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RESEARCH ARTICLE

A genetic reduction in antioxidant function causes elevated aggression in mice

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ABSTRACT

Male–male aggression can have a large influence on access to mates, particularly in highly territorial animals such as mice. It has been suggested that males with impaired antioxidant defence and a consequential increased susceptibility to oxidative stress may have a reduced ability to invest in aggressive behaviours, which could limit their mating opportunities and reproductive success. Oxidative stress occurs as a result of an uncontrolled over-production of reactive oxygen species (ROS) in relation to defence mechanisms (such as antioxidants), and can cause damage to a variety of different cellular components. Impairments in specific aspects of antioxidant defence, leading to oxidative stress, can limit investment in some reproductive traits in males, such as sperm quality and the production of sexual signals to attract mates. However, a direct effect of impaired antioxidant defence on aggressive behaviour has not, to our knowledge, been reported. In this study, we demonstrate that mice with experimentally elevated sensitivity to oxidative stress (through inhibition of copper–zinc superoxide dismutase, *Sod1*) actually show the opposite response to previous predictions. Males completely deficient in SOD1 are more aggressive than both wild-type males and males that express 50% of this antioxidant enzyme. They are also faster to attack another male. The cause of this increased aggression is unknown, but this result highlights that aggressive behaviour in mice is not highly constrained by inhibited *Sod1* expression, in contrast to other reproductive traits known to be impaired in this mouse model.

KEY WORDS: Life history, Fitness, Dominance, Mouse

INTRODUCTION

An organism's ability to survive and reproduce is governed by a range of environmental and physiological conditions (McNamara and Houston, 1996). One potentially important physiological factor is oxidative stress (Bize et al., 2008; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009), a process that can cause damage to proteins, lipids and DNA as a result of uncontrolled production of reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999). ROS are produced through normal metabolic processes, and organisms have a variety of defence systems, such as various antioxidants, that protect against ROS-induced damage (Valko et al., 2007). However, under certain conditions, defences can be overwhelmed and oxidative damage can result (Halliwell and Whiteman, 2004). Oxidative stress may play a major role in the ageing process (Beckman and Ames, 1998; Harman, 1956), can contribute to the onset of degenerative diseases (Dröge, 2002) and may limit investment in life history traits (Monaghan et al., 2009).

Oxidative stress impairs some aspects of male reproduction, particularly sperm quality (Agarwal et al., 2003; Saleh and Agarwal, 2002; Sikka et al., 1995) and, in some species, investment in aspects of sexual signalling used to attract mates (Garratt and Brooks, 2012; Metcalfe and Alonso-Alvarez, 2010). It has also been suggested that oxidative stress may limit a male's ability to invest fully in aggressive behaviours, and thus attain dominance and gain access to mates (Garratt and Brooks, 2012; Metcalfe and Alonso-Alvarez, 2010). Because engaging in aggressive behaviours can elevate metabolic rate (Briffa and Sneddon, 2007; deCarvalho et al., 2004; Haller, 1995; Smith and Taylor, 1993), Metcalfe and Alonso-Alvarez (Metcalfe and Alonso-Alvarez, 2010) predicted that males with good antioxidant defences may be able to sustain the highest levels of aggression. Garratt and Brooks (Garratt and Brooks, 2012) offered similar suggestions, and further highlighted that oxidative stress can impair bioenergetic function, which may reduce energy allocation to metabolically demanding reproductive behaviours.

