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Title: Between and within laboratory reliability of mouse behaviour recorded in home-cage and open-field

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Abstract: Background: Reproducibility of behavioural findings between laboratories is difficult due to behaviour being sensitive to environmental factors and interactions with genetics. The objective of this study was to investigate reproducibility of behavioural data between laboratories using the PhenoTyper home cage observation system and within laboratory reproducibility using different lighting regimes.
New Method: The ambulatory activity of C57BL/6 and DBA/2 mice was tested in PhenoTypers in two laboratories under near identical housing and testing conditions (Exp. 1). Additionally activity and anxiety were also assessed in the open-field test. Furthermore, testing in either a normal or inverted light/dark cycle was used to determine effects of lighting regime in a within-laboratory comparison in Aberdeen (Exp. 2).
Results: Using the PhenoTyper similar circadian rhythms were observed across laboratories. Higher levels of baseline and novelty-induced activity were evident in Aberdeen compared to Utrecht although strain differences were consistent between laboratories. Open field activity was also similar across laboratories whereas strain differences in anxiety were different. Within laboratory analysis of different lighting regimes revealed that behaviour of the mice was sensitive to changes in lighting.
Comparison with existing methods: Utilisation of a home cage observation system facilitates the reproducibility of activity but not anxiety-related behaviours across laboratories by eliminating environmental factors known to influence reproducibility in standard behavioural tests.
Conclusions: Standardisation of housing/test conditions resulted in reproducibility of home cage and open field activity but not anxiety-related phenotypes across laboratories with some behaviours more sensitive to environmental factors. Environmental factors include lighting and time of day.

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26th November 2017

Dear Professor Crunelli

Please find attached our revised manuscript titled 'Between and within laboratory reliability of mouse behaviour recorded in home-cage and open-field' we have addressed the comments of the reviewers and hope that the manuscript is now acceptable for publication in the Measuring Behavior special issue of Journal of Neuroscience Methods.

'I have read and have abided by the statement of ethical standards for manuscripts submitted to the Journal of Neuroscience Methods'

Thank you in advance for your consideration

Yours Sincerely

Lianne Robinson

Response to reviewers:

Reviewer 1

1. I do not understand figure 1. In fact two PhenoTyper home cages are represented for Aberdeen laboratory (and Utrecht??) and two open fields are represented for Utrecht. The design described is different: a comparison between PhenoTyper home cage between Aberdeen and Utrecht and a comparison between open field experiments between Aberdeen and Utrecht . Only in Aberdeen the AA have submitted the same animals after the open filed to another open field experiment with new lighting regime.

   Based on the comments and recommendations of both reviewers we have now removed Figure 1 so as to avoid any confusion. In doing so we have amended all figures, legends and references to them in the text accordingly.

2. It is very important to indicate the significance point by point in figure 2 and figure 5

   As suggested by the reviewer we have now indicated on the figures (now figure 1 and figure 4) the significance for each relevant data point.

3. How much the higher motor activity showed by Aberdeen animals can have an impact if I would like to study drugs that are believed to induce an induction of motor activity? There is the possibility that it will be very difficult to see an hyperactivity in these animals that are yet hyperactive. May the AA discuss this point?

   The reviewer has correctly pointed out that the Aberdeen animals do indeed display an increased activity compared to those tested in Utrecht however, as we have not tested drugs that induce hyperactivity in this present study it is difficult to confirm or comment on exactly how much of a further increase in activity we would be able to observe in these animals. It is reasonable to assume that we would still observe an increase following drug treatment however, it is likely that the extent of the increase in activity observed would be lower than that seen with less active animals (as in Utrecht) due to the already high baseline locomotor activity in the animals.

4. At page 13 of the discussion the AA declared : "Overall, these results clearly prove the suitability of the …….. However, the globally increased activity …….. must have other reasons and may not related to equipment, experimental design or origin of animals).Please the AA try to explain which other reason can give a so different result. There is a difference in housing room: housed in same room as home cage testing (Aberdeen) and housed and tested in separated room (Utrecht). Can the AA take in consideration this difference in the results found? If not can explain why?.

   The reviewer makes a very valid point and we have tried to address this issue and explain some of the reasons why we believe there is a overall increase in activity between the two facilities on page 15 - 17 of the discussion (section 4.2 Reproducibility within the Aberdeen laboratory). Here we discuss the differences between the testing conditions (working environment, noise etc) within the two facilities which we believe may account for the activity differences.

5. Literature always reported that DBA/2 mice are less active and more anxious. I totally disagree with the assumption of the AA at page 15 " we assume that some proxies such as activity are less susceptible to environmental factors than others as anxiety".
The AA provide then some literature discussion for this. I ask to the AA to cancel or reformulate this phrase in the discussion.

   We have addressed the concerns of the reviewer and have amended the relevant section of the discussion on page 15 by removing the phrase in question. We have however, retained the discussion of differences in activity and anxiety levels reported between the strains in support of our differential findings with DBA/2 and C57BL/6 mice in the present study.

Reviewer 2

1. Figure 1 is not really informative and may spur to misunderstanding as pictures of equipment are really different for the two laboratories.

   As mentioned above we have decided to remove Figure 1 so as to avoid any confusion and have amended the text, figure legends and figure labelling accordingly.

2. In Table 1 the lighting times are certainly wrong (for example: dark: 09h00-21h00) because elsewhere this is indicated dark at 08h00 (p6) and the onset of recording is at 08h00 (p5).

   We have amended the light timings in Table 1 and also throughout the text (Page 5 and 6 of the Materials and Methods) to ensure that all correctly state that onset of the dark phase was at 8 am and recording began at 8am.

3. p6: room temperature maintained at 23±2°C. This again different from what is written in Tables 1 and 2.

   The reviewer rightly pointed out that the temperature had been stated incorrectly in the text and we have now amended this to 21 ± 1°C and is now the same as both Table 1 and Table 2.

4. More importantly, the Open-field experiment is supposed to evaluate some anxiety trait in both laboratories. But we don't know which light intensity has been used (p7). As there are discrepancies between laboratories this is a crucial information.

   The reviewer is completely correct in stating that we need to include details of the light intensity used during the Open Field in both experiments. As already stated in Materials and Methods section (page 8) in Experiment 1 the open field was performed under red light conditions in both Aberdeen and Utrecht. Therefore the lighting between the two laboratories was identical. We have however, now included the light intensity (see page 8 of Materials and Methods) for experiment 2 when animals were then subsequently tested under normal lighting conditions in Aberdeen.

5. Result section: p10; activity and anxiety-related behaviour paragraph. Error on Figure 4A where the significance for C57 should translated to DBA.

   This figure is now Figure 3A and we have amended the significance to indicate a difference between DBA as opposed to C57BL/6

6. Exp 2: p11 Fig 5A instead of Fig 1A.

   We have corrected this error in the text and now refer to this as Figure 4A

7. Open field analysis first sentence p12: Fig 7A and Fig 7B instead of Fig 8A and Fig 8B.

   We have corrected this sentence on Page 12, and it now refers to Figure 6A and 6B.

8. Discussion: the discussion is far too long as the "take-home" is not really difficult to catch. However, although the "experimenter" influence on behavioural experiments was suggested in the introduction this point could be tackled in the discussion with the importance of the gender of the experimenter.

   We value the reviewers comment regards the discussion but feel that the discussion needs to be sufficient to consider all the factors that can affect the reproducibility of the data (both between and within the laboratories). The reviewer makes a very valid point regards experimenter gender influence on behavioural outcome and we have included this in the discussion on page 14 - 15 (with reference to the literature). We have also updated the reference list accordingly.

   Although the outcome of this paper does not make me very optimistic on the reproducibility of experiments conducted in different laboratories, I consider this is very important to publish such data to clearly emphasize that homogenization of environmental conditions, including housing conditions, are at least as important as the experimental procedure itself to augment the reproducibility of the results between laboratories.

