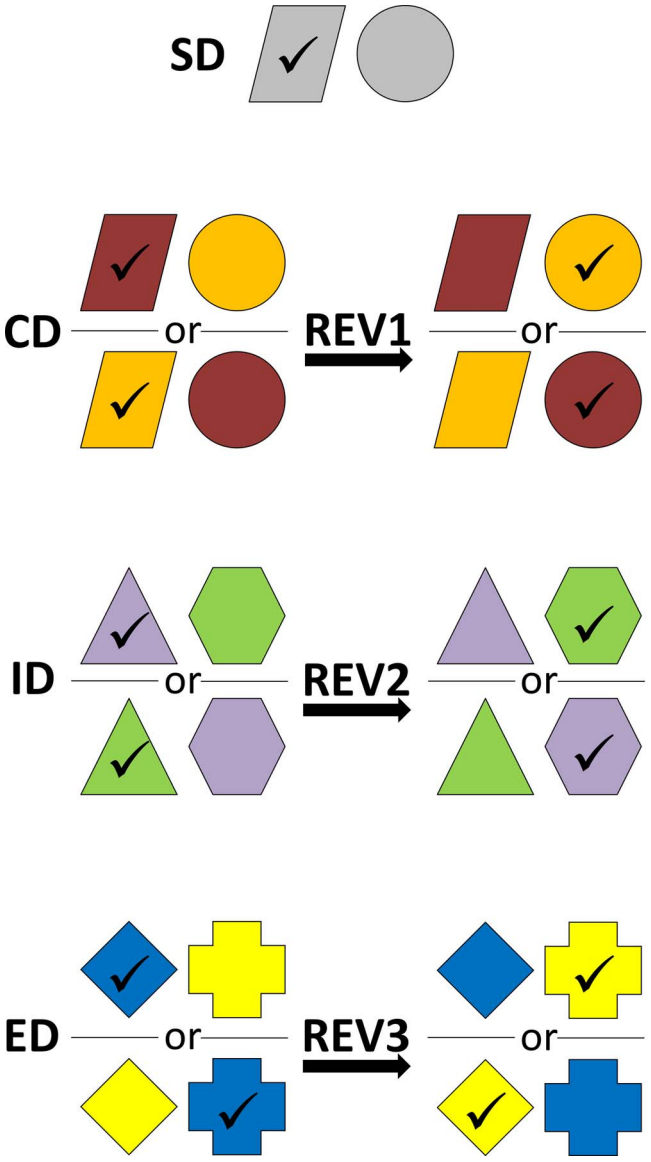

Fig. 2. The standard 7-stage task (depicted using visual stimuli for viewing ease). Rats solve a series of two-choice discriminations, where exemplars of one dimension predict reward 100% of the time (the relevant dimension), and exemplars of a second dimension are only rewarded 50% of the time (the irrelevant dimension). Novel stimuli are used at the SD/CD, ID and ED, with discriminations being solved using exemplars from the same dimension until the ED stage (shape in the above example), where exemplars from the previously irrelevant dimension become relevant (colour in the above example).

is not essential – in our initial studies, the rats' first exposure to the reward was in the arena during digging training (Birrell and Brown, 2000; McAlonan and Brown, 2003) – we have observed that rats dig more readily for the food if it is familiar. To that end, we currently leave bedding-filled bowls (containing the same sawdust as on the floor of the holding area of the apparatus; one per rat), with approximately six Honey Cheerios at the bottom, overnight in the rats' homecage prior to familiarisation with digging (Tait et al., 2007). The Honey Cheerios are always eaten by the following day.

Digging 'training' involves placing a rat in the testing arena and presenting it with bedding-filled bowls. If the rats have been habituated to the reward, they will have already dug in a bedding-filled bowl in their homecage, and therefore the novel component in this stage is the arena. We do not habituate the rats to the arena prior to digging training, and we find most rats will start investigating the bowls within a few minutes of being placed in the arena. Whilst our initial digging

training methods involved baiting the bottom of two bedding-filled bowls (one in each choice chamber) with half a Honey Loop, and re-baiting every 5–10 minutes (Birrell and Brown, 2000; McAlonan and Brown, 2003), we (Chase et al., 2012), and others, have observed that placing the reward at the surface, then progressively lower in the bowls' digging material, or alternatively, placing the reward in an empty bowl and gradually burying it in increasing amounts of digging material, over several trials results in reliable digging to the bottom of the bowl by trials 5 and 6. After they are reliably digging, the rats are exposed to discriminable bowls, only one of which is baited. Discrimination learning is discussed in detail in Section 4.5.

4.2. Task stages

The majority of published studies (currently 94 out of 137; Tait et al., 2014) use the standard 7-stage task (Fig. 2) originally described in Birrell and Brown (2000), and discussed elsewhere (see Brown and Tait, 2016; Tait et al., 2014) in detail.

4.2.1. Removing/replacing stages

Various stages have been removed or replaced, typically to test novel hypotheses investigating learning mechanisms. In principle there must, however, be sufficient stages to acquire the necessary experience of relevant versus irrelevant cues (Sutherland and Mackintosh, 1971) in order for an attentional set to form. To this end, although several studies have reported an ID/ED shift-cost with no reversal between the CD and the ID stages (Barense et al., 2002; Goetghebeur and Dias, 2009), the majority of experimental designs include this stage (so there are two compound learning stages before the ID) to help promote attentional set-formation (Tait et al., 2014). In designs where reversals are removed to investigate set-formation/shifting in their absence, multiple consecutive IDs are used to encourage attentional set-formation (Chase et al., 2012). It could be argued that the initial SD is unnecessary – in that it does not contrast the reward predictability of the relevant dimension with the irrelevant dimension – and therefore is not an essential component of set-formation. Indeed, we have observed rapid acquisition of a CD and a robust reversal-cost even when we have not included an SD before the CD (Dhawan et al., in press).