In this study, we manipulated antioxidant defence through molecular genetic techniques, to provide the first direct test of whether susceptibility to oxidative stress influences an individual male's ability/willingness to invest in aggressive behaviours, and attain dominance over another male. We used a genetically modified strain of mouse that does not express copper–zinc superoxide dismutase (*Sod1*), an antioxidant that has an important *in vivo* role in protecting against oxidative stress. We examined aggressive behaviours in homozygous fully deficient *Sod1* males ($Sod1^{-/-}$), heterozygous deficient *Sod1* males ($Sod1^{+/-}$; with 50% SOD1 enzymatic activity) and wild-type ($Sod1^{+/+}$) males. $Sod1^{-/-}$ males have been most frequently studied. When housed in standard laboratory conditions they show an elevation in oxidative damage in various tissues throughout adult life (Elchuri et al., 2005; Muller et al., 2006) and have a shorter lifespan (Elchuri et al., 2005). They have reduced sperm motility (Garratt et al., 2013; Tsunoda et al., 2012) and are subfertile when mated with at least one different strain of female (Garratt et al., 2013). Males of this strain are also less able to invest in some molecular and morphological components of olfactory sexual signalling known to be important in the attraction of mates (Garratt et al., 2014). Less is known about the phenotype of $Sod1^{+/-}$ males. No differences in oxidative damage or lifespan (Elchuri et al., 2005) have been detected in these animals in comparison with wild-type males. However, cells from $Sod1^{+/-}$ mice have a slightly increased sensitivity to paraquat, a molecule that increases the production of free radicals, and these mice also suffer lower motor neuron survival after induced injury (Reaume et al., 1996).

In mice, acts of overt aggression, such as trying to bite, chase and even kill competitor individuals, help males to attain dominance over other individuals and monopolise matings within a territory

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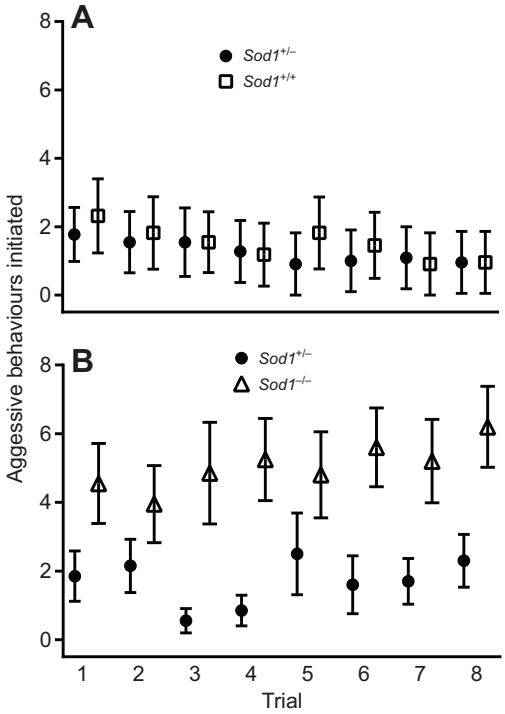

Fig. 1. The number of aggressive interactions initiated by males in each experimental trial. (A) Aggressive behaviours initiated by *Sod1*$^{+/+}$ males and *Sod1*$^{+/-}$ males that were paired together. (B) Aggressive behaviours initiated by *Sod1*$^{-/-}$ males and *Sod1*$^{+/-}$ males that were paired together. Trial numbers are successive, with trial one being the first trial conducted and trial eight being the last at the end of 3 weeks. The maximum number of aggressive interactions that males could initiate in each trial was 10.

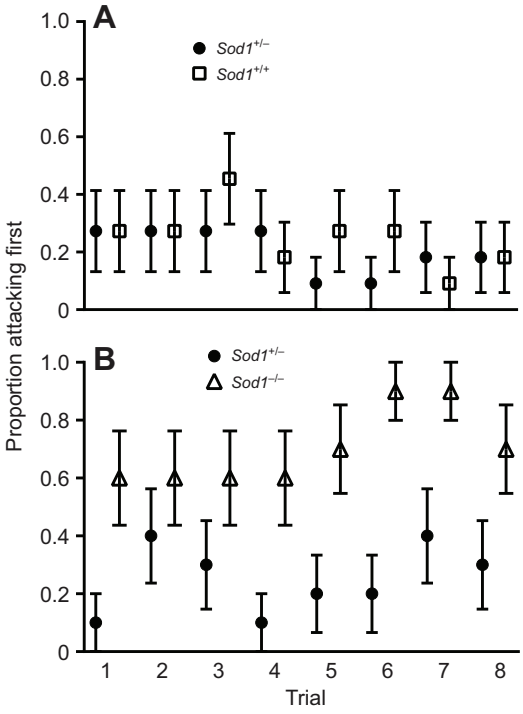

Fig. 2. The proportion of males that attacked first for each genotype in each experimental trial. (A) The proportion of *Sod1*$^{+/+}$ males and *Sod1*$^{+/-}$ males that attacked first in each trial. (B) The proportion of *Sod1*$^{-/-}$ males and *Sod1*$^{+/-}$ males that attacked first in each trial. Note that the values for the two genotypes in each trial do not always add up to one because in some trials neither male initiated an aggressive interaction, while in others both males mutually initiated an aggressive interaction at the same time.

