Pacing behaviour in laboratory macaques is an unreliable indicator of acute stress.

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Pacing behaviour, the most frequent stereotypic behaviour displayed by laboratory rhesus macaques (*Macaca mulatta*) is often used as an indicator of acute stress. In this study, we investigated how reliable this welfare indicator is by testing the reaction of macaques to the stressful event of being exposed to a fight between conspecifics housed in the same room but in a different cage. Pacing, agitated locomotion, and stress-related displacement behaviours were quantified before, during and after fight exposure, based on video recordings of 13 socially-housed macaques in their home cage. Displacement behaviours increased after fight exposure, confirming that the events were experienced as stressful by the focal individuals. The occurrence of pacing did not increase during or after the fights. Instead, agitated locomotion increased during the fights. These results suggest either, that pacing as an indicator of acute stress is prone to false negative results, increasing in some stressful situations but not others, or that agitated locomotion has been mistaken for pacing in previous studies and that pacing is in fact unrelated to current acute stress. Both interpretations lead to the conclusion that pacing is unreliable as an indicator of acute stress in laboratory rhesus macaques.

39 Of the laboratory animal species commonly used in biomedical research, non-human primates (NHPs)
40 are phylogenetically closest to humans. This proximity makes NHPs a crucial animal model in
41 biomedical science, but also makes their welfare of particular concern to the general public. Current
42 regulations reflect this public concern[1,2], and require that researchers ensure the best possible welfare
43 for the animals that they use. To achieve this goal, reliable welfare indicators are necessary.
44 Stereotypies, which are repetitive, unvarying and apparently functionless behaviours, are often used as
45 indicators of acute or chronic stress[3]. In rhesus macaques, one of the main NHP models used in
46 research, pacing is the most common stereotypic behaviour observed in the laboratory[4]. In this study
47 we sought to establish specifically whether pacing is a reliable indicator of acute stress in this species.

48 Pacing in rhesus macaques (hereafter macaques) is defined as repetitive walking of an
49 individual in the exact same pattern[4,5]. Previous studies have reported that macaques increase their
50 pacing during or immediately after short periods of acute stress (e.g. placing in a transport box for few
51 seconds, exposure to a 10-minute cue of impending social isolation)[6,7]. Injecting macaques with a high
52 dose of the anxiogenic drug FG7142 also increases pacing[8]. An increase in pacing frequency is thus
53 usually interpreted as an increase in acute stress[9,10], where the term 'stress' is used to refer to an
54 unpleasant, arousing affective state.

55 However, in a recent study where macaques were submitted to the human intruder test, a
56 classical test to induce acute stress, pacing frequency was found to decrease during the stressful
57 event[11]. This result suggests that pacing can increase or decrease with acute stress, depending on the
58 stressful situation. This finding is important from a welfare perspective, because if the frequency of a
59 behaviour can increase in some stressful situations and decrease in others, variation in the behaviour
60 in a new situation (whose stressful nature is unknown) cannot be interpreted unequivocally; in other
61 words, the behaviour is unreliable as an indicator of acute stress.

62 In the current study we sought to determine whether the behavioural response to the human
63 intruder test is an exceptional case, or whether other types of stressors also induce a decrease in
64 pacing. To do this, we needed to identify an alternative source of acute stress that occurs relatively
65 frequently in laboratory macaques and hence had the potential to explain the prevalence of pacing in
66 primate research facilities. We chose to investigate the effect of passive exposure to fights between

conspecifics on the assumption that this is stressful. Agonistic interactions in macaques are used to challenge or reassert the social position of protagonists and can induce severe injuries. In free-ranging macaque colonies, agonistic support (where one or several individuals help one of the protagonists) is frequent[12]. Hence, witnessing a fight implies potential imminent involvement in a stressful situation, which is likely to be stressful in itself. Exposure to fights has ecological relevance, since it occurs in research colonies where individuals are housed in pairs or small social groups, which is now the norm in European primate research facilities and an emerging trend in North America. Guided by ethical considerations, we made use of fights that occurred spontaneously in our research colony. We measured the occurrence of pacing before and during passive exposure to fights. In the recent intruder test study[11], the authors did not include behavioural observation of macaques after exposure to the acute stressor had ceased. It is thus possible that an increase of pacing frequency happened subsequently and was not recorded. To control for the possibility that the expected increase in pacing could be delayed, we also measured the occurrence of pacing after exposure to the fights.