In our experience, the critical ID-comparison stage needs to be preceded by a minimum of two compound learning stages (which could be a novel compound discrimination and a reversal, or two novel compound discriminations) in order to detect an ID advantage over a subsequent ED. It does not appear to be necessary to include a reversal of the ID discrimination before the ED stage: we have shown shift-costs in both multiple ID (Chase et al., 2012), and in multiple ED (Tait et al., 2009) tasks without an ID-reversal (REV2) stage, but the majority of studies – even those that omit the CD reversal (REV1; *e.g.*, Nicolle and Baxter, 2003), or replace it with an additional ID (*e.g.*, Broberg et al., 2008) – include an ID reversal (REV2). In task variants without a REV1, a REV2 provides the only indicator of reversal learning performance relative to on-set novel learning – and this is necessary to draw conclusions about the effects of a given manipulation on reversal learning and reversal-costs. The REV3 stage cannot provide the same information, because the relationship between the ED and REV3 is confounded by the presumed requirement for the rat to shift its attentional set at the ED: reversal-costs between the ED/REV3 should appear smaller in normal subjects; and reversal performance may be affected by impaired set-shifting/formation (*e.g.*, if set-shifting-impaired rats have not successfully formed a new attentional set upon completion of the ED stage).

To that end, the REV3 stage may appear to be unnecessary and it might be tempting to drop this stage. However, in cases where rats are to be repeatedly tested, all reversals serve an important role: within each test, at some point the rat will experience reward in all stimulus configurations (*i.e.*, every bowl will have contained reward at some point during testing, whether the rat was responding to the odour or the digging medium), so that on subsequent tests, all bowls will have been previously experienced as both baited and unbaited. This eliminates the need for counterbalancing to factor in previously-/never-rewarded exemplar status on subsequent tests.

4.2.2. Modifying stages

The bowl digging paradigm was first used by Dudchenko et al. (2000) – bowls of scented sand were used as stimuli for a memory (odour span) task. The fact that the digging bowls could be made 'multidimensional' – we initially varied digging media, odours and texture coverings – meant that the bowl digging paradigm was ideal for testing ID/ED attentional set-shifting. Although the standard 7-stage ID/ED task is most commonly used, there is potential for modifying stages and indeed it is important to do this for a number of reasons.

Firstly, most of the effects reported in the ID/ED ASST, from what might be considered a surprising diversity of manipulations, fall into one of three kinds: an increase in the trials at the ED stage; an increase in trials at the first reversal, sometimes accompanied by increased trials also at the second and/or third reversal; a reduction or abolition of the ID/ED difference (either because trials to criterion (TTC) at the ED is reduced, or TTC at the ID is increased, or both). Only rarely are other effects reported. It is often assumed that increased TTC at the ED stage must always reflect the same cognitive deficit, *i.e.*, an impairment in shifting attention. However, the converse should not be so readily assumed – decreased TTC at the ED stage has been explained in two ways: improved attentional shifting (*i.e.*, cognitive enhancement; *e.g.*, Hatcher et al., 2005; Medhurst et al., 2007; Tunbridge et al., 2004); and impaired set-formation (i.e., cognitive impairment; e.g., Chase et al., 2012; Tait et al., 2017). In such cases, the relationship between ID and ED performance should inform conclusions. A comparison of only TTC in each of the seven stages of the ID/ED ASST does not enable many possibilities to be tested. There is some scope for looking at the nature of errors, although there are typically very few errors so little information to be gained. Closer analysis of patterns of behaviour is also a possibility we are investigating. However, the most obvious way to tease out cognitive differences from identical behaviour is to challenge the behaviour by modifying elements of the task, for example, adding or removing stages as in the 4ID variant. McAlonan and Brown (2003) reported a severe reversal learning impairment following orbital prefrontal cortex (OFC) lesions, and what appeared to be no difference between the ID and ED stages. However, the data analysis was ambiguous and no conclusion could be drawn. Applying a task variant previously established in monkeys (Clarke et al., 2005) and mice (Bissonette et al., 2008), four consecutive ID stages (4ID task) with no reversals, revealed slower set-formation in the OFC-lesioned rats. Performance improved over successive IDs, providing evidence for attentional set. After set had formed, there was an impairment in TTC at the ED stage. The shift-cost between the last ID and the ED remains important, as any improvement in performance across the IDs, without a corresponding increase in trials at the ED stage could arise from a discrimination learning set (Harlow, 1949), rather than from the development of a perceptual attentional set.

In addition to changing the stages of the task, it is also possible to modify the stimuli at various stages. For example, at the reversal stages (Tait and Brown, 2007), one can remove the possibility of perseveration (by replacing the previously rewarded exemplar with a new, unrewarded, stimulus) or the impact of learned non-reward (by replacing the previously unrewarded exemplar with a new stimulus which is rewarded; Fig. 3). A similar approach could be taken at the ED shift stage, to contrast dimensional perseveration to learned irrelevance (Fig. 4). We are aware that the ED stage has been manipulated in this fashion in mice (Garner et al., 2006; Papaleo et al., 2008). To our knowledge, this has not been done systematically in rats, although other adaptations have. For example, novel exemplars of a third, previously non-discriminable, irrelevant dimension were introduced to challenge or potential distract (Cain et al., 2011; McGaughy et al., 2008). Having, or