area (Bronson, 1979). To explore the degree to which aggression is sensitive to impaired antioxidant defence, we used a previously published, standard protocol for assessing aggression and the ability to attain dominance in mice (Jones and Nowell, 1974; Jones and Nowell, 1989). This involved pairing two mice in a divided cage over 3 weeks, and allowing them to interact aggressively regularly over this period (see Materials and methods). To minimise animal numbers and maximise the comparisons we could make between genotypes, *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ males were each paired with *Sod1*$^{+/-}$ males. We could then determine whether *Sod1*$^{+/-}$ males were more likely to win or lose fights with either of the other genotypes. Because *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ males were paired with the same genotype of male (*Sod1*$^{+/-}$), we could also compare the aggression of these two genotypes of males against a standardised, equivalent competitor male.

RESULTS

Pairs of *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males were allowed to interact eight times over 3 weeks. Over this period, *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ males initiated a similar number of aggressive behaviours (attacks, chases or attempts to bite the other male) (χ^2=0.15, 1 d.f., P=0.70; Fig. 1A). The two genotypes of male were also just as likely to initiate the first aggressive behaviour in a given experimental interaction ($\chi^2 \leq 0.1$, 1 d.f., P=0.96; Fig. 2A). The number of aggressive behaviours exhibited decreased over the successive trials (χ^2=13.43, 1 d.f., P=0.0002; Fig. 1A) to a similar extent in the two genotypes (interaction between genotype and trial number: χ^2=0.07, 1 d.f., P=0.79).

When 10 *Sod1*$^{-/-}$ males were allowed to interact with paired *Sod1*$^{+/-}$ males over 3 weeks, *Sod1*$^{-/-}$ males initiated a greater number of aggressive behaviours in comparison to *Sod1*$^{+/-}$ males (χ^2=6.96, 1 d.f., P=0.0083; Fig. 1B). This effect was consistent over the 3 week duration of the experiment, with both genotypes of male increasing their levels of aggression over time (effect of trial number: χ^2=5.66, 1 d.f., P=0.017; interaction between genotype and trial number: χ^2=0.01, 1 d.f., P=0.89; Fig. 1B). *Sod1*$^{-/-}$ males were also much more likely to be the first to express an aggressive behaviour in a given experimental trial (effect of genotype: χ^2=9.45, 1 d.f., P=0.002; Fig. 2B).

Because *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ males were paired with equivalent *Sod1*$^{+/-}$ males over the same time period, in identical conditions, we also compared the aggressive behaviour shown by *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ males in their respective trials. When paired with *Sod1*$^{+/-}$ males, *Sod1*$^{-/-}$ males expressed a significantly greater number of aggressive behaviours than *Sod1*$^{+/+}$ males (χ^2=9.62, 1 d.f., P<0.002; Fig. 1), an effect that became progressively stronger over successive experimental trials (interaction between genotype and time: χ^2=22.54, 1 d.f., P<0.0001). *Sod1*$^{-/-}$ males were also much more likely to express the first aggressive behaviour in their experimental trials than *Sod1*$^{+/+}$ males (χ^2=8.73, 1 d.f., P<0.003; Fig. 2).

DISCUSSION

Our results reveal that males with experimentally impaired antioxidant defence (*Sod1*$^{-/-}$), through complete SOD1 deficiency, are more aggressive than both heterozygous knockout males (*Sod1*$^{+/-}$), which are 50% deficient in SOD1, and wild-type (*Sod1*$^{+/+}$) males. These results do not support the prediction that an increased susceptibility to oxidative stress reduces a male's ability to invest in aggressive behaviours, although we cannot exclude the possibility

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that constraints on fighting ability might be revealed if males are allowed to fight more frequently, or over a longer duration. However, in this experiment males were required to fight with another male eight times over a 3 week period and there was no evidence that the aggressive *Sod1*$^{-/-}$ phenotype abated: on the contrary, *Sod1*$^{-/-}$ males continued to become more aggressive, while wild-type males instead reduced their aggressive behaviour over progressive experimental trials.