Results

Fights

Video footage of home-cage behaviour of 13 focal individuals previously recorded for another purpose was used for this project. Fights were defined as any period lasting at least 10 s during which threatening/alarm vocalisations associated with loud object banging noises were heard. This identification was made based on the soundtrack of the video recordings with the video images concealed in order to avoid any selection bias caused by the behaviour of the focal individuals. Fourteen fights were identified. Analyses of video images confirmed that in all but one case the focal individuals were not involved in these fights. Data related to that fight were discarded. Video recordings of each focal individual were not always available for each fight. The number of fights for which video existed varied between 4 and 12 fights per individual, resulting in a total of 108 behavioural responses to fights in which the focal individual was not involved.

Pacing

Behaviour of focal individuals was scored in three time intervals relative to the fight: during each fight (the time interval designated as [0]), during the 15 min preceding the fight (designated [-15 – 0]) and during the 15 min following the end of the fight (designated [0 – 15]). The three time intervals were divided into 10-s time bins and the presence (1) or absence (0) of pacing within each time bin was recorded (for the operational definition of pacing, see table 1). Pacing behaviour was displayed by 7 out of 13 individuals. Among pacing individuals, occurrence of pacing varied from 0.7 to 46.7 % (percentage of 10-s time bins in which pacing occurred).

Generalised linear mixed models of pacing data revealed a significant effect of time interval relative to fight (Fig. 1; Table 1, model 1 vs. model 0, χ^2 (2) = 11.2, P = 0.004), driven by a decrease in occurrence of pacing after the fights, compared to before (Table 2, model 1: β (s.e.) = -0.24 (0.07), Z = -3.4, P < 0.001). There was no difference in occurrence of pacing before and during the fights (Table 2, model 1: β (s.e.) < 0.01 (0.34), Z = 0.005, P > 0.99).

Exposure to a stressful event induces an immediate increase in catecholamines and a slower increase in cortisol, associated with behavioural responses occurring at different time scales[13–15]. Our previous analyses focused on testing the hypothesis that the fast released cathecholamines would induce an increase of pacing. However, if an increase of pacing is triggered by cortisol, one would expect it to take place 20 to 30 minutes after the stressful event, when cortisol is at its peak. To take into account this possibility, we subsequently analysed additional videos of pacing individuals corresponding to the time interval [15 – 40] min post-fight. The analysis of the dataset including the four time intervals ([-15 – 0], [0], [0 – 15] and [15 – 40]) revealed a significant effect of time interval (Table 2, model 2 vs. model 0, χ^2 (3) = 93.9, P < 0.001) due to a decrease in occurrence of pacing during the two time intervals after the fights, compared to before (Table 2, model 2: [0 – 15] vs. [-15 – 0]: β (s.e.) = -0.23 (0.07), Z = -3.3, P < 0.001; [15 – 40] vs. [-15 – 0]: β (s.e.) = -0.61 (0.07), Z = -9.5, P < 0.001).

Finally, we tested the possibility that the decrease in pacing might be due to an effect of absolute time of the day, because we have unpublished data suggesting that occurrence of pacing follows a circadian rhythm. Despite the fact that our analyses were restricted to a narrow time window of 55 min, circadian changes might still potentially confound the effect of time intervals relative to

fight. Including time of day as an additional fixed effect significantly improved model fit (Table 2, model 3 vs. model 2, χ^2 (1) = 5.9, P = 0.02). Occurrence of pacing was found to decrease with time of day (Table 2, model 3: β (s.e.) = -14.84 (6.04), Z = -2.46, P = 0.01) and the effect of time interval relative to fight in the new model was no longer significant (Table 2, model 4 vs. model 3: χ^2 (3) = 2.9, P = 0.41).

