Fluctuating Asymmetry in Relation to Stress and Social Status in Inbred Male Lewis Rats

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Summary

Environmental or intrinsic stressors acting on growing animals and humans may be expressed as small, random deviations from symmetry in otherwise bilaterally symmetrical characters – a phenomenon known as fluctuating asymmetry (FA), the mechanism behind which is not yet clear. In this study, we investigated the effects of two known stressors (grid floor and single housing) on the development of FA in young male Lewis rats compared to housing under normal conditions (bedding) or an enriched environment. It was found that such environmental factors have an impact on FA in rats. Initially, FA was found to be high in all rats. In bedding and in enrichment groups, FA decreased throughout the study ($P<0.05$ in bedding group and $P<0.001$ in enrichment group from five to eleven weeks of age). FA in singly housed rats and in rats on a grid floor did not change significantly throughout the study. FA in these rats was considerably higher than in rats housed on bedding with or without environmental enrichment ($P<0.001$). Moreover, the influence of social status on FA was evaluated. Dominant rats housed in the enriched environment were found to have a higher FA of combined traits than subordinate rats at eight weeks of age ($P<0.01$), but except for this result, no relationship between FA and dominance was found. Singly housed rats showed significantly higher FA than dominant as well as subordinate rats ($P<0.001$). In conclusion, FA of selected traits may hold a potential for measuring stress influences in laboratory animals, which can be of some importance in welfare research.

Introduction

Harmful conditions such as environmentally and intrinsically induced stress in growing animals and humans may be expressed as small, random deviations from symmetry in otherwise bilaterally symmetrical characters (*Wilson & Manning, 1996*); a phenomenon known as fluctuating asymmetry (FA) (*Parsons, 1992*). The mechanism behind this is not yet known, but it may be seen as a result of developmental instability, which reflects the impaired ability of the body to maintain a stable development under differing conditions. An important theory is that different kinds of stress disturb stable development, thus increasing the level of FA. FA may thus be used to quantify the reduced efficiency of developmental processes. However, the effect on individual traits is weak, and the traits differ in sensitivity (*Stub et al., 2002b*), so the use of composite FA analysis using multiple traits is recommended (*Leung et al., 2000*). It is also unclear to what extent the sensitivity of FA is high enough to allow its use as an indicator for any type of stress, but in controlled studies with inbred laboratory animals the variation may be kept low enough to allow a method

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measuring those discrete changes as a tool.

We have previously shown, that it is possible to measure FA reproducibly in rats and mice (*Stub et al., 2002a*), and that it may be used to demonstrate stress-related impact on laboratory animals, *e.g.* due to social environment (Stub et al., 2004c), spontaneous disease such as renal epithelial hyperplasia in rats (*Stub et al., 2004b*) or transgenetically induced disease, such as cystic fibrosis in mice (*Stub et al., 2004a*). However, it would also be of interest to study whether stress caused by external, environmental factors could be shown to have an impact on FA in laboratory animals.

In this study, we measured the development of FA in rats housed either individually or on a grid floor, which in earlier studies were found to stress the rats illustrated by increased plasma corticosterone (*Heidbreder et al., 2000; McCormick et al., 1998*), behavioural changes (*Heidbreder et al., 2000; Eskola & Kaliste-Korhonen, 1998*) and elevated blood pressure and heart rate (*Krohn et al., 2003*). Furthermore, grid floors are known to be deselected by rats in preference testing (*Blom et al., 1996; Krohn & Hansen, 2001*). Moreover, social status within a group is known to influence a range of behavioural and pathophysiological parameters (*Sørensen et al., 2004*), and therefore the impact of social status on FA was evaluated.

Materials and Methods

Animals and housing

The subjects were 74 Lew/Mol male rats, microbiologically defined according to the FELASA guidelines (*Nicklas et al., 2002*). The rats were pair-housed in transparent standard macrolon type III cages measuring 425x266x185 mm (Tecniplast, Buggugiate, Italy) with a grid lid. The cage contained 300 gr. (2 L) aspen bedding, 50 gr. (2 L) aspen nest material (wood wool) and an aspen gnawing stick (about 5x1x1 cm) all sourced from Tapvei (Tapvei, Vaikkojoentie 33 FIN-73620, Kortteinen). Cages were changed twice a week. Room temperature was maintained at 20°C and with a relative humidity of 55-80% and ten to fifteen air changes per hour. Since rats are nocturnal animals, they were housed under a reversed 12:12 hour light:dark cycle with no natural light to ensure that they were awake and active during the working day. The light period started at 22.00 h and lights were turned off at 10.00 h. The rats had free access to food (Altromin 1324 from Altromin Denmark, Chr. Petersen A/S, DK-4100 Ringsted, Denmark) and tap wate. The rats were purchased at four weeks of age, and a group of 18 rats were single housed with bedding (single housing) during the acclimatization period and onward. At five weeks of age following one week of acclimatization, the remaining rats were randomly allocated to one of three groups: 18 rats were pair housed with aspen wood shavings for bedding, wooden wool and a stick, all sourced from Tapvei (enrichment), 18 rats were pair housed with bedding from Tapvei (control), and 18 rats were pair housed on grid floors, grids measuring 2mm with 6.5 mm space between (grid floor). The grid was placed on the solid bottom of the cage. Two rats, used as intruder rats in the tests for dominance, were pair housed in an enriched environment. The rats were measured for body weight, FA and social status (social status was not tested in single housed rats) at five, eight and eleven weeks of age. The rats were euthanised at eleven weeks of age.