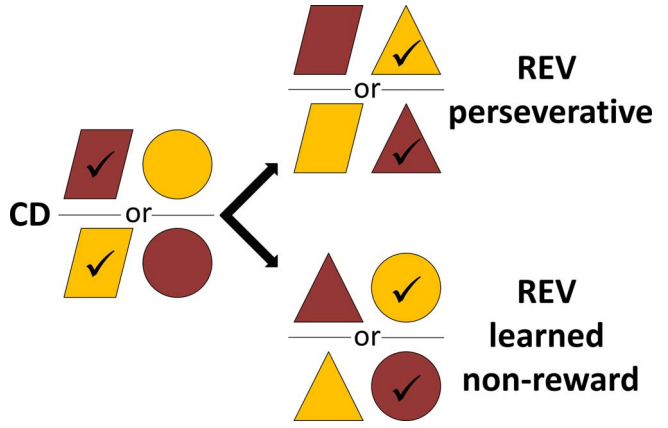

Fig. 3. Modified stages to investigate perseveration and learned non-reward during reversal learning (depicted using visual stimuli). In the perseveration reversal rats can perseverate, but cannot exhibit learned non-reward – the previously rewarded shape becomes incorrect, with the previously incorrect shape replaced by a novel correct exemplar. In the learned non-reward reversal, rats can exhibit learned non-reward, but cannot perseverate – the previously incorrect exemplar becomes rewarded, with the previously correct exemplar replaced by a novel incorrect exemplar.

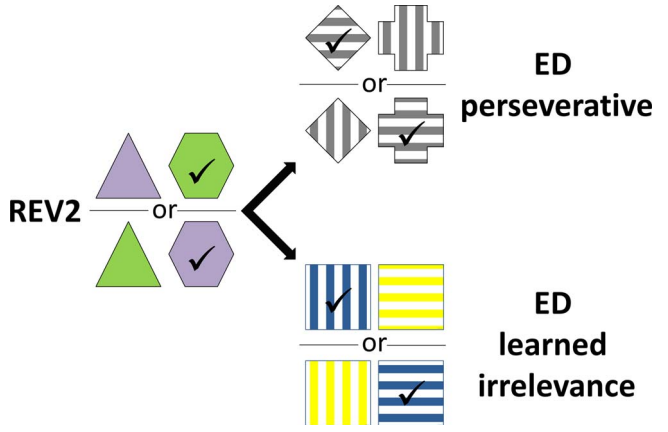

Fig. 4. Modified stages to investigate perseveration and learned irrelevance during ED shifting (depicted using visual stimuli). In the perseveration ED rats can perseverate, but cannot exhibit learned irrelevance – the previously relevant dimension becomes irrelevant, with the previously irrelevant dimension replaced by a novel relevant dimension. In the learned irrelevance ED, rats can exhibit learned irrelevance, but cannot perseverate – the previously irrelevant dimension becomes relevant, with the previously irrelevant dimension replaced by a novel irrelevant dimension.

Table 2
An example of exemplar order in our standard 7-stage attentional set-shifting task.

	Relevant dimension exemplars	Irrelevant dimension exemplars
Discrimination		
Simple (SD)	**M1**/M2	
Compound (CD)	**M1**/M2	O1/O2
Reversal (REV1)	**M2**/M1	O1/O2
Intradimensional (ID) acquisition	**M3**/M4	O3/O4
Reversal (REV2)	**M4**/M3	O3/O4
Extradimensional (ED) shift acquisition	**O5**/O6	M5/M6
Reversal (REV3)	**O6**/O5	M5/M6

In this example of exemplar order in our standard 7-stage attentional set-shifting task, rats undertake a shift-direction of Medium → Odour; pairing-order of 1 → 2 → 3 (see Table 1); and within-pair exemplar-rewarding of Odd → Even. The rewarded exemplar is bolded.

introducing, a third dimension (such as bowl texture) also gives the possibility of testing multiple ED stages within a single session. We have introduced bowl colour (black versus white) with the specific intention that this would be a particularly difficult ED, as the rats had no prior experience of a visual discrimination in the task (Tait et al., 2009). Nevertheless, the rat's limited visual ability restricts the value of bowl colour as a dimension.

4.3. Counterbalancing

4.3.1. The standard 7-stage task

There are three principle ways that exposure to the exemplars need to be counterbalanced within a group of rats: 1) shift-direction; 2) order of presentation of different pairs of stimuli (referred to as pairing-order); and 3) the exemplar that is rewarded (see Table 2 for an example).

Shift-direction describes whether the rats initially learn odour or digging medium as relevant, and therefore shift to digging medium or odour at the ED stage (O → M and M → O respectively). Within a cohort of rats, typically equal numbers encounter each shift-direction (Fox et al., 2003; Lapiz et al., 2007; Nikiforuk et al., 2010; Tait et al., 2009), although some studies use only one shift-direction (Kim et al., 2016; McLean et al., 2008). We have not observed any effect of shift-direction on either ID or ED performance (see Section 4.3.2).

Pairing-order describes the order in which the three exemplar pairings are presented during the ASST. The SD/CD/REV1 stages all use the same pairings, the ID/REV2 stages use a second pairing, and the ED/REV3 stages use a third pairing. Whilst some studies describe this as a randomised design (*i.e.*, the order is chosen at random from the different possibilities; *e.g.*, Lapiz et al., 2007), we attempt to balance the presentation of these stimulus pairings using a 3 × 3 Latin square design. Therefore as much as possible, an equal number of rats within a group undertake pairing-orders 1 → 2 → 3, 2 → 3 → 1, and 3 → 1 → 2, such that each pairing of exemplars occurs an equal number of times at each stage of testing, but without full counterbalancing.

For exemplar-rewarded counterbalancing, as each acquisition is followed by a reversal, all exemplars will have reward in them at some point. If a rat starts the ASST at the SD with O1 rewarded, and O2 unrewarded, then O3 will be rewarded at the ID, and M5 will be rewarded at the ED shift. Hence rats are assigned as 'Odd → Even' (an odd-numbered exemplar is always correct during novel learning, whereas an even-numbered exemplar is always correct during reversals), or 'Even → Odd' (the opposite). This within-pair counterbalancing procedure minimises any potential bias arising from one exemplar within a pair being more aversive/preferred than the other.