Agitated locomotion

In addition to pacing behaviour, we also assessed the frequency of agitated locomotion, a behaviour defined as 'moving rapidly between locations, often via jumps, with a stiff un-relaxed gait' (Table 1). In our macaque facility where individuals are housed in large and enriched cages, this behaviour differs from pacing not only in the gait of the subject (which is stiff in agitated locomotion and elastic in pacing) but also in the flexibility of the path used within and between occurrences, making the two behaviours easy to distinguish (the inter-observer reliability measured with Kappa scores were of 1 for pacing and 0.97 for agitated locomotion in the present study). However, it is possible that when macaques are housed in small, barren cages, the two behaviours could be conflated. We thus hypothesised *a priori* that agitated locomotion might be the behaviour increasing during or after exposure to an acute stressor.

Presence (1) or absence (0) of agitated locomotion within each time bin was recorded and was displayed by 11 out of 13 individuals, including all pacers (N = 7) and 4 non-pacers. The occurrence of agitated locomotion among individuals that displayed the behaviour at least once varied from 0.03 to 1.1 %. Occurrence of agitated locomotion displayed by each individual and occurrence of pacing were not correlated (r = 0.002, t (11) < 0.01, P > 0.99).

Generalised linear mixed models of the occurrence of agitated locomotion revealed a significant effect of time interval relative to fight (Table 2, model 6 vs. model 5: χ^2 (3) = 149, P < 0.001), driven by an increase in agitated locomotion during fights, compared to before (Table 2, model 6: [0] vs. [-15 – 0]: β (s.e.) = 4.1 (0.28), Z = 14.4, P < 0.001). There was no difference in occurrence of agitated locomotion before and after the fights ([0 – 15] vs. [-15 – 0]: β (s.e.) = -0.48 (0.28), Z = -1.69, P = 0.09; [15 – 40] vs. [-15 – 0]: β (s.e.) = 0.12 (0.24), Z = 0.48, P = 0.63). Generalised linear mixed

models applied separately to pacers and non-pacers revealed similar effects of time interval relative to fight in both groups (Fig. 2, pacers: χ^2 (3) = 120, $P < 0.001$, [0] vs. [-15 – 0]: β (s.e.) = 4.28 (0.344), Z = 12.7, $P < 0.001$; non-pacers: χ^2 (3) = 34, $P < 0.001$, [0] vs. [-15 – 0]: β (s.e.) = 3.57 (0.60), Z= 5.9, $P < 0.001$). Adding the absolute time of day as an additional fixed effect did not improve model fit (Table 2, model 7 vs. model 6: χ^2 (1) = 0.5, P = 0.49).

Displacement behaviours

To verify that fight exposure was experienced as stressful by the macaques, we recorded and analysed the occurrence of displacement behaviours[16] displayed by macaques, namely scratching, body shaking and self-grooming (Table 1). These behaviours were chosen based on their pharmacological validation as indicators of stress or anxiety by Schino and colleagues[17]. Following these authors approach, scratching, body shaking and self-grooming were combined to create a single category of 'displacement behaviours'. Presence of at least one of the behaviours (1) or absence of all of them (0) within each time bin was recorded.

Displacement behaviours were displayed by all individuals, with occurrence varying between 5.1 and 26.9 %. Generalised linear mixed models of occurrence of displacement behaviours revealed an effect of time interval relative to fight (Table 2, model 9 vs. model 8: χ^2 (3) = 43.0, $P < 0.001$), driven by an increase in displacement behaviour frequency in the second time interval after the fight, compared to before ([15 – 40] vs. [-15 – 0]: β (s.e.) = 0.34 (0.06), Z= 6.0, $P < 0.001$). Adding the absolute time of the day as an additional fixed effect did not improve model fit (Table 2, model 10 vs. model 9: χ^2 (1) = 1.5, P = 0.2).