Between and within laboratory reliability of mouse behaviour recorded in home-cage and open-field

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Highlights (for review)

- Home cage activity of mice is reproducible across laboratories
- Anxiety- related behaviours are more susceptible to environmental factors
- Behaviour of the mice is sensitive to changes in lighting regimes

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Abstract

Background: Reproducibility of behavioural findings between laboratories is difficult due to behaviour being sensitive to environmental factors and interactions with genetics. The objective of this study was to investigate reproducibility of behavioural data between laboratories using the PhenoTyper home cage observation system and within laboratory reproducibility using different lighting regimes.

New Method: The ambulatory activity of C57BL/6 and DBA/2 mice was tested in PhenoTypers in two laboratories under near identical housing and testing conditions (Exp. 1). Additionally activity and anxiety were also assessed in the open-field test. Furthermore, testing in either a normal or inverted light/dark cycle was used to determine effects of lighting regime in a within-laboratory comparison in Aberdeen (Exp. 2).

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Comparison with existing methods: Utilisation of a home cage observation system facilitates the reproducibility of activity but not anxiety-related behaviours across laboratories by eliminating environmental factors known to influence reproducibility in standard behavioural tests.

Conclusions: Standardisation of housing/test conditions resulted in reproducibility of home cage and open field activity but not anxiety-related phenotypes across laboratories with some behaviours more sensitive to environmental factors. Environmental factors include lighting and time of day.

Keywords: Home-cage, behavior, anxiety, mice, reproducibility

1. Introduction

Differences in phenotyping results between research laboratories and lack of reproducibility is a major concern for behavioural testing and for preclinical studies in general (Wahlsten et al. 2003). Multiple attempts have been made to overcome this issue and suggestions for more standardised reporting is proposed to lead to higher reproducibility between laboratories (Jarvis and Williams, 2016). But even efforts, in which experimental factors were fully standardised across laboratories (Crabbe et al. 1999) have not been completely successful. For many researchers, this does not come as a surprise given that behavioural testing is sensitive to environmental factors (Sousa et al. 2006) such as housing conditions (background noise, olfactory cues), experimenter interactions (Bohlen et al. 2014), and also experimental design (Avey et al. 2016). A number of multi-laboratory studies have observed significant differences between mouse strains across laboratories and also interactions of genotype x laboratory despite efforts to rigorously standardise both housing conditions and experimental design (Richter et al. 2011; Wolfer et al. 2004). Chesler and colleagues (2002) reported that environmental factors including experimenter interactions, handling, time of day and order of testing all can influence reproducibility of behavioural experiments. Others have successfully reproduced behavioural findings across time and laboratories (Mandillo et al. 2008; Wahlsten et al. 2006; Kafkafi et al. 2003) although it has been suggested that reproducibility may be test dependent (Wahlsten et al. 2006).

One of the main factors affecting robustness and reproducibility of behavioural testing between (and even within) laboratories is that of human/experimenter intervention. This factor is difficult to control since the majority of behavioural assays require handling of the animal, when it is removed from its' home cage and placed in the test environment, and often repeatedly moved during trials. Behavioural phenotyping in an automated home-cage environment (Casadesus et al. 2001; Spruijt 1992; de Visser et al. 2006; Kas and van Ree, 2004) has a number of advantages over conventional

behavioural assays and could assist with reliability and reproducibility of behavioural outcomes. Eliminating the stress of environmental novelty and handling is expected to lower variability during long-term continuous monitoring of animals (Tecott and Nestler 2004) and therefore circadian rhythms and gross locomotor baseline activity may be assessed free from such confounders (Tang et al. 2002).

Differences in home cage behaviour (Tang and Sanford 2005; Tang et al. 2002) and circadian activity patterns (Loos et al. 2014; de Visser et al. 2006; Kopp 2001; Ebihara et al. 1978; Oliverio and Malorni 1979; Schwartz and Zimmerman 1990) have been observed between inbred mouse strains. C57BL/6 mice robustly display higher levels of general activity compared to DBA/2 mice in home-cages (deVisser et al. 2006; Krackow et al. 2010), and also in the open field (Kafkafi et al. 2005; Logue et al. 1997; Wolfer et al. 2004). Other conventional tests have also revealed differences in anxiety and locomotor activity related behaviours in inbred strains of mice (See Crawley et al. 1997 for review). DBA/2 mice are characterised by heightened levels of emotionality (Bouwknecht and Paylor, 2002; Rogers et al. 1999; Moy et al. 2007; Yilmazer-Hanke et al 2003; Ohl et al. 2003), which could explain their lower locomotor activity (Cabib et al. 2002; Lad et al. 2010; Crabbe et al. 1986) compared to C57BL/6. However, behavioural differences seem to be test dependent (Crabbe et al. 1986; Griebel et al. 2000).

1.1 Aim

The overall aim of this present study was to investigate between and within laboratory reproducibility. The home-cage appears to deliver the simplest way of harmonisation across laboratories, experiment 1 included the recording of baseline ambulatory activity, circadian rhythms and anxiety-related behaviour of two inbred mouse strains (C57BL/6 and DBA/2) in handling-free cages across two laboratories (Utrecht and Aberdeen). As an extension to this experiment, animals were subsequently tested in the open field that required short intervals of handling. We

hypothesized that implementation of standardised home-cage observations will eliminate environmental factors that could influence behavioural outcomes and as a consequence similar results will be observed across laboratories. Whereas, results may appear more variable for the open field test. In experiment 2, we repeated the behavioural experiments in Aberdeen using the same animals.

1.2 Experimental Design

Harmonisation was achieved by controlling the majority of factors that we predicted would influence the experiment. Animals were ordered from Harlan UK (now Envigo) and randomly selected from a cohort of age and gender matched littermates. Half of the animals were sent to Aberdeen, whilst the other half were shipped to Utrecht (in the same week) where following acclimatisation to an inverted day-night cycle, the behavioural testing commenced. For the home-cage observations, we utilised 30 PhenoTyper cages in each laboratory and the onset of recording was at 8 am (Greenwich time) on the same Friday (Utrecht time adjusted by 1 hr for CET). Following the completion of testing (see Methods for details), the Aberdeen cohort were then re-aligned to the normal day-night rhythm and measured again for within-laboratory reproducibility. We predicted that there would be minor differences in the home-cage observations, but a substantial difference in the open field would emerge given that mice are nocturnally active and experimenting during their light phase would disrupt their sleep patterns (Hawkins and Golledge, 2016).

2. Materials and methods

2.1 Subjects

C57BL/6JOlaHsd and DBA/2OlaHsd male mice were supplied by a commercial breeder (Harlan UK – now Envigo). In Exp. 1, N=30 male mice (N=15 of each strain) aged 10 weeks were delivered to the research facility at the University of Aberdeen, UK via truck on the same day, and the other 30 (N=15 per strain) to Utrecht University, Netherlands, by air and truck over two days. All animals were selected randomly from a much bigger pool of age-matched littermates. Their Health status was SPF (Specific Pathogen Free) at delivery. Following their arrival in the respective facilities, the animals were individually housed under an inverted 12 hour day/night cycle (lights on at 20.00 and off at 08.00) for two weeks. Housing conditions between the two facilities were similar (see Table 1 for comparisons) with only a few exceptions including Macrolon II cages in Utrecht whilst there were shoebox cages in Aberdeen and enrichment of either tissue (Utrecht) or wood shavings (Aberdeen). In Exp. 2, the 30 mice (N=15 DBA and N=15 C57BL/6) delivered to the research facility at the University of Aberdeen for Exp. 1 were returned to the endogenous day/night cycle (lights on at 08.00 and off at 20.00) for four weeks prior to testing. The only difference between the two testing sessions was the lighting cycle (see Table 2 for comparisons). In both research facilities water and food were provided ad libitum, ambient room temperature was maintained at 21 ± 1°C and 40–55% relative humidity. All experiments followed ARRIVE guidelines and were ethically approved whilst adhering to the standards outlined in the European Communities Council Directive (63/2010/EU) and a project license under the UK Scientific Procedures Act (1986).