Combining these three counterbalancing techniques results in a cohort of 12 rats each undertaking a unique series of discriminations, with each shift-direction, pairing-order and exemplar-rewarded being equally represented.

4.3.2. The effects of shift-direction on performance

To confirm our lack of observation of an effect of shift-direction in individual groups of rats, we assembled data from 375 Lister Hooded rats, most of which (288) were sourced from Charles River (UK), with the rest (76) from Harlan (UK) or bred in-house (11; from Charles River stock). All data were collected in accordance with the UK Animals (Scientific Procedures) Act, 1986, and EU Directive 2010/63/EU (where applicable). The rats were tested in 22 different experiments (14 of which have been published), conducted over 18 years. The data were collected by nine different investigators, in some cases with assistance from students. Of the 375 rats, most were male, but females were used in two experiments (13 cases). There was no obvious effect of sex on the pattern of data, although the numbers are small and therefore statistical verification of a lack of difference would not be meaningful. The cases had in common that they were used as experimental controls, which had either received sham surgery (and/or subcutaneous,

intraperitoneal, or oral administration of a vehicle) or no intervention, and were naïve to behavioural testing at the time of their first test.

An overall analysis of the data confirmed a robust difference between the ID and the ED acquisition stage, which reflects the behavioural cost of shifting attentional set. In three of the experiments, the stimuli comprised an additional texture dimension, but most (337) were tested with compound stimuli with just two dimensions (odour or medium). There was an effect of 'number of dimensions', with initial learning (SD and CD) and all three reversals requiring more trials when there were three, compared to when there were only two, dimensions. However, neither the ID nor the ED stages, nor the difference between them (the shift-cost) differed as a function of number of dimensions. There was, however, a small effect of source, with Harlan-sourced rats taking slightly fewer trials to learn most stages (other than SD and CD) than the Charles River-sourced rats (main effect of Source: $F_{(2,372)} = 8.89$, $p < 0.05$, partial Eta squared $\eta_p^2 = 0.05$; Stage by Source interaction, Huynh-Feldt-corrected for sphericity: $F_{(10.43, 1940.54)}$, $\eta = 1.97$, $p < 0.05$, $\eta_p^2 = 0.01$). The effect sizes are small, and would require a sample size of over 80 rats (G*Power, v. 3.1; Faul et al., 2007) to observe the main effect – substantially more than ASST studies typically employ.

Of the rats tested with just two dimensions, a nearly equal number started discriminating on the basis of odour and then shifted to medium (168) as discriminating between media before shifting to odour (169). The effect of discrimination type was dependent on the particular stage of the test (interaction of Stage and Shift-direction, Huynh-Feldt-corrected for sphericity: $F_{(5.17,1733.40)} = 5.94$, $p < 0.05$, $\eta_p^2 = 0.02$), so the data were analysed further to look for simple main effects (Winer, 1971). There was no effect of discrimination type on any acquisition stage: the SD, CD and ID stages were acquired equally rapidly whether the discrimination was between media or odours. As expected, there was a robust difference between ID and ED acquisition (main effect of Stage: $F_{(1,1733.40)} = 39.75$, $p < 0.05$, $\eta_p^2 = 0.15$) but shift-direction did not affect either stage ($F_{(1, 1734.51)} < 1.0$, not significant (ns)), confirming that shifting between discrimination types was also equal. There was, however, an effect of discrimination type at the first two reversals: both REV1 ($F_{(1,1733.40)} = 25.34$, $p < 0.05$, $\eta_p^2 = 0.04$) and REV2 ($F_{(1,1733.40)} = 5.18$, $p < 0.05$, $\eta_p^2 = 0.01$) required more trials when the discrimination was based on odours compared to media (see Fig. 5). This effect of discrimination type was not observed at the third

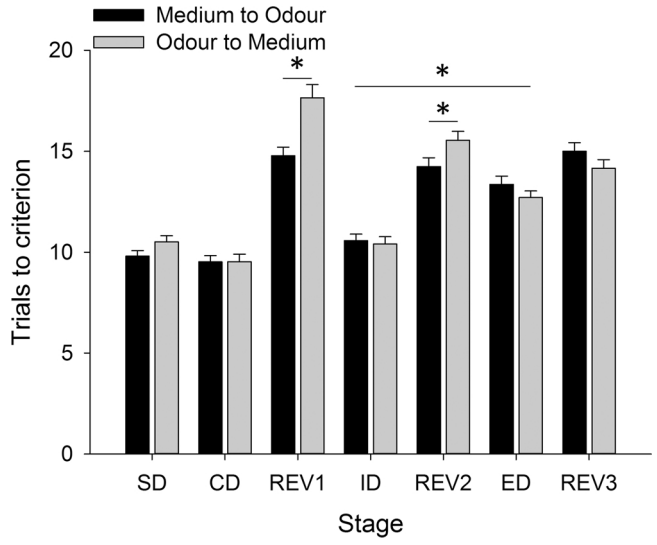

Fig. 5. Mean + SEM trials to criterion for rats (M → O, $n = 169$; O → M, $n = 168$) tested as controls on their first test. There is no effect of dimension on performance at novel learning stages (SD/CD, ID and ED), with the ED requiring more trials than the ID regardless of shift-direction. Performance was significantly worse when the initial reversals (REV1 and REV2) were odour-based, compared to medium-based, discriminations.

Table 3
An example of exemplar order in our 4ID attentional set-shifting task.