Discussion

This study investigated the behavioural reaction of laboratory rhesus macaques to fights occurring between conspecifics housed in separate, but nearby cages within the same room. Displacement behaviours, which are pharmacologically validated indicators of stress or anxiety, increased after the fights, confirming that fights were perceived as stressful by the macaques. Our study reveals that stereotypic pacing did not increase during or after the fights. Peterson and colleagues previously

showed that pacing did not increase during an intruder test, a paradigm established to induce stress in macaques[11]. Here we extend this result by testing a different stressful situation (passive exposure to a fight between conspecifics) and by providing a more comprehensive assessment of pacing by testing its frequency not only during the stressful event but also during a sustained period of time (up to 40 min) after the stressful event. These data contrast with previous studies where pacing was found to increase with acute stress[6,7]. These conflicting results can be interpreted in two different ways that we describe in the next paragraphs.

The first possible explanation for the discrepancy in results from different studies is that pacing frequency increases in some stressful situations but not in others. If this is the case, it means that pacing when used as an indicator of acute stress is prone to false negative results, where macaques experience acute stress but do not express it by an increase in pacing (Fig. 3). From a welfare perspective, this possibility is problematic for two reasons. First, using pacing as an indicator of acute stress might lead stressful husbandry or experimental procedures to fail to be identified as such, preventing researchers, veterinarians and/or technical staff from refining them and thus improving animal welfare. Second, false-negative results also undermine the use of an absence of pacing frequency increase during or after a procedure as a reliable indicator that the procedure is not stressful. This is problematic because determining which husbandry and scientific procedures are not experienced as stressful is as important as identifying those which are stressful. It should also be noted that whether pacing increases after positively-valenced (i.e. pleasant) arousing events has never been investigated. The potential of pacing to generate false positive results (Fig. 3) when used as an indicator of acute stress is thus currently unknown. As a consequence, when pacing frequency is found to be increased by an event or procedure, one cannot conclude with confidence that this event or procedure induced acute stress rather than a pleasant arousing emotion. Because optimising the welfare of animals means reducing negative emotions but also improving positive ones, attempts to reduce pacing frequency could be counter-productive if increased pacing has been caused by positively-valenced arousal.

Alternatively, the fact that pacing seems to increase during some stressful situations[6,7], but not others (ref 11, our study), might be due to the possible conflation between pacing and agitated

207 locomotion in previous studies. Indeed, our study revealed an increase in agitated locomotion during
208 fights. Differences between pacing and agitated locomotion are summarised in Table 1. One notable
209 difference is that agitated locomotion is not stereotypic, the path used by an individual varying from
210 one occurrence to another. In our research facility where macaques are housed in big cages (15 m^3)
211 with shelves and ropes, the two behaviours are easy to distinguish. However, it is possible that in
212 small cages agitated locomotion appears stereotypic due to the lack of options in the paths individuals
213 can walk, making it visually indistinguishable from true pacing. Interestingly, studies reporting an
214 increase in pacing frequency induced by acute stress, including the anxiogenic drug FG7142[6–8], all
215 come from macaques housed in very small cages (below 1.3 m^3). It is thus possible that existing
216 literature linking acute stress and pacing should be reinterpreted as showing a link between acute
217 stress and agitated locomotion. If this is the case, pacing would never be related to present acute
218 stress.

219 In some individuals, occurrence of pacing was found to be high before, during and after the fights
220 (fig. 1B). Source(s) of acute stress during the whole period could not be identified. This result
221 indicates that pacing is likely to be caused by factor(s) other than current acute stress. We have
222 recently reviewed these potential factors[18]. Our review suggests that pacing might be the consequence
223 of brain abnormalities induced by a past stressful event, and be unrelated to the current state of the
224 individual. Alternatively, pacing might be caused by chronic stress, boredom or a specific need to
225 walk. These different hypotheses have different welfare consequences with the welfare of pacing
226 macaques being either better, worse or equivalent to that of non-pacing individuals[18]. As long as the
227 cause(s) of pacing remain unresolved, we argue that pacing should not be used as an indicator of acute
228 or chronic stress, especially in the absence of other indicators of poor welfare.