2.2 Behavioural Apparatus

Home-cage activity analysis was performed using the video based observation system PhenoTyper (Noldus IT, Wageningen, The Netherlands) (de Visser et al, 2006; Riedel et al 2009; Robinson et al 2013; Robinson and Riedel 2014). Each box (30 x 30 x 35 cm made of transparent Perspex) contained

a shelter (10 x 10 x 5 cm; distal right corner), feeder and water bottle (front panel; see Riedel et al 2009 for a complete description of the set up). Continuous ambulatory activity of the mice was recorded as X-Y coordinates by built in infrared sensitive video cameras and the video tracking software Ethovision 3.1 (Noldus IT, Wageningen, The Netherlands). In total, 30 PhenoTypers were used simultaneously using 15 PhenoTyper cages (connected via quad units) per PC in Aberdeen, and 4 PhenoTyper cages per PC in Utrecht.

Anxiety like behaviour in the mice was tested in a separate room using a circular 80 cm diameter (40cm high) grey PVC open field arena positioned on a white base. Behaviour in the open field was recorded using an overhead video camera and the tracking software Ethovision 3.1.

2.3 Testing

In total, two experiments were conducted. Exp. 1 constituted an inter-laboratory assessment conducted at exactly the same time (start on Friday 8 am Aberdeen time by placement of mice into PhenoTyper boxes and start of recording) under inverted light conditions (standard for Utrecht) until completion of open field test. Exp. 2 extended this by returning the Aberdeen cohort to normo-dian lighting and conducting a second test (intra-laboratory assessment).

In both experiments home cage observations were performed for 4 days using a similar protocol to that of de Visser and colleagues (2006). Days 1 – 2 constituted habituation, whereas day 4 represented normal baseline activity of each individual. In the reverse lighting conditions the mice were placed into the PhenoTypers at the start of the dark cycle, whereas when tested under the normal light cycle in Exp. 2b recording was started 3 hours prior to the start of the dark cycle. Ambulatory activity was recorded as X-Y coordinates and multiple parameters were extracted from the raw data including: i) total distance moved in open areas; ii) duration of time spent in open areas; iii) time in shelter. Data were either averaged into hourly bins or pooled together and

contrasted for the 12 hour dark/light periods respectively. Following on from home cage assessment, mice were removed from the PhenoTypers and returned into their original home cages (shoeboxes in Aberdeen, Macrolon II in Utrecht) where they were allowed to habituate for 1 week prior to testing in the open field. The testing conditions for the open field were dependent on the light/dark cycle. For Exp. 1, testing in the open field was performed under red light conditions in both laboratories during the early phase of the dark cycle (09.00 – 14.00). For Exp. 2, the open field was performed under normal lighting (09.00 – 14.00) in a dimly lit room (95 lux light intensity). Animals were transported individually to the testing room, habituated (10mins) and released into the centre of the arena. Ambulatory activity was recorded for 15 minutes after which the mice were returned to their home cages and the arena cleaned with warm water between animals. For analysis purposes three zones were defined by the software (Ethovision 3.1): i) outer circle, 6cm ring from the arena wall; ii) inner circle, 68cm ring and iii) centre, 12cm circle in the middle of the arena. In addition to recording distance moved as an overall measure of locomotor activity, the time spent in the inner circle was taken as a proxy of anxiety related behaviour.

2.4 Statistical Analysis

Statistical analysis was performed using the statistical software GraphPad Prism version 5.0. Circadian activity of each strain was analysed for 24 hours (day 4 of the PhenoTyper recording), with laboratory (Utrecht/Aberdeen) and strain (C57BL/6, DBA/2) as factors in Exp. 1. By contrast, light/dark cycles and strain constituted the factors for the analysis of home-cage data of Exp. 2 against Exp. 1 in Aberdeen. Similar contrasts applied for the results of the open field.

Data were analysed using factorial Analysis of Variance (ANOVA), followed by Student t-tests and appropriate post hoc comparisons. Outliers were determined by the Grubbs method and alpha set to 5 %. Only reliable analyses are given for clarity.

3. Results

Experiment 1: Between-laboratory reproducibility of home cage and anxiety related behaviour in C57BL/6 and DBA/2 mice.

The overarching experimental design along with details pertaining to housing, the conditions of the holding facilities and handling and care are summarised in Table 1. Apart from the difference in delivery method from UK, there were only a few minor differences in holding conditions such as cage type, the enrichment used, and the fact that animals in Aberdeen were continuously housed in the same room, in which the PhenoTyper experiment took place. Although the research facility in Aberdeen was operating on a normal day-night cycle, the holding room lights were inverted to match the light regime of the Utrecht facility and all experiments were aligned in time and calendar week. We reasoned that these minor variations in holding conditions are unlikely to have a significant bearing on the overall experimental outcome. Although a strain comparison is of interest, in this present study particular emphasis was placed on the comparison between laboratories whilst strain differences were secondary.

Once animals were placed in the PhenoTyper cages (Friday), their activity was monitored. Habituation was considered to last for 2 days and the first weekday (Monday) was considered to return baseline activity levels (Fig. 1). Circadian activity of DBA/2 and C57BL/6 mice in both laboratories was increased during periods of darkness (Fig. 1, grey background) and declined to virtual absence of activity during the light phase. Despite overall higher ambulatory activity in the Aberdeen cohorts, similar activity peaks at the beginning and end of the dark phase were obtained in both laboratories. This was particularly apparent with the DBA/2 mice (Fig. 1A), but also observed for C57BL/6 (Fig. 1B).

These data were further collapsed into 12 hour bins reporting on the activity pattern of the light and dark phases separately (Fig. 2). Novelty induced activity was derived from the first dark cycle, and factorial repeated measures Analysis of Variance returned a significant main effect of laboratory on

distance moved (F(1,54)=19.866; p≤0.001); with both strains more active in Aberdeen compared to Utrecht (Fig. 2A and 2B). However, there was no main effect of strain and no interaction between factors. Analysis of the time spent in the shelter confirmed the main effect of laboratory (F(1,54)=7.497; p≤0.009); with both strains spending more of the light phase in the shelter however, in Utrecht they also spent increased amounts of the night phase in the shelter compared to Aberdeen (Fig. 2 C,D).

Activity and anxiety related behaviour

Following the completion of the PhenoTyper recording, animals were tested in the open field. As proxies, we extracted the distance moved as activity and time spent in the inner circle of the arena as an anxiety parameter (Fig. 3). Both parameters interacted reliably with strain and laboratory (F(1,57)=9.547; p≤0.003, Fig. 3A; and F(1,57)=21.323; p≤0.001, Fig. 3B). C57BL/6 mice displayed no differences in activity between laboratories, but DBA/2 mice were much more active in the open field in Utrecht compared to Aberdeen (t=4.236; df=28; p≤0.001; Fig. 3A). Aberdeen C57BL/6 mice were less anxious and ventured more into the centre than the Utrecht cohort (t=2.837; df=28; p≤0.009); whereas the opposite was observed for DBA/2 mice (t=4.095; df=28; p≤0.001). In contrast to the home cage observations, both laboratories measured heightened levels of activity in DBA/2 mice compared to C57BL/6 (Utrecht: t=9.047; df=28; p≤0.001 and Aberdeen: t=2.836; df=28; p≤0.008), while the strain effect for time in centre was only significant in Aberdeen. Here, DBA/2 mice spent less time in the centre than C57BL/6 (t=6.223; df=28; p≤0.001), which is indicative of anxiety-like behaviour.

Experiment 2: Within laboratory reliability: comparison in endogenous and inverted day/night cycles.

The experimental and housing conditions used for this within laboratory comparison are detailed in Table 2 with timing of the light/dark cycle being the main variable that was changed. It was proposed that circadian activity in the home cage would be similar independent of whether the day/ night rhythm followed the endogenous day-night-time or was inverted. Circadian home cage activity of both DBA/2 and C57BL/6 mice are displayed in Fig. 4. In both testing conditions the two strains displayed a normal circadian rhythm with increased levels of activity during the dark phase and vastly reduced levels of activity in the light phase (Fig. 4: main effect of time: Fig 4A, DBA/2 ($F(23,46)=62$; $p<0.0001$; Fig. 4B, C57BL/6 ($F(23,46)=32.30$; $p<0.0001$). DBA/2 mice displayed significantly higher ambulatory activity during dark hours compared to C57BL/6 when tested under the endogenous day/night rhythm (main effect of strain: ($F(1,48)=5.61$; $p=0.02$)), but there was no clear difference from the inverted day/night cycle. Nevertheless, we obtained a main effect of testing condition for both strains (C57BL/6: $F(1,46)=37.43$; $p=0.03$; DBA/2: $F(1,48)=8.20$; $p=0.0062$).