	Relevant dimension exemplars	Irrelevant dimension exemplars
Discrimination		
Simple (SD)	**O4**/O3	
Compound (CD)	**O4**/O3	M3/M4
Intradimensional (ID1) acquisition	**O6**/O5	M5/M6
Intradimensional (ID2) acquisition	**O8**/O7	M7/M8
Intradimensional (ID3) acquisition	**O10**/O9	M9/M10
Intradimensional (ID4) acquisition	**O12**/O11	M11/M12
Extradimensional (ED) shift acquisition	**M2**/M1	O1/O2

In this example of exemplar order in our 4ID attentional set-shifting task, rats undertake a shift-direction of Odour → Medium; a pairing-order of 2 → 3 → 4 → 5 → 6 → 1 (see Table 1); and within-pairing exemplar-rewarding Even → Odd (although with no reversal stages). The rewarded exemplar is bolded.

reversal. It should be noted that the effect is small ($\eta_p^2 = 0.04$ for REV1) and therefore it is unlikely to be detected in ASST experiments with group sizes that we typically use: a power analysis suggests groups of over 200 would be required.

Despite the lack of effect of shift-direction on ID and ED performance, we nevertheless suggest that counterbalancing shift-direction is good practice. Not only does it increase confidence in the data, in light of the numerous small differences that may be introduced in a task with non-standardised exemplars, but it also enables the experimenter to rule out the possibility that an experimental manipulation might have impacted one, and not the other, direction of shift (for example, as a result of a specific sensory impairment).

4.3.3. Task variants

Counterbalancing task variants to achieve an equality of pairing-order and exemplar-rewarded is typically not possible (*e.g.*, the 4ID task in Chase et al., 2012). With four ID stages, six exemplar pairings are required – SD/CD → ID1 → ID2 → ID3 → ID4→ ED. Without increasing the number of rats used well beyond the power needed to observe expected effects, it is not possible for all exemplars to be rewarded equally. We therefore counterbalance normally for shift-direction, and pseudo-randomly assign the pairing order to maximise the spread of exemplar pairings across the stages (see Table 3 for an example).

4.4. Retesting

When rats are retested on the standard 7-stage task, consistent results are seen in unoperated controls (Wallace et al., 2014) and across multiple manipulations (Cain et al., 2011; Chase et al., 2012; Tait et al., 2013, 2009). We (Chase et al., 2012) and others (Wallace et al., 2014) have sometimes seen a statistically reliable effect of 'test number' within a cohort, but this is a general improvement in performance in the second and/or subsequent tests. We have not seen a differential improvement at any particular stage and nor have we seen interactions with any manipulations. Indeed, in our dataset of control rats (see Section 4.3.2), 99 of the rats (from six experiments) were tested twice consecutively (first and second test data only) under control conditions (Fig. 6). The pattern of data was consistent over the two tests (the *F*-ratio was < 1.0 for both the main effect of Test, and the interaction between Stage and Test). Furthermore, there were no consistent correlations between any stages, or within or between tests, indicating that for individual rats no element of performance (*i.e.*, neither acquisition, nor reversal, nor shifting) in Test 1 predicted any element of that rat's performance in Test 2. The test-retest reliability was very low for both shift-costs (Cronbach's $\alpha = 0.15$) and reversal costs (Cronbach's

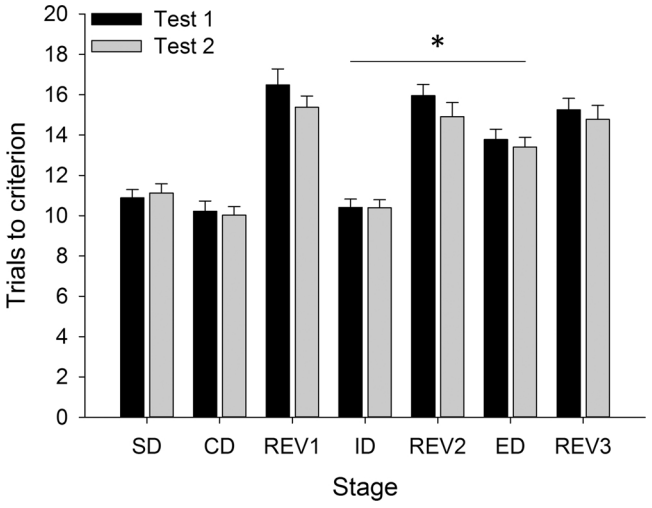

Fig. 6. Mean + SEM trials to criterion for $n = 99$ rats tested as controls on their first and second tests. There is no effect of repeated testing on rats' trials to criterion performance between the first and second tests, with the ID/ED difference being equally robust across tests.

$\alpha = 0.3$), suggesting that the task is not measuring an intrinsic stable cognitive ability (for example, a 'flexibility quotient'), but rather that behavioural flexibility varies both within and between animals.

It is not possible to fully counterbalance pairing-order, and shift-direction within a group when retesting. Nevertheless, for an individual rat we try to avoid repeated exposure to particular orders of stimulus pairs and particular shift-directions (for further discussion, see Tait et al., 2014). Doing so results in consistent and replicable effects in both medial prefrontal cortex-lesioned/control rats (Tait et al., 2009), and aging rats (Tait et al., 2013) after as many as six tests, which is sufficient to obtain baseline measures and a drug dose-response curve.

To mitigate the effects of always/never-rewarded exemplars in the 4ID task, we performed 7-stage tests between 4ID tests (Chase et al., 2012) – although we use some exemplar pairings in the 4ID task that are not used in the 7-stage task. We have also given rats the opportunity to dig for reward from all exemplars in their 'simple' form twice (once from each choice chamber) to reduce the possibility of them learning that some exemplars are never rewarded.