229 Our results raise the possibility that agitated locomotion could be a useful indicator of acute
230 stress. In sharp contrast with pacing behaviour, agitated locomotion increased during the fights but
231 was virtually non-existent outside periods of fights, when no source of acute stress could be detected.
232 These results however need to be replicated with other stressful situations. If agitated locomotion is
233 systematically observed during different stressful situations (i.e. if no/few false negative results are
234 observed), this behaviour might be a convenient welfare marker since it is easy to identify (at least in

large, enriched cages) and its baseline frequency near to zero makes any increase easy to detect. Whether agitated locomotion also increases during positively-valenced arousing events (susceptibility to false positive results) will also need to be determined.

To our knowledge, the precise time scale of behavioural indicators of acute stress in macaques has never been systematically investigated. From an applied perspective, this information might help in designing better behavioural observation protocols. At the physiological level, acute stress induces a well-established cascade of events, including the release of different hormones over different time scales. In the present study, we used this knowledge to guide our observation periods. Agitated locomotion increased during the very brief period of fights, suggesting that this behaviour might be triggered by the fast-released catecholamines. By contrast, displacement behaviours increase was observed during the 15 - 40 minute interval after the stressor, suggesting that these behaviours might be induced by the slowly-released cortisol. Displacement behaviours, however, correspond to a combination of different behaviours (scratching, self-grooming and body shaking). Further studies will need to determine whether the exact timing of these three behaviours is similar or not.

To conclude, by showing that pacing does not increase following exposure to conspecific fights this study shows that pacing behaviour is not a reliable indicator of acute stress. Our data also suggest that agitated locomotion might potentially be a useful indicator of acute stress, or at least arousal, but more studies are needed to confirm this. Welfare research on captive animals is often done using experimental paradigms that involve manipulating the affective state of the animals by imposing a stressor[19,20]. While this approach is undoubtedly the most powerful, the current study illustrates how new knowledge can also be gained by using observations of animals without inducing additional stress.

Materials and methods

Subjects and ethical statement

Subjects were 13 male rhesus macaques (*Macaca mulatta*) aged between 4 and 10 years (weight range: 5 to 18 kg). Individuals had been raised in a British breeding centre, first with their mother for

262 at least 6 months and then with other juveniles in large rooms. When adolescent, subjects were moved
263 to the Newcastle University research facility, which complies with the NC3Rs Guidelines for
264 'Primate Accommodation, care and use' (www.nc3rs.org.uk/primatesguidelines). There, they were
265 housed in small social groups (2 to 3 individuals) in large cages (2.1 x 3.0 x 2.4 m) exceeding the
266 minimal space requirement under the UK legislation (1.8 m^3 per animal). The height of the cages and
267 the presence of high perches and shelves allowed macaques, if alarmed, to flee upwards. Besides the
268 presence of at least one cage mate, enrichment was provided by daily foraging opportunity (the food
269 was scattered in the litter on the floor), wooden shelves, swings, ropes, objects to manipulate (changed
270 on a regular basis) and natural light. Cages were located in a large room housing more than 40
271 individuals. Focal individuals were thus in visual and auditory contact with many other conspecifics
272 in addition to their cage mates. Some of the individuals were routinely involved in neuroscience
273 experiments while others had not yet been subjected to any experimental procedures. This study
274 consisted of behavioural observation of macaque home cage behaviour recorded via remotely-
275 controlled cameras, inducing no interference with the animals. Females were not included in this
276 study for the sole reason that video recordings of these individuals were not available in sufficient
277 quantity. The stressor consisted of passive exposure to fights occurring naturally between other
278 animals; the frequency of fights was not manipulated for this study. No licence was required for this
279 study.
280
281 *Behavioural observation*
282 Videos were recorded with remotely-controlled cameras (Cube HD 1080, Y-cam Solutions Limited,
283 Twickenham, UK). Recordings from between 1600 and 1800 on Saturdays and Sundays between
284 January and December 2015 were analysed. This period of the week was chosen as it was the only
285 period when macaques were in their home cage, awake and no people were present in the facility.
286 This strategy avoided the necessity of having to control for the potentially stressful effects of human
287 presence and cage mate absence.
288
289 *Behavioural scoring and analysis*