Analysis of each strains performance across the two conditions are depicted in Fig. 5. DBA/2 mice displayed increased activity in normal compared to inverted lighting regimes (Fig. 5A) for both light and dark phases of testing (all p's$\leq$0.03) whilst also spending less time in the shelter (days 1 and 2 only: $p\leq0.03$; Fig.5C). By contrast, C57BL/6 mice displayed an increased distance moved in inverted compared to normal testing conditions, but only for the dark hours of testing (all days: p's<0.001; Fig. 5B) whilst also spending more time in the shelter during normal hours of darkness versus inverted ($p\leq0.005$; Fig. 5D).

While there was clear habituation to novelty in both strains in the inverted condition such that animals were more active during the night phase of day 1 relative to the following days (all p's<0.0001), this was different in the normal light setting (Fig. 5A and B). Levels of activity were constant throughout the 4 days in normal light regimes (all p's >0.05).

Open Field analysis of activity and anxiety related behaviours

In the open field, analysis of distance moved (Fig. 6A) and time in centre (Fig. 6B) revealed differences in relation to testing condition. There was a significant interaction of strain and lighting condition for distance moved ($F(1,28)=11.57$; $p=0.002$) and a main effect of lighting ($F(1,28)=126.05$;$p<0.0001$). Post hoc analysis confirmed that DBA/2 (df=28; t=6.42; $p<0.0001$) and C57BL/6 mice (df=28; T=4.84; $P<0.0001$) were more active in the inverted compared to normal lighting regimes. These data are to be considered in the context that when recorded under the inverted light/dark cycle, all animals are in their natural activity cycle and awake. As a consequence, the higher general activity levels during this condition are not surprising. Finally, DBA/2 mice were more active than C57BL/6 when housed and tested under inverted lights (df=28; t=3.50; p=0.0016).

Similar to distance moved, a reliable difference between testing conditions was evident for time in centre ($F(1,28)=21.09$;$p<0.0001$) with each strain spending higher amounts of time in the centre during the inverted lighting regime (C57BL/6: df=28; t=2.25; p=0.03 and DBA/2: df=28; t=2.34; p=0.03). Moreover, C57BL/6 mice were less anxious than DBA/2 and spent significantly more time in the centre ($F(1,28)=44.64$; $p<0.0001$) independent of the lighting condition they were tested in (Fig. 6B; see asterisks). Post hoc analysis confirmed that DBA/2 mice spent less time in the inner circle compared to C57BL6's for both conditions (normal: df=28; t=5.63; $p<0.0001$ and inverted: df= 28; t=5.97; $p<0.0001$).

4. Discussion

The overarching aim of this study was to evaluate between and within laboratory reproducibility of home cage behaviour with two inbred mouse strains. When testing across the two laboratories we predicted that minimising the differences between cohorts (see Table 1) and utilising identical equipment (hardware and software) would facilitate reproducibility of data. We further took great care that the recording was experimenter independent and all recording parameters (sampling rate, start and end) as well as analytical settings (summarising data, pooling over days etc.) were

matched. Whilst considerable differences are evident between laboratories when using conventional behavioural tests (Jarvis and Williams 2016), we proposed that home cage activity in a standardised testing apparatus would be less sensitive to such variations.

4.1 Reproducibility across laboratories – Aberdeen versus Utrecht

Our home cage analysis of circadian rhythmicity and baseline activity of both strains are in agreement with previous findings (Kopp 2001; Tang et al. 2002; Tang and Sanford 2005; de Visser et al. 2006); both strains displayed heightened activity during the dark cycle when mice are more active and exploratory compared to the light phase. Circadian activity peaks were also evident during the dark cycle, one immediately following onset of and the other shortly before the end of the dark cycle in anticipation of the light period (Loos et al, 2014; de Visser et al. 2006 Tang et al. 2002). These circadian rhythms were similar across laboratories but much higher levels of activity were observed in Aberdeen during the dark cycle of testing. No differences between the two mouse strains were observed in each laboratory (no main effect of strain or interaction). Overall, these results clearly prove the suitability of the equipment for reproducibility studies. However, the globally increased activity in Aberdeen must have other reasons and may not be related to equipment, experimental design or origin of animals (see below).

Previous studies have reported a 'novelty induced activity' during the first dark period of testing (de Visser et al. 2006; Tang et al. 2002), especially for C57BL/6 mice. Matching results were obtained in this study such that relative to the overall activity on days 2-4, both laboratories reproduced a heightened activity in the dark phase on day 1 for both strains (see fig. 2) further underlining the reproducibility of the approach. Reciprocal results for time in shelter and presumably sleep time are thus not at all surprising. But despite this high similarity in results between Utrecht and Aberdeen laboratories, others have reported pronounced differences between DBA/2 and C57BL/6 strains in their home cage analyses; with C57BL/6 mice typically expressing more ambulatory activity than

DBA/2 mice (de Visser et al. 2006; Krackow et al. 2010; Tang et al 2002). Although our primary goal was reproducibility between laboratories with strain differences as a secondary outcome measure, possible explanations for these differences between our findings and the historical data may arise from i) different providers for the rodents; ii) different lighting regimes; ii) different equipment using single or community housing (IntelliCage); iv) different recording and analysis proxies (movement versus entries to activity corner).

Following the completion of the home cage test, all animals were placed in the open field and video-observed for 15 minutes. In terms of activity, DBA/2 mice presented with higher path lengths than C57BL/6 mice in both laboratories confirming again the reproducibility of this parameter across laboratories. According to the literature, the opposite would be expected (Lad et al. 2010; Rogers et al. 1999; Cabib et al. 2002; Crawley 1997), but Crabbe and co-workers (1986) rightly suggested that the locomotor activity of inbred mouse strains in the open field is highly sensitive to testing conditions and apparatus. Although we undertook great efforts to keep them identical, it is clear that both room cues and environmental sensory cues differed between Aberdeen and Utrecht (and all historical work). Yet, reproducibility between our two laboratories suggests that these external factors may exert less of an influence than previously expected (Kafkafi et al. 2003).

However, DBA/2 mice expressed heightened anxiety levels in Aberdeen, but lower levels in Utrecht relative to C57BL/6 mice (Fig. 3). These between laboratory variations are more in line with performances in conventional behavioural tests, which were not reproducible across laboratories and instead observed laboratory x strain interactions (Wolfer et al. 2004; Crabbe et al. 1999) and were explained by either non-standardisable idiosyncratic handling, testing environments (Crabbe et al. 1999), experimenter gender differences (Sorge et al. 2014) or possibly a result of within-strain variability or individuality (Lathe 2004). Heightened individuality scatter has been reported for DBA/2 mice by Loos and colleagues (2015), but it is not readily obvious to us how this would explain the contrast between our data and the work of others. The parameter anxiety in general appears

more prone to between laboratory and experimenter differences than activity. Sorge and colleagues (2014) reported that anxiety like behaviour in mice could be influenced by experimenter gender with increased anxiety evident following exposure to a male as opposed to a female experimenter. A lack of reproducibility across laboratories has been previously revealed by Wahlsten and colleagues (2006). In agreement with our study, Wahlsten et al. also reported reliable locomotor phenotypes across laboratories, but different anxiety traits. Consequently, differences in anxiety levels between DBA/2 and C57BL/6 mice as seen in Aberdeen have been reported previously (Lad et al. 2010; Rogers et al. 1999) in standard anxiety assays including the elevated plus maze (Moy et al. 1997; Yilmazer-Hanke et al. 2003), the hole board task (Ohl et al. 2003), the mirror chamber (Paterson et al. 2010), the elevated zero maze (Tang et al. 2002) and the light/dark box (Crawley 2008). Utilisation of different equipment will produce different forms of anxiety (for example state or trait anxiety; Robinson et al., unpublished observations) and can readily explain different levels of anxiety evident between strains, and also between laboratories (Crabbe et al., 1999).