4.5. Discrimination learning

4.5.1. Bowl placement

Each trial within each stage requires the rat to choose which of two bowls to dig in for reward. In a simple discrimination, bowls differ by only one stimulus dimension, thus a set of two bowls is required. For compound discriminations, bowls differ by two or more stimulus dimensions, therefore a set of four bowls is required, presented in two pairs. On each trial, all exemplars are present – thus, for our exemplar pairings, if cinnamon and ginger were the correct/incorrect exemplars in the SD, during the first two trials of the CD, rats would be presented with the choice of cinnamon in coarse tea versus ginger in fine tea (sides switched between trials one and two), then cinnamon in fine tea versus ginger in coarse tea in the next two trials (sides switched between trials three and four). Using this technique, within the exploratory trials (where the rat should obtain reward on each trial), the rat should obtain reward from each of the two possible baited bowls twice – once from each choice chamber. The exemplars are pseudo-randomly presented to the rats to reduce the chance that a non-perceptual solution is tried (*e.g.*, spatial location), with the proviso that no particular pair of bowls is presented more than twice consecutively, and the baited bowl is not in the same location on more than three consecutive trials (two trials in the case of SDs). Specific details of bowl placement are seldom fully described in published methods sections, with most papers describing such as random (Lapiz and Morilak, 2006; Nikiforuk et al., 2010) or pseudo-random (Birrell and Brown, 2000; McLean et al., 2008).

4.5.2. Assessing 'digging'

A trial is deemed correct or incorrect when the rat digs in one of the bowls. The exact determination of a 'digging response' is subjective, and when authors do give precise operational definitions, there is some variety. We have previously described what we regard as a 'dig' as "when the digging medium was significantly displaced" (McAlonan and Brown, 2003), but in the majority of our publications, we have not provided explicit details (Birrell and Brown, 2000; Chase et al., 2012; Tait et al., 2009). Many other publications also do not give a specific description of a 'dig' (Barense et al., 2002; Featherstone et al., 2007; Goetghebeur and Dias, 2009; Lapiz and Morilak, 2006), although some have described it as "any distinct displacement of the digging media with either the paw or the nose" (Nikiforuk et al., 2010), or "a vigorous displacement of the digging medium" (Rodefer et al., 2005). Other researchers have given what might seem to be more conservative descriptions, for example, Cao et al. (2012) describe a 'dig' as "moving the digging medium with the paws or nose". Currently, we consider pawing at the surface of the media, which can lead to limited displacement of the media, but not enough to expose or detect the reward, as an 'investigation'. Vigorous digging, on the other hand is almost always considered a 'dig'. However, we do see individual variation in rats' digging style: some rats do not dig 'vigorously', but rather cautiously paw at the media repeatedly to expose the reward in the correct bowl. Others might 'investigate' a bowl 'vigorously', but pull back from the bowl very quickly once they have determined it is the incorrect medium. The experimenter needs, therefore, to observe the behaviour of individual rats carefully and not apply too rigid a rule when deciding if a given rat is 'digging' or merely 'investigating' the bowl.

4.5.3. Learning criterion

Trials to criterion (TTC) for learning a discrimination is six consecutively correct trials (chance probability $p < 0.016$) in the vast majority of published research (Tait et al., 2014). The majority of studies also permit four 'exploratory' trials at the beginning of each stage – whereby if the rat digs in the incorrect bowl, it is allowed to 'self-correct' and dig in the correct bowl for the reward – which are included in the TTC. On all subsequent trials, whenever the rat makes a digging response, access to the other bowl is immediately blocked. The purpose of the exploratory trials is to allow the rat to experience both bowls early on during a stage – to achieve a level of equivalence with human/monkey visual-based ASSTs, where both stimuli are available to the subject prior to making a decision to respond. We originally chose four exploratory trials in Birrell and Brown (2000) because, whilst the rat was equally likely to dig in the unbaited bowl, they would have the opportunity to obtain a reward from each of the two baited bowls in each of the choice chambers twice (see Section 4.5.1 above). The majority of publications use four exploratory trials (Brooks et al., 2012; Cain et al., 2011; Cheng and Li, 2013; McCoy et al., 2007; Nikiforuk et al., 2010), although some do not report whether they have any (Lapiz and Morilak, 2006), and some use fewer (Broberg et al., 2008; Goetghebeur and Dias, 2009).

Some studies deviate slightly from this, either by not including exploratory trials in the TTC (Hatcher et al., 2005; Izquierdo et al., 2010; Nikiforuk et al., 2010), or by training to ten consecutively correct trials, but using six as the criterion during testing (Redrobe et al., 2012). To our knowledge, only Newman and McGaughy (2011) have used a criterion other than six consecutively correct trials in rats, instead requiring seven consecutively correct trials after completion of four exploratory trials. Seven trials for criterion was chosen over six in this particular instance to partially counter the likelihood of '*n*' consecutively correct trials occurring by chance as number of trials

increases – with the subjects (adolescent rats) taking substantially more trials than normal adults (McGaughy, personal communications). There are also mice studies in which 8 out of 10 correct (chance probability $p = 0.044$) is used as a criterion (Garner et al., 2006). Excluding exploratory trials from TTC inflates the estimate of ID learning and possibly obscures an ID/ED difference. This problem is discussed by McLean et al. (2008), who did not observe a shift-cost in their control rats, having not included the exploratory trials in their TTC analysis. Given the fundamental necessity of demonstrating that the ED is a measure of attentional set-shifting – that only a shift-cost in control subjects can provide – excluding the exploratory trials from data should be avoided.