Statistical analyses were performed in R, version 3.3.2[21] using the *lme4* package[22]. Data were analysed using generalised linear mixed models (*glmer* function) with binomial error distribution and logistic link function. The *time interval relative to fight* was declared as a fixed effect (categorical effect with 3 or 4 levels), while *subjects* and *fights* were declared as random effects, with *fights* being nested within *subjects*. Maximum-likelihood estimation was employed throughout. Note that these models cope with highly unbalanced designs such as this one (the amount of data in the [0] interval being much less than in the other time intervals) and non-independence due to repeated measures of subjects and fights. Significance testing was carried out by the likelihood ratio test, which compares the change in deviance when a term is excluded from the model with the χ^2 distribution.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare no competing interests.

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Figure captions

Figure 1: Occurrence of pacing as a function of the time interval relative to fight. Occurrence of pacing corresponds to the proportion of 10-s time bins when the behaviour was displayed (with 1 corresponding to 100 %). The dotted line indicates the ‘during fight’ interval. a: Data averaged over fights and subjects. b: Data averaged over fights. c: Data per fight and per subject. Note that points

appear darker when data from several fights are superimposed. The n value indicated in the corner of each individual plot corresponds to the number of fights. For the legend of the x axis, see plots a and b.

Figure 2: Occurrence of agitated locomotion as a function of the time interval relative to fight in pacers (a-c) and non-pacers (d-f). Occurrence of agitated locomotion corresponds to the proportion of time bins when the behaviour was displayed (with 1 corresponding to 100 %). The dotted line indicates the 'during fight' interval. a/d: Data averaged over fights and subjects. b/e: Data averaged over fights. c/f: Data per fight and per subject. Note that points appear darker when data from several fights are superimposed. The n value indicated on each plot corresponds to the number of fights. For the legend of the x axis, see plots a/d and b/e.

Figure 3: Different possible relationships between pacing frequency and acute stress.

Table 1: Ethogram

Behaviour	Description
Pacing	Repetitive walking of the same path (at least twice)
Agitated Locomotion	Moving fast between locations with a stiff un-relaxed gait
Self-grooming	Stroking, picking, or otherwise manipulating own body surface
Body shaking	Dog-like shake of whole body
Self-scratching	Scratching the skin vigorously with nails

Table 2: Generalised linear mixed models

Dependent variable	Model	Fixed predictors	Degrees of freedom
Pacing	0	none	3
	1	Time interval relative to fights (3 levels)	5
	2	Time interval relative to fights (4 levels)	6
	3	Time interval relative to fights (4 levels); Time of day	7