4.2 Reproducibility within the Aberdeen laboratory

Since Exp. 1 revealed high levels of reproducibility for home cage exploration measures, but the overall level of horizontal activity still differed significantly, this could arise from the local set-up of laboratories and their integral placement within our animal facility. An alternative explanation could be the workings of the facility, which normally operates under endogenous lighting conditions whereas, Exp. 1 utilised an inverted lighting regime for harmonisation with Utrecht. As for circadian activity recorded as home cage exploration, however, the exact lighting regime should not impinge on global activity (provided ample habituation is provided to each lighting rhythm) unless external factors critically modulate these measures. Thus, Exp. 2 compared the PhenoTyper activity in Aberdeen under inverted lighting (Exp. 1: lights on at 20.00h) with a second recording after returning the animals to an endogenous lighting regime (lights on at 8.00h). As pointed out for the between

laboratory study, both DBA/2 and C57BL/6 mice showed heightened exploration during the dark phase in both inverted and endogenous lighting rhythms and we thereby replicated similar work reported by others (Kopp 2001; de Visser et al. 2006; Krackow et al. 2010). A more refined analysis over 4 days, however, presented strain differences in horizontal activity such that DBA/2 mice were more active during the endogeneous while C57BL/6 were more active during the inverted lighting regime. Since we have not found an exact match to our behavioural assessment in the literature, it is difficult to draw any significant conclusions from this behaviour. And other interpretations that have been brought forward to explain strain-related differences such as differential anxiety profiles or stress responses (Cabib and Bonaventura, 1997; Mineur et al. 2006, see discussion below) are difficult to reconcile given this is a repeat test in the home cage with little or no interference. Nevertheless, it is not entirely impossible that factors pertaining to the running of the animal unit are responsible for the differences observed in behaviour under the two lighting regimes. The Aberdeen animal house is a working facility in which breeding, maintenance, experimental work and tissue harvesting occur side by side with no specific sectors identified for each activity. A corollary of normal working hours and the endogenous lighting regime (with simulated dawn and dusk) leads to an overall high activity level during the day time (9.00 – 16.00h). This would coincide with the endogenous activity profile of the mouse as a nocturnal species if an inverted light cycle is implemented. Increased sensory stimulation (people walking corridors, speaking and shouting, telephone ringing, etc.) would be easier to handle by the less anxious C57BL/6 mice, who are responsive to these repeated interferences (consequently high levels of activity) in line with previous work (Lad et al. 2010; Rogers et al. 1999; Cabib et al. 2002; Crawley et al 1997). By contrast, the more anxious DBA/2 line (Tang et al. 2002; Lad et al. 2010; Crawley 2008; Yilmazer-Hanke et al. 2003) spent increased time in shelter especially during the light phase for this recording regime (Fig. 5) thereby lowering exploratory activity. The opposite applies for the endogenous light cycle in which the animal unit is active when the mice typical reduce exploratory activity, but are interrupted by external stimuli. It appears that DBA/2 mice are more sensitive to such stimuli and react with

bouts of exploration, but also appreciate the darkness which is co-incident with the closure of the unit. This period of quiescence results in continued exploratory behaviour in DBA/2 mice but overall 'boredom' in C57BL/6. A similar explanation would parsimoniously explain the global activity difference observed in Exp. 1, in which Aberdeen animals displayed much higher horizontal activity relative to the laboratory in Utrecht.

Milligan and colleagues (1993) reported that behavioural outcomes are affected when increased sound levels are evident within facilities during the day due to human intervention and activities. This can be observed in all animal units and may lead to stress induced anomalies in activity and anxiety-related behaviours in both C57BL/6 and DBA/2 mice (Mineur et al 2006). Qualitative assessment of DBA/2 mice in Aberdeen by the experimenter found that they were more hyperactive and anxious when handled compared to observations in Utrecht with similar reports of an irritable and jumpy phenotype with DBA/2 mice in other studies (Rogers et al. 1999). Although standardisation of experimental conditions and homogenisation of study populations in order to reduce within experiment variation was previously considered to improve sensitivity and reproducibility across laboratories (Wahlsten 2001) the interaction of mouse genetics with environmental conditions (Cabib et al. 2000; Crabbe et al. 1999) has questioned whether environmental standardisation has an impact on external validity and reproducibility of results ('standardization fallacy', Wurbel 2000; 2002) with multi-laboratory studies using standardization protocols and obtaining results that were idiosyncratic to a single laboratory (Crabbe et al. 1999; Richter et al. 2011; Wolfer et al. 2004). Studies have therefore suggested that heterogenisation and systemic variation of genetic and environmental conditions as opposed to excessive harmonisation is necessary to detect interactions between genetic and environmental factors and as a consequence improve reproducibility (Richter et al. 2011; Richter et al. 2009; Chesler et al. 2002; Wurbel 2002).

4.3 Conclusions

Standardisation of housing and test conditions along with utilisation of a home cage observation system allowed us to eliminate some of the environmental factors that can influence reproducibility of behavioural outcomes across laboratories (Exp. 1). As expected we were able to observe reproducibility of both home cage and open field activity but not anxiety-related phenotypes across laboratories. Strain differences between the two laboratories were consistent and comparable; however, within strain differences between laboratories remained evident but are explained by external factors pertaining to the set-up of the respective animal unit in Aberdeen compared to Utrecht. Moreover, within laboratory analysis (Exp. 2) of different lighting regimes on activity and anxiety related traits revealed that the behaviour of mice strains are sensitive to changes in lighting conditions. These data further support the contention of an interaction between i) equipment, ii) recording and analysis tools and iii) external factors which needs to be controlled to reveal a better reproducibility and robustness of behavioural outcomes.

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Figure Legends

Figure 1. Between laboratory analysis of circadian rhythms for two different mouse strains DBA/2 (A) and C57BL/6 (B). Circadian activity (averages presented in hourly bins) under baseline conditions (mean ± SEM) are expressed as time spent in the open area of the PhenoTyper during a period of 24 hrs. The shaded area represents the 12 hour dark period of testing. Note that both mouse strains show heightened activity levels during hours of darkness, but that global horizontal activity was much higher in Aberdeen than in Utrecht.

Figure 2. Home cage activity and shelter times in between laboratory comparison. All graphs include 4 consecutive recording days with dark (D) and light (L) phase activity pooled over 12 hours for DBA/2 mice (A + C) and C57BL/6 mice (B + D). Heightened activity (distance moved) in Aberdeen was observed during all 4 night cycles for DBA/2 (A) and C57BL/6 (B) mice. Reciprocal observations were made for time in shelter such that higher activity correlated with less time in shelter in both strains. Means + SEM. Asterisks denote $p<0.05$, t-test.

Figure 3. Open field analysis of activity and anxiety related behaviours in mouse strains following testing in different laboratories. Distance moved (A) and time spent in the centre (B) by the two mouse strains. Note the overall heightened activity levels in DBA/2 mice compared with C57BL/6 independent of laboratory. However, anxiety levels differed between the strains in Aberdeen, but not in Utrecht. Means + SEM. Asterisks denote $p<0.05$, t-test.

Figure 4. Within laboratory analysis of circadian rhythms for DBA/2 (A) and C57BL/6 (B) mice in Aberdeen comparing inverted and endogenous light cycles. Circadian activity (averages presented in hourly bins) under baseline conditions (mean ± SEM) are expressed as time spent in the open area

of the PhenoTyper during a period of 24 hrs. The shaded area represents the 12 hour dark period of testing. Note the reproducibility of the circadian behaviour in DBA/2 mice, but the altered activity profile in C57BL/6 mice is dependent on the lighting regime.