Fluctuating asymmetry

FA was measured using a digital slide caliper with constant pressure to the nearest 0.01 mm. (Mitutoyo, Mitutoyo Corporation, Japan). The traits were measured on the surface of a fully awake animal. The traits measured were the medio-lateral width of the carpal bones, the medio-lateral width of the joint between tibia and tarsal bones and the dorsopalmar width of the joint between the third metatarsal bone and the third digital bone on the hind paw. These traits (*i.e.* carpal joint, tibio-tarsal joint and hindpaw) were in earlier studies found to express FA (*Stub et al., 2002a*). Each side was measured twice and trait size was determined by calculating the mean of the two measurements.

Absolute asymmetry was defined as right-minus-left trait size, and follows a normal distribution with a mean of zero. Relative FA of a trait was defined as absolute asymmetry divided by mean trait size (relative FA= absolute asymmetry/[½ x size of right side + ½ x size of left side]). Relative FA was calculated for each trait.

Dominance testing

Three different dominance tests were performed at five, eight and eleven weeks of age in pair-housed rats only. Each test was performed once at each age level. Testing was done between 2 am and 6 am, the inactive period of the animals, as this is normal procedure (*Barclay, 2001*). First, an intruder test was performed in the home cage of pair-housed rats only. The first resident rat to approach the intruder rat was noted as the dominant (*Barclay, 2001*). In the second test, the first rat accessing a box on a grid floor placed in a Macrolon type III cage was noted as the dominant. Both rats had been individually habituated to the grid floor and the box immediately before the test. In the last test, the rat first accessing a known attractive piece of food placed on the bottom of the cage were noted to be the dominant. At each age tested, the rat being dominant in a minimum of two of the three tests was considered to be the dominant rat at the specified age. FA in single housed rats were calculated as well and compared to FA in dominant and subordinate rats in the control group (as they had the same physical housing conditions as the single housed).

Statistical analysis

All statistics were made in MINITAB 12.1 (Minitab Inc.). Absolute asymmetry of the individual traits (*i.e.* carpal joint, tibio-tarsal joint and hindpaw) was tested for normality using the Kolmogorov-Smirnov test for normality ($\alpha<0.05$). ANOVA was used to test whether the mean of absolute asymmetry differed from zero. Numeric values of relative fluctuating asymmetry (FA) do not follow a normal distribution, so FA of rats housed under different housing conditions were compared using the Kruskal-Wallis test and if a significant difference was revealed it was followed by a Mann-Whitney U-test comparing the groups two by two. FA of dominant and subordinate rats was also compared using the Kruskal-Wallis and Mann-Whitney U-test in each kind of housing conditions. Body weight was tested for normality using the Kolmogorov-Smirnov test for normality ($\alpha<0.05$), and it was found to be normally distributed. Body weight was then compared between treatments using ANOVA. Body weight of dominant and subordinate rats versus housing conditions was also compared using ANOVA.

Results

All traits (*i.e.* carpal joint, tibio-tarsal joint and hindpaw) measured fulfilled the demands for absolute asymmetry as they followed a normal distribution and had a mean of zero. Body weight at five, eight and eleven weeks of age did not differ between the groups (data not shown).

Development of FA in all three individual traits as well as in traits combined is shown in Table 1. Significant differences between the various housing groups existed for carpal bones and tibio-tarsal joint at 11 weeks of age, and for the combined traits at 8 and 11 weeks of age. Grid floors gave significantly higher FA of the carpal bones at 11 weeks of age, as compared to the enrichment group. Single housing gave significantly higher values than the control, enrichment and grid floor groups. At 11 weeks of age, the FA of the tibio-tarsal joint in animals on grid floors was significantly higher than the enrichment and control groups. Single housing gave a significant increase of the tibio-tarsal joint value as compared to the enriched situation. For the combined traits, grid floor and single housing caused significantly higher FA values as compared to the enriched situation at 8 and 11 weeks of age. Grid floor and single housing also caused a significant increase in the FA of the combined traits as compared to the control housing situation at 11 weeks of age.