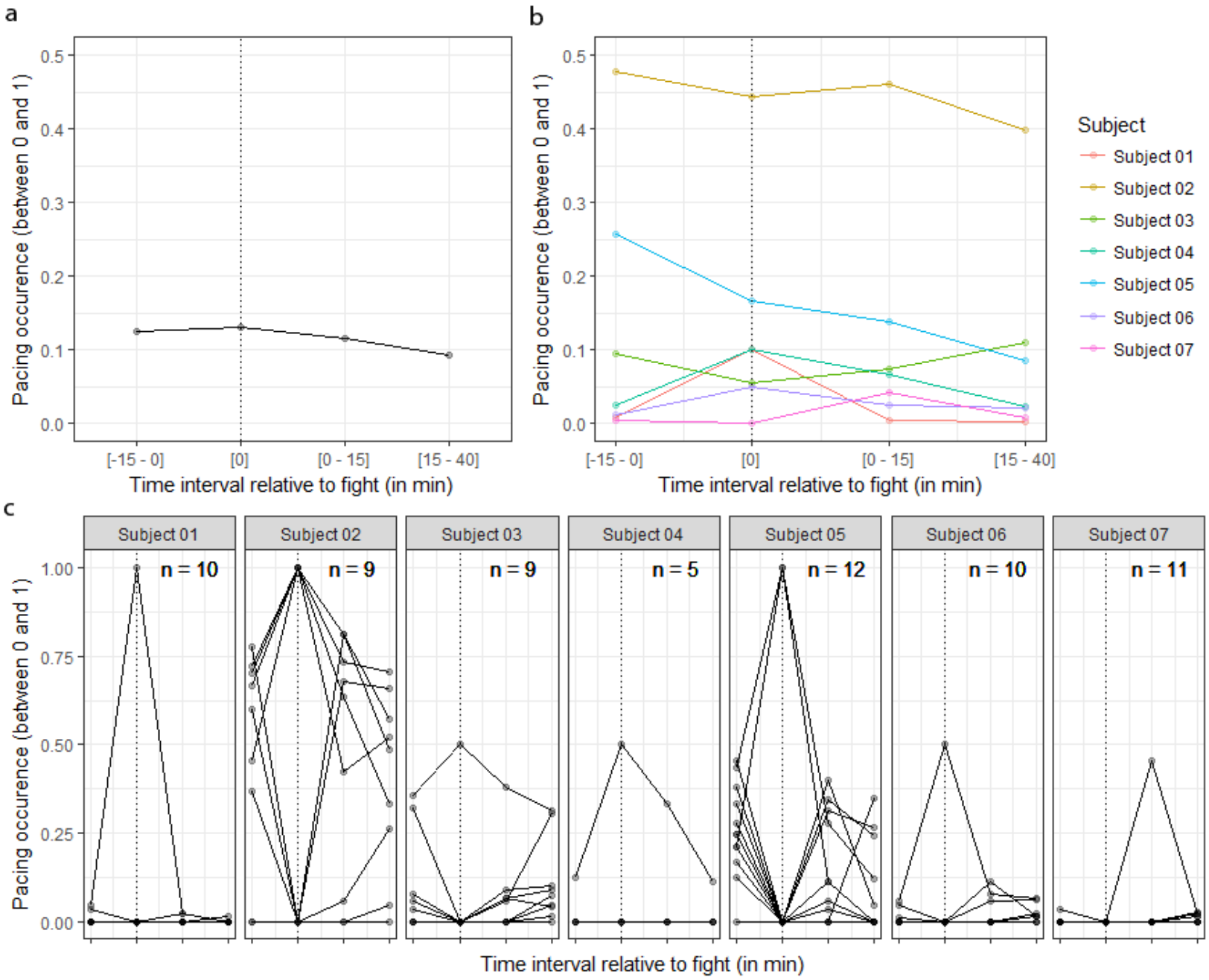
	4	Time of day	4
Agitated locomotion	5	none	3
	6	Time interval relative to fights (4 levels)	6
	7	Time interval relative to fights (4 levels); Time of day	7
Displacement behaviours	8	none	3
	9	Time interval relative to fights (4 levels)	6
	10	Time interval relative to fights (4 levels); Time of day	7