Figure 5. Home cage observations in Aberdeen during inverted and endogenous light/dark cycles. All graphs include 4 consecutive recording days with dark (D) and light (L) phase activity pooled over 12 hours for DBA/2 mice (A + C) and C57BL/6 mice (B + D). Heightened activity (distance moved) under endogenous lighting was observed for DBA/2, but not C57BL/6 mice. Globally, the negative correlation between activity and time in shelter was maintained in either condition. Means + SEM. Asterisks denote $p<0.05$, t-test.

Figure 6. Open field analysis of activity and anxiety related behaviours in mouse strains following testing in Aberdeen under different day/night cycles. Distance moved (A) and time spent in the centre (B) by the two mouse strains. Note that activity levels in DBA/2 mice and C57BL/6 mice are equal when measured under the endogenous light cycle, but are significantly elevated when recordings took place under an inverted cycle (A). DBA/2 mice were more active then C57BL/6 under inverted conditions, but showed higher anxiety levels than C57BL/6 mice independent of cycle. Means + SEM. Asterisks denote $p<0.05$, t-test.

Table 1. Housing and testing conditions implemented in Aberdeen and Utrecht laboratories (Experiment 1).

Table 2. Housing and testing conditions implemented in Aberdeen for the within-laboratory study (Experiment 2).

Table 1

	Aberdeen	**Utrecht**
Animals		
Strains	C57BL/6JOlaHsd DBA/2OlaHsd	C57BL/6JOlaHsd DBA/2OlaHsd
Supplier	Harlan UK	Harlan UK
Transport and time	by truck, several hours	by air and truck, 2 days
Housing		
Cage type	'shoe box' cage, similar dimensions to Macrolon type I elongated	Macrolon type II
Bedding	Aspen chips (medium size)	Aspen chips (small size)
Enrichment	wood shavings	tissue
Lighting	dark: 08.00 – 20.00 white: 20.00 – 08.00	red: 08.00 – 20.00 white: 20.00 – 08.00
Temperature	21°C ± 1	21°C ± 1
Humidity	45 – 55%	40 – 50%
Room	housed in same room as home cage testing	housed and tested in separate rooms
Open Field testing		
Room:	different room to home cage	different room to home cage
Lighting and time:	red light, early dark cycle (09:00 – 14:00)	red light, early dark cycle (09:00 – 14:00)
Testing:	consistent testing order	consistent testing order
Handling and care		
Handling	once a week	once a week
Cage cleaning	once a week under red light	once a week under red light
Food and water	twice per week	twice per week
Food type	SDS CRM (P)	SDS CRM (E)

Table 2

	Aberdeen - A	**Aberdeen - B**
Animals		
Strains:	C57BL/6JOlaHsd DBA/2OlaHsd	C57BL/6JOlaHsd DBA/2OlaHsd
Supplier:	Harlan UK	Harlan UK
Transport and time:	by truck, several hours	by truck, several hours
Housing		
Cage type:	'shoe box' cage, similar dimensions to Macrolon type I elongated	'shoe box' cage, similar dimensions to Macrolon type I elongated
Bedding:	Aspen chips (medium size)	Aspen chips (medium size)
Enrichment:	wood shavings	wood shavings
Lighting:	Inverted dark: 08.00 – 20.00 white: 20.00 – 08.00	Normal white: 08.00 – 20.00 dark: 20.00 – 08.00
Temperature:	21°C ± 1	21°C ± 1
Humidity:	45 – 55%	45 – 55%
Room:	housed in same room as home cage testing	housed in same room as home cage testing
Open Field testing		
Room:	different room to home cage	different room to home cage
Lighting and time:	red light, early dark cycle (09:00 – 14:00)	white light, early light cycle (09:00 – 14:00)
Testing:	consistent testing order	consistent testing order
Handling and care		
Handling:	once a week	once a week
Cage cleaning:	once a week	once a week
Food and water:	twice per week	twice per week
Food type:	SDS CRM (P)	SDS CRM (P)