Although aggressive behaviour is not negatively affected by SOD1 deficiency, at least not in a noticeable manner here, other aspects of reproduction are known to be impaired in this mouse model. Female fertility in *Sod1*$^{-/-}$ mice is markedly reduced, with females only producing a couple of small-sized litters when allowed to breed over 3–6 months (Ho et al., 1998; Matzuk et al., 1998). Sperm motility and fertilisation success are reduced in *Sod1*$^{-/-}$ male mice under certain conditions (Garratt et al., 2013; Tsunoda et al., 2012). Particular molecular (major urinary proteins) and morphological (preputial glands) aspects of sexual signalling are also depressed in *Sod1*$^{-/-}$ males (Garratt et al., 2014). In contrast to these declines in physiological aspects of reproduction, behavioural investment in scent marking is not noticeably affected by SOD1 deficiency (Garratt et al., 2014), and investment in aggressive behaviour actually seems to increase. There is, therefore, substantial variability in the degree to which different aspects of reproduction are affected by oxidative stress. We speculate that investment in behavioural aspects of reproduction may be less physiologically constrained by oxidative stress, allowing males to increase their investment in such traits more freely than in molecular and morphological aspects of reproduction. Although behavioural traits require energy they do not require the synthesis and maintenance of specific molecular components that could be susceptible to oxidative damage (over that required for muscular function). Other reproductive traits, such as sperm production, require efficient generation and maintenance of cellular components that are easily damaged by uncontrolled production of ROS. The degree to which molecular and cellular components involved in a specific aspect of reproduction are susceptible to oxidative damage might influence whether that aspect of reproductive allocation is negatively impacted by oxidative stress.

Instead of the predicted decrease in aggression in *Sod1*$^{-/-}$ mice, we found that these males exhibit a greater number of attacks and are quicker to attack their opponent in staged trials. The increased aggression with oxidative stress we report here was unexpected, but several previous studies have provided evidence that male aggression can correlate with a marker of oxidative stress or antioxidant defence (measured after the assessment of aggression). In a reptile species, White's skinks (*Egernia whitii*), male aggression is positively correlated with a marker of oxidative damage in plasma (Isaksson et al., 2011). In laboratory mice, Rammal et al. (Rammal et al., 2010) demonstrated a negative correlation between latency to attack an intruder and intracellular redox status (with a higher status expected to signify greater oxidative stress) of peripheral blood granulocytes. A recent study in humans has also revealed a positive correlation between a patient's history of aggression and two markers of oxidative stress in plasma (Coccaro et al., 2014). These studies have been interpreted in various different ways, with oxidative stress being suggested as either a cause (Coccaro et al., 2014) or a consequence (Isaksson et al., 2011; Rammal et al., 2010) of differences in aggressive behaviour. Our study provides the first experimental evidence that oxidative stress can directly alter investment in aggressive behaviours, although the underlying cause of the increased aggression with oxidative stress requires further investigation.

Investment in aggression is influenced by both intrinsic and extrinsic factors. We think that it is unlikely that these males have some elevated physiological function (e.g. intrinsic factor) that increases the capacity for aggressive behaviours, particularly as *Sod1*$^{-/-}$ males show impairments in muscle mass and function that increase with age (Muller et al., 2006; Vasilaki et al., 2010). It is possible, however, that impaired *Sod1*$^{-/-}$ expression causes some pathophysiological change, in the brain, for example, that increases the expression of aggressive behaviours, or willingness to fight. In this scenario, the increased aggression in *Sod1*$^{-/-}$ males is not an adaptive response, rather a negative consequence of oxidative damage. This could include any hypothetical damage to known pathways directly associated with aggressive behaviour, such as serotonin, dopamine or glutamate pathways (Coccaro et al., 2014). Oxidative damage in the brain could affect aggression indirectly, by influencing emotional status in some manner, such as by altering anxiety, which has been linked both positively (König et al., 1996) and negatively (Nyberg et al., 2003) to offensive aggression. Experimental manipulations that alter oxidative stress have previously been linked to changes in the expression of anxiety-linked behaviours (Berry and Cirulli, 2013; Hovatta et al., 2005), generally suggesting that oxidative stress increases anxiety (Bouayed et al., 2009). As a consequence, an effect of oxidative stress on anxiety, although not previously noted in this mouse model, offers one plausible pathway through which this aspect of physiology and aggressive behaviour are linked.