Table 1 Fluctuating asymmetry (medians, min - max)) of each of the three traits and the three traits combined. Multiple Kruskal-Wallis test for all housing groups within a trait/age: ** $p < 0.01$, *** $p < 0.001$

- One group compared with control group (same trait/age) by Mann-Whitney U-test # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$
- One group compared with enrichment group (same trait/age) by Mann-Whitney U-test ¤ ¤ ¤ < 0.01, ¤ ¤ ¤ ¤ < 0.001
- Single housed group compared with grid floor housed group (same trait/age) by Mann-Whitney U-test + $p < 0.05$

	Five weeks	Eight weeks	Eleven weeks
	Med (min-max)	Med (min-max)	Med (min-max)
Carpal bones			***
Control	0.051 (0.005-0.1)	0.022 (0.0- 0.052)	0.021 (0.001- 0.053)
Enrichment	0.029 (0.002- 0.101)	0.016 (0.001- 0.075)	0.012 (0.005- 0.052)
Grid floors	0.025 (0.002- 0.0740)	0.038 (0.04- 0.076)	0.03 (0.002- 0.056 ¤ ¤
Single housing	0.031 (0.002- 0.111)	0.036 (0.001- 0.065)	0.043 (0.015- 0.088) ### ¤ ¤ ¤ +
Tibio-tarsal joint			**
Control	0.010 (0.0- 0.06)	0.023 (0.0- 0.058)	0.016 (0.0- 0.074)
Enrichment	0.025 (0.00- 0.098)	0.008 (0.001- 0.037)	0.016 (0.002- 0.049)
Grid floors	0.018 (0.005- 0.037)	0.026 (0.002- 0.064)	0.035 (0.008- 0.064) # ¤ ¤
Single housing	0.030 (0.004- 0.075)	0.022 (0.002- 0.051)	0.023 (0.003- 0.073) ¤ ¤
Hind paw			
Control	0.051 (0.0- 0.127)	0.041 (0.0- 0.123)	0.021 (0.0- 0.076)
Enrichment	0.039 (0.0- 0.099)	0.024 (0.0- 0.064)	0.025 (0.0- 0.099)
Grid floors	0.049 (0.012- 0.114)	0.034 (0.0- 0.145)	0.043 (0.003- 0.079)
Single housing	0.031 (0.0- 0.12)	0.054 (0.0- 0.11)	0.037 (0.001- 0.104)
Combined traits		**	***
Control	0.0430 (0.004- 0.074)	0.029 (0.009- 0.065)	0.0230 (0.012 – 0.043)
Enrichment	0.037 (0.013- 0.063)	0.021 (0.008- 0.055)	0.0190 (0.007 – 0.041)
Grid floors	0.037 (0.008- 0.063)	0.036 (0.013- 0.080) ¤ ¤	0.0340 (0.015 – 0.053) ## ¤ ¤ ¤
Single housing	0.028 (0.015- 0.074)	0.035 (0.021- 0.07) ¤ ¤ ¤	0.0385 (0.021 – 0.071) ### ¤ ¤ ¤

FA of the combined traits was relatively high in all rats at five weeks of age. In control and enrichment groups, FA of the combined traits decreased throughout the study ($P<0.05$ in control group and $P<0.001$ in enrichment group from five to eleven weeks of age). FA in singly housed rats and rats on grid floors did not change significantly throughout the study.

FA of the combined traits in relation to dominance status at different ages is shown in Figure 1. The hierarchic structure among the rats changed during the study, but the actual status was used for comparison at each age. Dominant rats housed in an enriched environment were found to have a higher