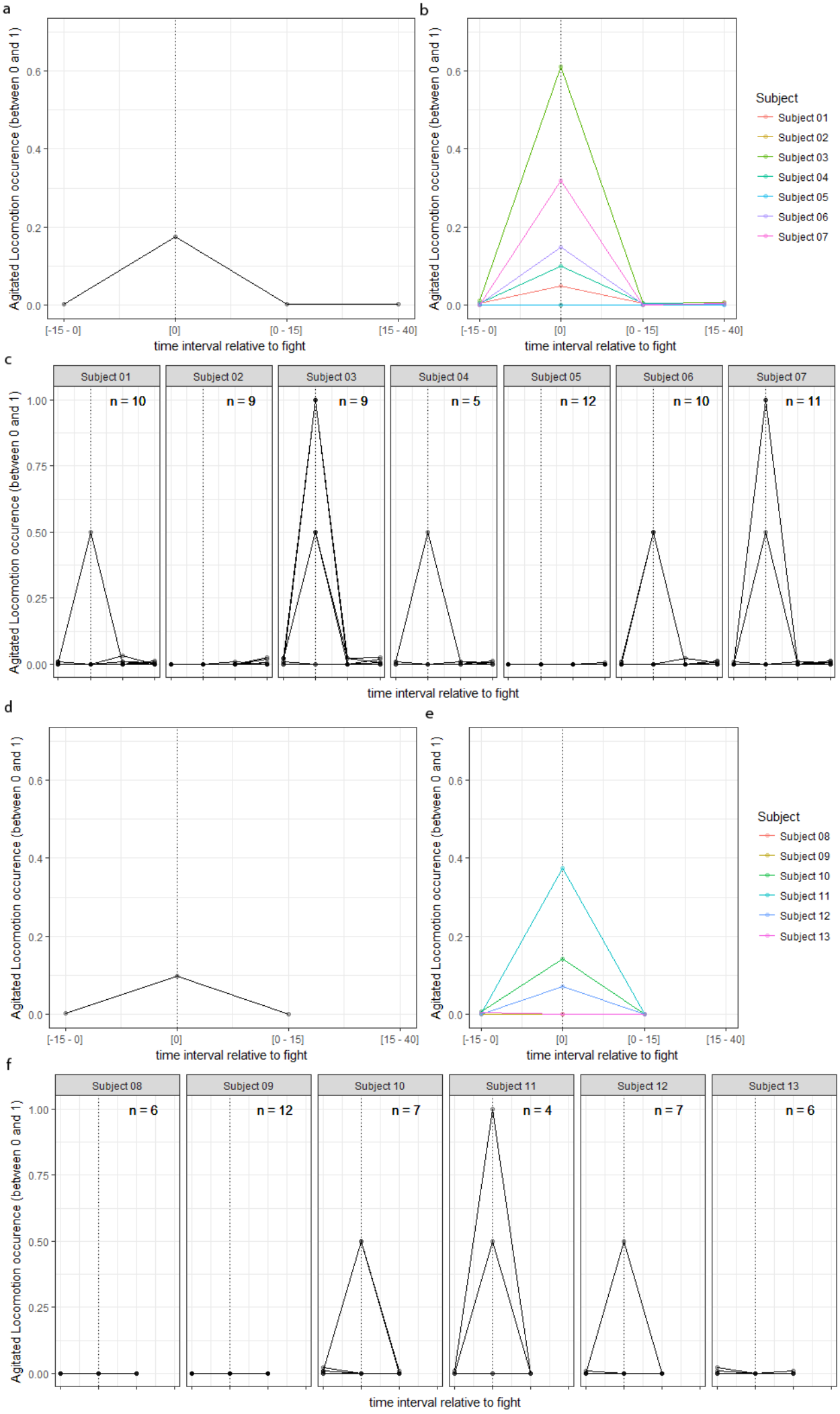
All models include random effects for focal subject and fight to control for non-independence arising from repeated measures on subjects and fights.

Table 3: Differences between Pacing and Agitated Locomotion

	Pacing	**Agitated locomotion**
Form	Elastic gait	Inelastic gait
Flexibility	Invariant path	Variable path
Prevalence[1]	About half of individuals (7/13)	Almost all individuals (11/13)
Frequency[1]	Frequent (up to 46.7%)	Rare (up to 1.1 %)
Duration[1]	Long (up to 10 min)	Short (≤ 20 s)
Context[1]	Before, during and after fights	Almost exclusively during fights

[1] in this study


a
b
c
Pacing occurence (between 0 and 1)
Time interval relative to fight (in min)
[-15 - 0]
[0]
[0 - 15]
[15 - 40]
Subject
Subject 01
Subject 02
Subject 03
Subject 04
Subject 05
Subject 06
Subject 07
n = 10
n = 9
n = 9
n = 5
n = 12
n = 10
n = 11


a
b
c
d
e
f
Agitated Locomotion occurence (between 0 and 1)
time interval relative to fight
[-15 - 0]
[0]
[0 - 15]
[15 - 40]
Subject
Subject 01
Subject 02
Subject 03
Subject 04
Subject 05
Subject 06
Subject 07
Subject 08
Subject 09
Subject 10
Subject 11
Subject 12
Subject 13
n = 10
n = 9
n = 9
n = 5
n = 12
n = 10
n = 11
n = 6
n = 12
n = 7
n = 4
n = 7
n = 6

		Induction of acute stress	
		Yes	No
Increase in pacing frequency	Yes	True positives	False positives
	No	False negatives	True negatives