Extrinsic factors that influence aggression include winner and loser effects and territory possession (which are controlled for in this experiment), in addition to other factors that increase motivation to fight (Dugatkin, 1997). It is interesting to note that previous experiments have observed increased aggression levels with manipulations that would be expected to reduce male physiological function. Male field crickets (*Gryllus integer*) (Pölkki et al., 2013) with an experimentally activated immune response show heightened aggression and dominance, which the authors suggest might be a plastic response to the survival threat presented by the immune challenge: a form of 'terminal investment' in reproduction, which has also been observed in relation to other components of reproductive behaviour (Candolin, 2000; Clutton-Brock, 1984). In white-footed mice (*Peromyscus leucopus*), wild-caught males that are parasitised with bot flies (*Cuterebra fontinella*) are more aggressive in staged trials in the laboratory than non-parasitised males, another example of increased aggression with reduced survival prospects (Cramer and Cameron, 2007).

As *Sod1*$^{-/-}$ male mice have repeatedly been shown to have a lifespan that is shorter by about 30% (Elchuri et al., 2005; Pérez et al., 2009; Zhang et al., 2013), increased aggressive behaviour by *Sod1*$^{-/-}$ males could possibly also reflect a terminal investment strategy, with males investing more in aggressive behaviour in an effort to increase their immediate reproductive success. Another possibility is that *Sod1*$^{-/-}$ males show elevated levels of aggression because they are compensating for their reduced ability to invest in olfactory signalling. When male mice win fights and become dominant, their investment in olfactory signalling changes, with particular volatile molecules increasing in concentration in urine (Novotny et al., 1990). Several of these volatiles are produced in the preputial glands, which have been found to be smaller in *Sod1*$^{-/-}$ males when housed in a competitive environment (Garratt et al., 2014). It is feasible that increased aggression by *Sod1*$^{-/-}$ males partially compensates for a reduced ability to produce volatile molecules that signal their dominance to male conspecifics.

Our result of increased aggression in *Sod1*$^{-/-}$ males adds to a complex picture of how increased susceptibility to oxidative stress

influences male reproductive effort. Further studies in additional mouse models and other taxa may help to adjudicate the generality of the result we reveal. There are a range of different mouse models with genetically impaired antioxidant defence and varying degrees of associated pathophysiology (Pérez et al., 2009); examination of sexual signalling and aggression in these mice may reveal the general sensitivity of these reproductive traits to perturbations in redox status. Conditional knockouts of antioxidant defence, where the expression of a particular gene in that defence process can be manipulated over a specific period of an animal's life cycle, may be particularly helpful in determining the impact of oxidative stress on these traits during a specific period of adulthood (Hamilton et al., 2012). Ultimately, however, direct links between oxidative stress and aggression need to be tested for in organisms other than biomedical models, as these model animals show alterations in their behaviour and life history as a result of selective breeding in laboratory conditions. Further exploration of the direct effects of oxidative stress on aggression in a more diverse range of species, perhaps through manipulation of ROS production (genetic manipulation of antioxidant defence is only available in model organisms), may help to confirm whether oxidative stress has a sufficient impact on aggression in wild animals that it affects their ability to attain dominance and mating success.