It should be noted that the learning criteria chosen to date have been based on frequentist statistical tests in which the null hypothesis is random responding with respect to the correct choice. Typically, the criteria are based on *n*-correct-in-a-row or *x*-correct-out-of-the-last-*n*-trials, and learning is judged to occur when the null hypothesis of random responding can be rejected below a specified *p*-value. These criteria have the advantage that the *p*-values are straightforward to calculate and they align well with learning. However, these criteria have three limitations: First, as the number of trials within a stage increases, the likelihood of a false positive increases due to multiple tests, as noted above. For example, in the 6-correct-in-a-row criterion, there is a ~12% chance of a false positive in the first 20 trials. For the 8-out-of-10 criterion, the corresponding value is ~20%. The increased likelihood of a false positive as the number of trials increases will cause differences between control animals and experimental animals with wholly impaired learning to be *underestimated*. Indeed, if the latter are tested over enough trials, the probability that they will satisfy the learning criterion approaches certainty. Second, both types of criterion are subject to false positives because they use a sliding fixed window of trials. Consider a case in which the 6-correct-in-a-row criterion is used, and an animal makes five correct responses, followed by one error, followed by five more correct responses. The animal would not have satisfied the learning criterion in spite of choosing the bowl correctly in 10 of the last 11 trials ($p < 0.006$ over the 11 trials). Third, even if a *p*-value allows the null hypothesis of random responding to be rejected, the alternative hypothesis that learning has occurred cannot be fully accepted unless other confounding alternative hypotheses, such as responding to one side, can be eliminated *within the window of trials*. We believe that simple *Bayesian* analysis might help overcome these three limitations and this will be the topic of a future paper.

4.5.4. Responses

There are three types of response that a rat can make in the ASST – correct, incorrect, and 'non-dig'. In our experience, the majority of rats make responses within one minute of the initiation of a trial, although time taken to self-correct during an exploratory trial in a reversal stage can be much longer. However, it is quite feasible that some manipulations might result in the rat taking considerably longer. As the task is self-paced, the behaviour of interest can be measured even if the rat is slow overall. On tests subsequent to the first, the time taken for most rats to both initiate digging and to complete each stage is much reduced. We generally allow a maximum trial time of 10 min to make a response, but there is substantial variability in the limit used by others. Whilst many do not specify the time that the rats have to make a response (Egerton et al., 2005b; Featherstone et al., 2007; Wallace et al., 2014), some permit only 60 s on non-exploratory trials (Brooks et al., 2012; McCoy et al., 2007; McGaughy et al., 2008), some two minutes (Leuner and Gould, 2010), some three minutes (Gastambide et al., 2012), and some permit up to 15 min (Broberg et al., 2008). If a rat does not make a response in the allotted time, the trial is recorded as a non-dig trial and we replace the barriers and prepare the next trial. The non-dig trial is not included in the TTC, thus TTC data only reflect trials where the rat could have learned something about one or both of the bowls. If the rat does not dig for three consecutive trials, it is given a break from testing – typically an additional 30 min, before we attempt to continue. The majority of publications do not describe their process for non-dig trials, although those that offer a 15 min response limit describe a 30 min break in the rat's homecage before continuing with the test (Broberg et al., 2008). After such a break, we typically see two patterns of behaviour: if the rat stopped because it was sated, it tends to 'pick up where it left off'; if the rat stopped because it would not self-correct during reversal learning, and its response to the previously correct bowl was extinguished, it tends to sample either bowl. We have observed similar patterns whether the break was 30 min, or overnight.

4.6. Data collected

The ASST provides several types of data for determining performance. We typically analyse TTC as the principal measure of discrimination learning, although we also collect errors to criterion (ETC) data, as well as recording the number of non-digs, the latency for the rat to make a response, and whether the rat encountered one or both bowls before making a response. Data are then typically reported as TTC, although we have occasionally had cause to analyse ETC as well (Tait and Brown, 2007, 2008). ETC typically show a similar pattern to TTC, although variability can be higher than TTC, and statistical evidence from ETC is therefore less robust. The low numbers of errors relative to human/monkey ASST performance (Roberts et al., 1988), as well as the availability of exploratory trials, also makes it more difficult to categorise errors by their types, as is often done in human/monkey studies (Dias et al., 1996; Owen et al., 1991). Rats are permitted to sample the correct stimulus after an error during exploratory trials, meaning that they are less likely to reject that stimulus the next time they encounter it.

Latency to respond is a potentially interesting, but ultimately unreliable, indicator of performance, due to its high variability, and gradual, but dramatic reduction within the testing session and over multiple testing sessions. The speed at which a rat makes a response reduces as it becomes more familiar with the stimuli and their correct/incorrect status (*i.e.,* within-stage variability), and as it becomes more familiar with the rules of the task (*i.e.,* within- and between-stages and between-sessions variability). Indeed the principal reason that the time taken to run the ASST reduces with repeated testing is the reduced latency to respond, as well as the reduction in non-dig trials, particularly during reversal learning. Latency is also affected by whether the rat encounters one or both bowls during each trial: latency is obviously reduced if the rat approaches the 'correct' bowl first and can respond without having to check the other bowl.

Recording whether the rat encountered one or both bowls prior to making a response is useful: a rat correctly rejecting a bowl is more informative, because this suggests the rat is rejecting the unbaited bowl. When the rat digs in the first bowl it encounters, it could be because the rat 'knows' it is baited, or because it is making a 'lucky guess'.

Until recently, we have collected all data on paper sheets premarked with exemplar pairings and trial order. However, we now collect data electronically only, using software to write the rats' responses into a spreadsheet – making novel analysis techniques, such as the Bayesian analysis mentioned in Section 4.5.3, easier to apply.

4.7. Troubleshooting

There are four circumstances which render interpretation of data difficult: discriminations may be solved within six trials; discriminations may not be solved after a very large number of trials; there may be no ID/ED difference; or rats simply may not dig.