Figure

Fig. 1_Robinson et al

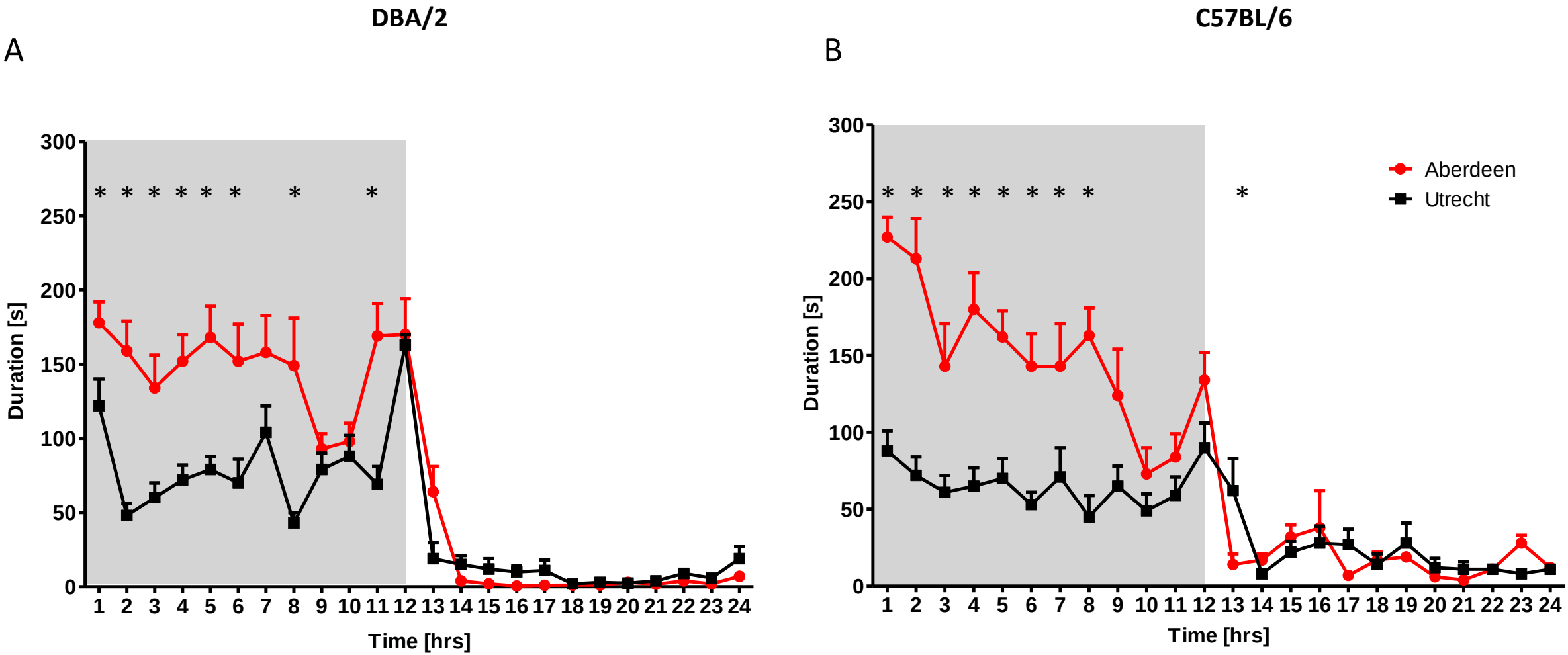
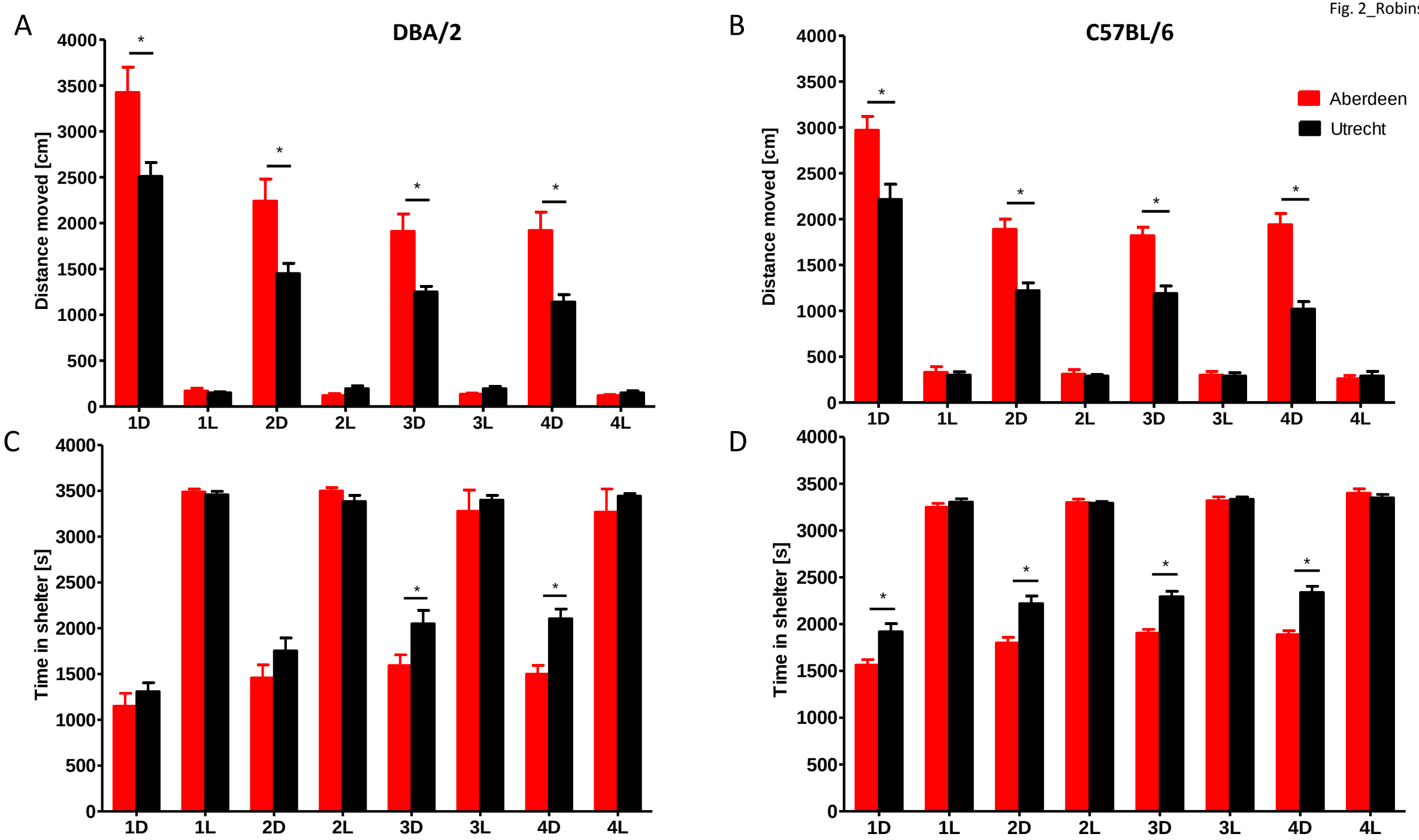
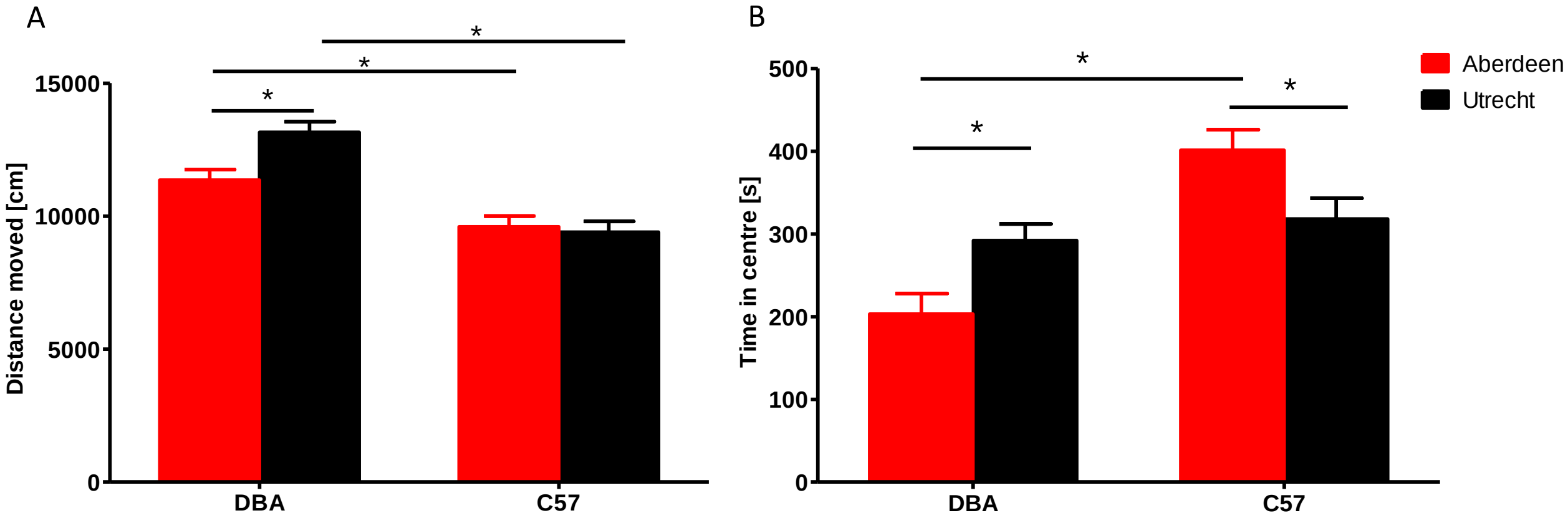


Fig. 2_Robinson et al




A
B
Distance moved [cm]
15000
10000
5000
0
DBA
C57
*
*
*
Time in centre [s]
500
400
300
200
100
0
DBA
C57
*
*
Aberdeen
Utrecht