MATERIALS AND METHODS

Animals

The *Sod1* line of mice were maintained on a C57BL/6 background. The generation of this knockout strain (Kostrominova et al., 2007; Muller et al., 2006) and details of our breeding colony (Garratt et al., 2013) have been reported previously. Briefly, the line of *Sod1* mice used in these experiments was derived from three pairs of $Sod1^{+/-}$ mice imported from the Jackson Laboratories (Bar Harbor, ME, USA) and used to create a SPF breeding colony at the Australian BioResource Centre in Mossvale, NSW, Australia. Experimental mice were the progeny of matings between $Sod1^{+/-}$ pairs; the *Sod1* genotypes of offspring were determined by genotyping a small sample of ear tissue collected at weaning. Genotyping was conducted by the mouse genotyping service at the Australian Cancer Research Foundation (ACRF), the Garvan Institute, using a combination of real-time PCR and melting curve analysis. When 6–8 weeks old, experimental mice were transported to the University of New South Wales (UNSW) and housed in conventional facilities. The mice were maintained at 22°C under a 12 h light:12 h dark cycle. All experimental procedures and aggression trials were conducted in the dark period under dim red light. Two weeks prior to the experiment, males were housed singly in cages (53×35×18 cm) and were regularly exposed to the odour and presence of males and females of the CBA strain (CBA/CaHAusb) to ensure the development of normal reproductive behaviour. Food (stock feed from Gordon's Specialty Stockfeeds, Yanderra, NSW, Australia) and water were provided *ad libitum*. All experimental procedures were approved by the UNSW animal ethics committee (approval number: 12/30A).

Aggressive interactions

We created 11 pairs of $Sod1^{+/+}$ males and $Sod1^{+/-}$ males. At the same time, a second set of paired males consisted of 10 $Sod1^{-/-}$ and $Sod1^{+/-}$ males. One male in each pair was marked with a fur clip on the back to allow individual identification of the males during aggressive interactions. The genotype of the male that was marked was randomised, and the experimenter that recorded the aggressive interactions was unaware of each male's genotype (blind to both the genotypes of the males in the cages and which cages contained $Sod1^{-/-}$ and $Sod1^{+/+}$ males), ensuring unbiased assessment of aggressive behaviour.

After 2 weeks of single housing, each pair of males was housed in cages (53×35×18 cm) divided by a perforated plastic barrier, with a male on either side; this barrier allowed continuous visual and olfactory contact between the males but did not permit direct physical contact between the pairs. Males were housed in these conditions for 3 weeks, and over this period males were allowed to interact aggressively eight times. There was at least one rest day (i.e. when the males did not fight) between each aggressive interaction.

On each day of aggressive interactions, the barrier between the males was removed. Males were allowed to interact directly for a 15 min period or until 10 aggressive interactions between the males had occurred, whichever was first. The trial was then stopped and the barrier and bedding returned to the cage. During the trials, an experimenter was always present to split up any fights that were persistent (aggressive interactions that continued for more than 10 s) or involved obvious biting, following previous protocols (Garratt et al., 2012). This ensured none of the males were injured during the experiment. This experimenter also documented the time until each male initiated an aggressive behaviour and the number of aggressive behaviours initiated and received by each male. An aggressive behaviour was defined as when a male attempted or succeeded in biting, chasing or kicking the other male. Separate aggressive interactions were recorded when there was a period of 3 s between any of these behaviours. If one mouse was aggressive towards the other, and the attack was broken up by the experimenter, then the mouse immediately initiated another aggressive interaction, this was counted as two separate events. As 10 aggressive interactions were permitted before the trial was terminated, the maximum number of aggressive interactions each male could initiate or receive was 10. If males mutually initiated an aggressive behaviour, both males were considered to have initiated and neither male was considered to have received an aggressive behaviour.

Data analysis

To test for differences between genotypes in the expression of aggressive behaviours, we constructed Generalised Linear Mixed Effect Models using the lme4 package in R. Models that explored the number of aggressive behaviours expressed by each male were fitted with a Poisson distribution. Models that examined which male was first to initiate an aggressive behaviour were fitted with a binomial distribution, with males initiating the first behaviour scored as '1' and those that did not scored as '0' (if both males initiated a behaviour at the same time they both received '1' and if neither attacked they both received '0'). Male genotype and trial number were added as fixed effects. Male ID and pair were added as random effects, to control for repeated assessment of male behaviours and the non-independence between individuals in each pair. The significance of the genotype and trial number effect, and the interaction between the two, were tested by comparing models with and without a particular term using a log-likelihood ratio test.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.G. and R.C.B. conceived the study. M.G. conducted the study and wrote the manuscript.

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