If the discriminations are solved in consistently few trials, the discrimination might be 'too easy', which implies that they are not actually performing discrimination learning, perhaps because they are able to discern the odour of the reward from within the digging media. Such behaviour is typified by very low TTC, or no errors at all, even during

reversal learning. In our 375 control subjects (see Section 4.3.2), efficiency of reversal learning does not reliably predict efficiency of performance at any other stage. Nevertheless, it should be noted that there are unequal variances at the reversal stages compared to, in particular, the ID stage. At the ID stage, 17% (65/375) made a 'lucky guess' on the first trial and made no errors before reaching criterion, while another 12% (45/375) made an error only on the first trial. Thus, 29% of the rats completed the ID stage in six or seven trials and 64% made no errors after the 4th trial. For reversal learning, by contrast, a significantly smaller proportion (< 30%) made no errors after the 4th trial. As the reversal stages are uncued, it is expected that the rat should only be aware that the contingencies have changed when it digs in a bowl in which it expects to find food but does not. Therefore, the first trial of a reversal ought to be an error and this is what we found: none of the 375 rats performed the first reversal without any errors and only five rats (1.3%) made only one error. It is possible that a rat could make an 'incorrect choice' on what happens to be the first trial of a reversal, and thereby the rat would chance upon reward, nevertheless this should be unusual. This database contains 1422 examples of reversal learning (including data from second tests) and on only five occasions (0.4%) did a rat solve the reversal without making any errors. In a typical experiment, with group sizes of 6–12, it should not be treated as a chance occurrence if it is observed more than once or twice. Rather, the possibility that the rat is 'cheating' (using means other than the intended perceptual cues – such as the scent of the reward – to solve the discriminations) should be considered. Selecting media that are sufficiently densely packed eliminates this problem, although several groups report using crushed reward mixed through the media such that all bowls smell of the reward regardless of whether they are correct or incorrect (Barense et al., 2002; Brooks et al., 2012; Leuner and Gould, 2010; McGaughy et al., 2008; Mohamed et al., 2011). If TTC are consistently low only within specific exemplar pairings, then it is likely that the differences between the exemplars within the pairing are too great or the rat has a preference for one of the odours or media. This is more likely to be the case with digging media than with odours, as there are more perceptual features that can be used to distinguish them and greater effort is required to dig in media of greater density.

High TTC most likely arise when specific exemplar pairings are difficult to discriminate. This is more likely to occur with digging media pairings that use the same physical material (to reduce the risk of unintended odour/visual cues), where the distinction between 'coarse' and 'fine' is insufficient. If TTC are high for only one exemplar within a pair, and low for the other, the implication must be that one of the exemplars is aversive (for example, overly fine 'dusty' media can be an irritant), and should be changed.

If rats do not exhibit a shift-cost – *i.e.*, ED TTC are not higher than ID TTC – then, outwith the possibility that the rats are cheating, there are two probable causes. The first is that there are insufficient stages reinforcing relevant versus irrelevant dimensions before the ID stage that is to be compared to the ED (see Sutherland and Mackintosh, 1971). We have already discussed our recommendation of two compound stages prior to the ID that is to be compared to the ED (see Section 4.2.1), but depending on strain or manipulation (Chase et al., 2012), more stages may be necessary to form an attentional set.

The second potential reason that rats might not show a shift-cost is that the relative salience of the exemplars between the stimulus dimensions is skewed, or as mentioned previously, discriminations can be solved by cues from either dimension. Whilst the latter typically arises because digging media have different inherent odours that can be used to discriminate them, the former is likely to arise if too much of a strong odour is added to the digging media. We have never specified in our previously published methods how much herb/spice is added to the digging media, because each is dependent on a specific herb/spice odour strength, and the particulate density of the paired digging medium. A general rule of thumb is to add only enough herb/spice that the experimenter can tell the bowls apart by their odour. Too much, and salience-driven attentional processing can overcome an established perceptual attentional set – and rats solve the ED apparently too easily. Full counterbalancing between shift-direction, pairing-order and exemplar-pairing reward-status should mitigate exemplar-related shift-cost difficulties when only one pairing is an occasional problem – although novel exemplars should be sought if such is consistent.

If rats refuse to dig, it can be for several reasons – some already mentioned above – *i.e.*, satiety; or in the initial trials of reversal learning, having encountered unexpected non-reward. Rats can also refuse to dig because they find particular exemplars aversive or because they are risk averse when novel exemplars are encountered – *i.e.*, there is a 50% chance of digging in an unbaited bowl, as new stimuli signal that the food could be in either bowl. This uncertainty might cause an initial reluctance to dig in risk averse rats. We choose exemplars that are not aversive to the majority of rats, and choose rat strains that are naturally inquisitive about their environment and motivated to seek appetitive reward. However, not all rats are equal, even within strains: some rats take more trials to learn how to dig for reward; some rats need time to habituate to the arena; some rats find some exemplars aversive; and some rats find some exemplars preferable to others. All of these problems can be managed and overcome to a certain degree. If rats persist in refraining from digging, we typically wait until they resume and, as mentioned above, we typically see the same patterns of behaviour regardless of how long it takes the rat to respond. When investigating acute pharmacological effects, we typically run a single test before starting the pharmacological manipulation, as rats generally respond more quickly during tests after the first –and are less likely to stop responding during reversal stages. If some rats find some exemplars preferable/aversive, then counterbalancing should reduce the impact of this at any particular stage – although exemplars should be changed only if there is consistent preference/aversiveness.

5. Conclusion

When well-implemented, the rat ASST provides a robust and repeatable measure of the cognitive processes involved in attentional set-shifting behaviour and thus convergent validity with the CANTAB ID/ED task for humans and monkeys. Whilst throughput can initially be limited by time taken to conduct the task, with repeated testing and within-subjects designs, the task can be completed in under an hour, allowing dose response curves to be established for acute pharmacological interventions, as well as chronic and subchronic manipulations. There remains, however, a need for comparisons between methodologies, to confirm that the various commonly used differences (*e.g.*, odoured bowl rims instead of odoured digging media) provide data that reflect the same cognitive processes. We must also further develop the ASST to help discern, and understand, the exact nature of those processes: the mechanisms involved in shifting behaviour are yet to be fully described. To that end, we are willing to share our raw data with other researchers and would be pleased to facilitate wider data sharing.

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Conflicts of interest

None.

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