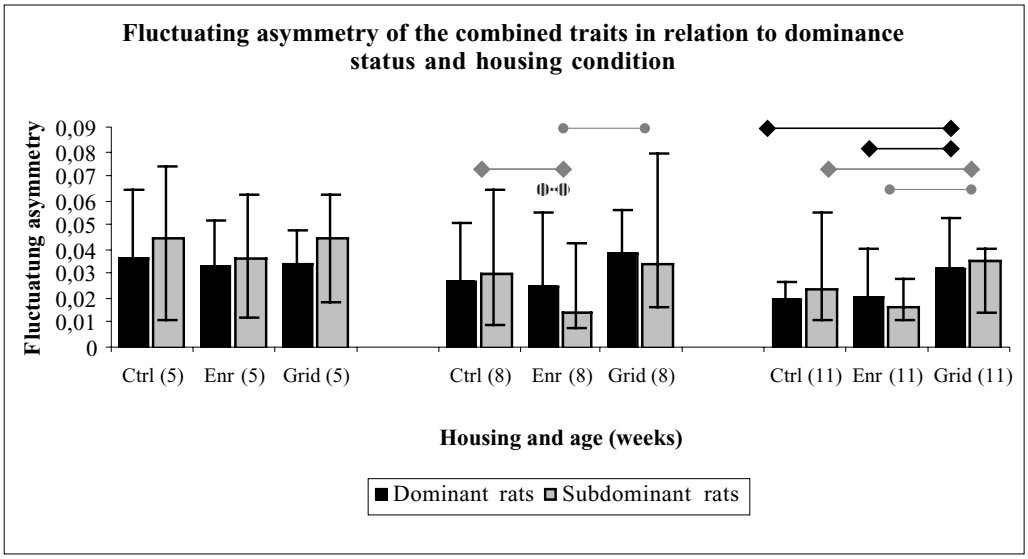

Figure 1: Fluctuating asymmetry of the combined traits within each housing conditions (control (ctrl), enrichment (enr) and grid floor (grid) in relation to dominance status and age (5, 8 and 11 weeks of age) in Lew/Mol rats in three groups of 18 rats (single housed rats not included). Significant difference between dominant and subordinate males within housing condition: Dotted bar, $p < 0.01$
Black bar with squares: Comparison of housing condition in dominant males; $p<0.05$
Grey bar with squares: Comparison of housing condition in subordinate males; $p<0.05$
Grey bar with circles: Comparison of housing condition in subordinate males; $p<0.01$

FA at eight weeks of age compared to their subordinate cage mates ($P<0.01$). Differences within the status groups are shown in Figure 1. At 8 weeks of age, the subordinate rats of the control and grid floor group have significantly higher relative FA values than the enriched rats. At 11 weeks of age, the grid floor housed subordinate rats have significantly higher relative FA values than the control and enriched counterparts. Of the dominant rats, at 11 weeks of age a similar significantly higher relative FA value in grid-housed rats as compared to control rats and enriched rats was found.

Singly housed rats were obviously not tested for social status; however, since their physical housing condition were similar to that of the control group, FA in single housed rats were compared to that of pair housed rats in the control group. Single housed rats showed a higher FA than dominant as well as subordinate rats of the control group ($P<0.001$) at eleven weeks of age.

Discussion

This study clearly indicates that environmental stress, such as the stress caused by non-ideal housing, may have an impact on FA, as rats housed on grid floor or singly were, in contrast to control or enriched rats, unable to decrease the initial high relative FA of young animals over time. The effect on relative FA was seen most strongly in the combined traits (Table 1), although the same trend was found in all individually measured leg traits as well (Table 1).

Dominance status itself had little influence on FA, but although it should not be over-interpreted it is worth noting that FA in enriched rats differed significantly in relation to their dominance status at 8

weeks of age (Figure 1). Male rats mature sexually at 7-9 weeks of age, and it is possible that establishing a hierarchy is more stressful for the dominant male, establishing and defending their position, than to the subordinate males. The resulting FA is only shown under enriched conditions, which could be due to the presence of attractive enrichment objects, further increasing the competition among the males. These findings should be studied further in order to shed light on the discussion, whether enrichment may increase variation. At 11 weeks of age, animals housed on grid floors show a higher level of FA, but at this age, no effect of social status is shown in relation to FA. According to the present study FA seems to be usable for stress measurements independent of dominance status.

The important feature of this study is, therefore, the indication that FA may provide a valuable parameter within research in laboratory animal welfare. It is not likely that FA may become a common tool for diagnosing stress in the individual animal in daily clinical work, *e.g.* for the animal welfare officer, but we have previously shown that it may be used as a research tool for showing the impact of social environment, spontaneous disease and induced disease (*Stub et al., 2004c,b,a*). In inbred BN rats, which have an extremely high prevalence of hematuria caused by local hyperplasia on the renal pelvic epithelium, FA has been used to show that the rats are affected clinically by the condition, i.e. it may indicate a reduced well-being in the individual rat (*Stub et al., 2004b*). With the common application of transgenesis as a means of inducing genetic disease models, tools for assessing the welfare impact of these diseases on the animals have become increasingly important. For example, FA has been applied to show the impact of cystic fibrosis induced by a combination of microinjection and homologous recombination in mice (*Stub et al., 2004a*). The present study shows that FA may also be used for assessing the influence of environmental factors, such as housing conditions, on the animals. The application of FA monitoring in relation to grid floors and single housing confirms previous observations on these well known stressors by other methods (*Krohn et al., 2003; Heidbreder et al., 2000, McCormick et al., 1998*) It is, however, important to emphasize that judgments within animal welfare can never be based upon just one parameter, and, therefore, FA should be considered as only one tool along with other physiological as well as ethological tests. This point is emphasized by the fact that no differences were found in FA between rats housed in an enriched environment and rats housed on bedding alone. On the other hand, the method did find differences in FA relating to social status in a resource-rich environment.

In conclusion, FA of selected traits seems to hold some potential as a future research tool for assessing stress and identifying stressors in laboratory rats, which in combination with a range of ethological and physiological tests may reveal useful information on the impact of the experiments and the environment on the animals.

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