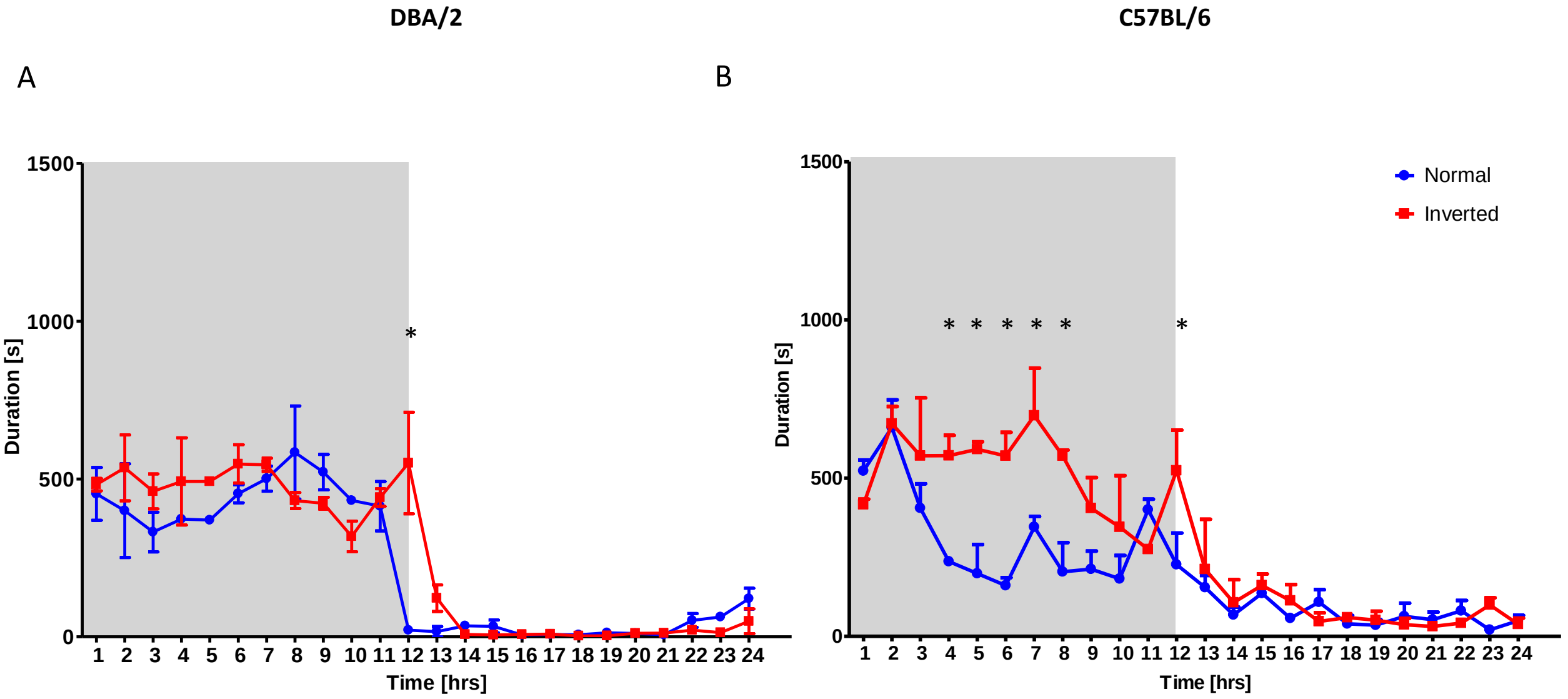
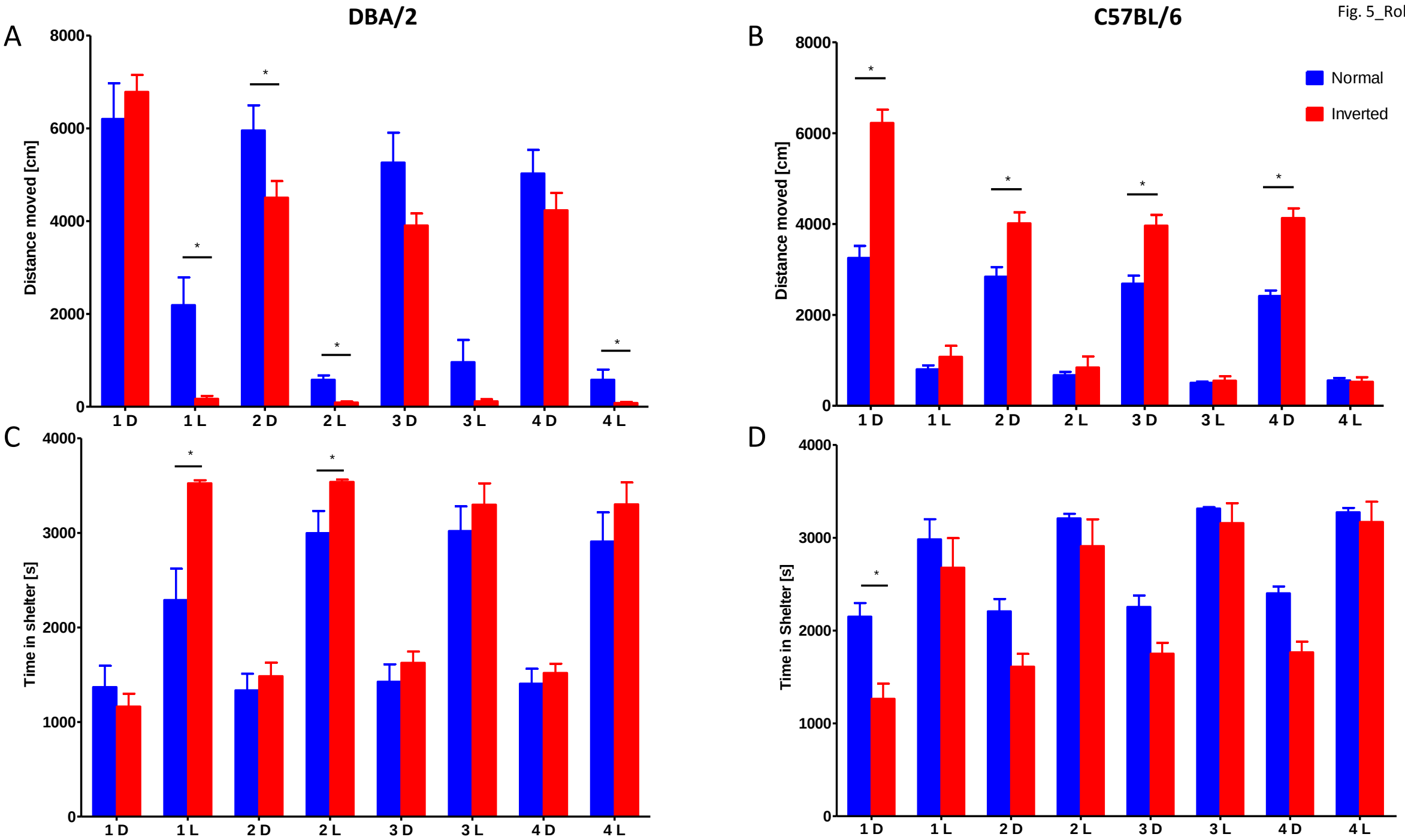
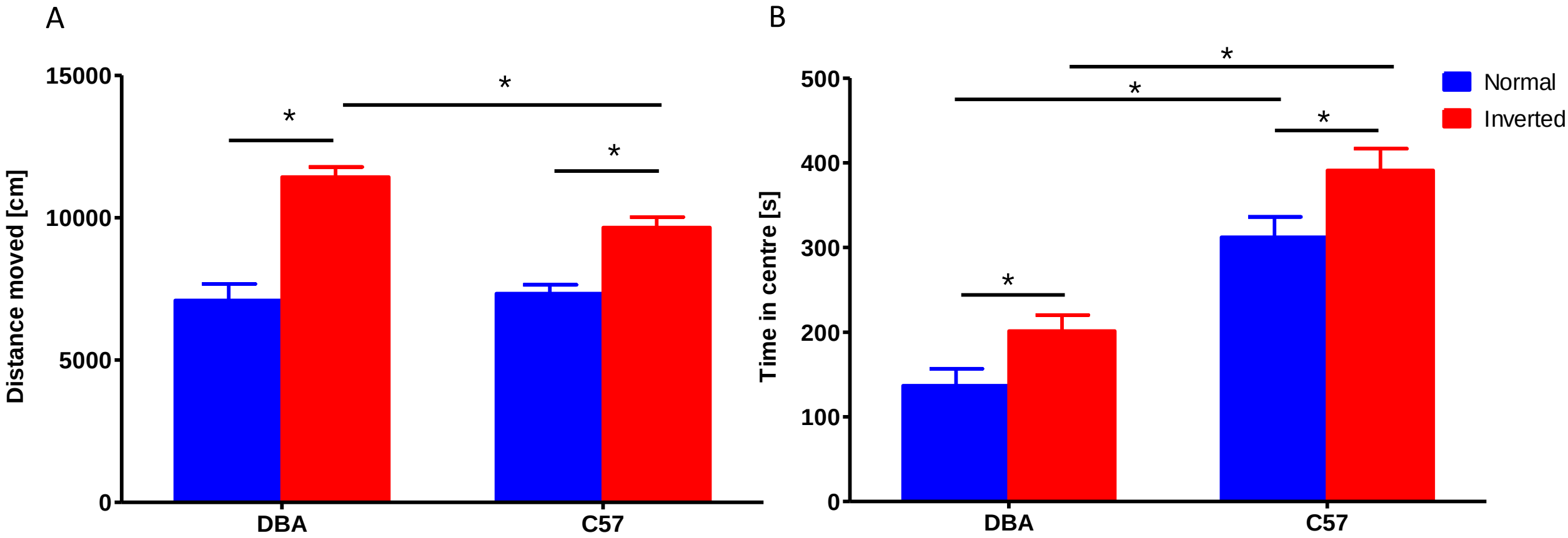

DBA/2
C57BL/6
A
B
Duration [s]
Time [hrs]
Normal
Inverted
0
500
1000
1500
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
*
* * * * *

Fig. 5_Robinson et al




A
B
Distance moved [cm]
15000
10000
5000
0
DBA
C57
*
*
*
Time in centre [s]
500
400
300
200
100
0
DBA
C57
*
*
*
Normal
Inverted