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# Hair cortisol concentrations in New Zealand white rabbits subjected to surgery

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## Abstract

*The aim of this study was to assess hair cortisol concentrations in New Zealand white rabbits (*Oryctolagus cuniculus*) that were subjected to relocation and surgery to evaluate HPA-axis activity; in addition, we used this marker of cortisol secretion to evaluate the allostatic load of animals undergoing surgery. After a period of acclimatisation, which lasted 40 days from their arrival at the enclosure, 19 rabbits were subjected to T1–T12 dorsal arthrodesis (RS), 19 were sham-operated (SS), and 19 were non-operated (CON). Hair samples were collected at the time of arrival (ST1) at the animal facility, and seven other sets of hair samples were collected at 40-day intervals from the same area of skin for a period of 240 days as re-shaved hair (anagen phase): immediately before surgery (ST2) and after the surgery (ST3, ST4, ST5, ST6, ST7, and ST8). The transition from the rabbitry to the animal breeding facility led to a significant increase in cortisol concentration (ST2) in all of the groups. At ST3, the RS group presented higher cortisol concentrations than those of the SS group and the CON group. At ST4, the experimental groups showed similar values that remained constant until ST8. The results show that the management of rabbits undergoing surgery should be evaluated very carefully, and hair cortisol concentrations may provide a means of avoiding the dangerous cumulative effects of additional stressors close to surgery.*

**Keywords**: *animal welfare, cortisol, hair, HPA axis, rabbit, surgery*

## Introduction

Cortisol is involved in metabolic homeostasis and the regulation of many physiological processes. It is the end-product of HPA-axis stimulation and is the primary glucocorticoid in most mammalian species, whereas in rodents, birds and reptiles corticosterone is the primary glucocorticoid. Rabbits secrete both corticosterone and cortisol in a circadian rhythm. Szeto *et al* (2004) found that the values of both glucocorticoids were significantly correlated, suggesting that these hormones are regulated in a similar fashion and exhibit similar response profiles.

Cortisol can be measured in the blood or, non-invasively, in faeces (Teskey-Gerstl *et al* 2000), urine (Walker *et al* 2009), milk (Gygax *et al* 2006) and saliva (Negrao *et al* 2004). These methods provide a measurement of the cortisol concentration either at a single point in time or within the previous 12–24 h (Russell *et al* 2012). Cortisol concentrations in hair provide an integrated rather than a single-time-point measure of HPA-axis activity (Meyer & Novak 2012). The collection of hair is simple and non-invasive. Furthermore, hair samples do not decompose as body fluids or other tissues (Balikova 2005). The precise mechanisms by which lipophilic steroid hormones are incorporated into hair are still not fully understood. There are studies to support both a systemic and a local derivation. It is known that brain and skin communicate with each other (Zmijewski & Slominski 2011; Chen & Lyga 2014) to regulate global homeostasis through the systemic release and/or local production of hormones, neuropeptides, neurotransmitters and biological regulators (Slominski & Wortsman 2000; Slominski 2005; Zmijewski & Slominski 2011). Hair follicles, being surrounded by a dense and continuous plexus of capillaries (Montagna & Ellis 1958) and as one of the most densely and intricately innervated of all peripheral tissues (Paus & Foitzik 2004), are an ideal model to study the inter-system communication characteristic of stress responses. The presence of several neuropeptides and other biologically active compounds in the skin and its appendages is due to the transport of them from blood, to the release of them from nerve-endings or to the migrating immune cells but also to their local synthesis (Slominski & Wortsman 2000; Slominski 2005; Zmijewski & Slominski 2011) since the skin expresses an equivalent of the HPA axis (Slominski & Mihm 1996). A possible supply in the hair due to local cortisol production has been described *in vitro* (Ito *et al* 2005; Slominski *et al* 2007; Russell *et al* 2014) or *in vivo* as a consequence of a local stimulation (Sharpley *et al* 2009, 2010; Stubsjøen *et al* 2015; Salaberger *et al* 2016). Other studies provide support for the systemic model



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of hair cortisol incorporation. The detection of several synthetic glucocorticoids in human hair (Bévalot *et al* 2000; Cirimele *et al* 2000; Gaillard *et al* 2000) and both direct (Davenport *et al* 2006; Accorsi *et al* 2008; Bennett & Hayssen 2010) and indirect (Kirschbaum *et al* 2009; Thomson *et al* 2010; Manenschijn *et al* 2011; Noppe *et al* 2014) validation studies, as those investigating patients with Cushing's syndrome and Addison's disease, support the assumption that hair cortisol concentrations provide a valid marker of systemic cortisol concentrations. Such studies suggest that hair cortisol concentrations are not subject to diurnal variation and are not acutely influenced by sweat (for a review, see Stalder & Kirschbaum 2012; Grass *et al* 2015) and that local follicular cortisol production may contribute *in vivo* only marginally to hair cortisol concentration (Grass *et al* 2015). Cattet *et al* (2014), in a study in which cortisol has been determined on guard hairs plucked with the follicles removed, suggest that hair cortisol concentrations may also be influenced by short-term or acute stressors. The results observed by them could be influenced by presence of elongation, pre-keratinised and hardening zones in the sample that are still close to the capillary bed, to the sebaceous gland and to nerve-endings (Popescu & Hocker 2007). Differently, a clipped or shaved sample provides only the solidly keratinised part of the hair shaft.

Hair cortisol, as a marker of chronic cortisol secretion, is used to evaluate the allostatic load, the cumulative result of an allostatic state. The allostatic state refers to altered and sustained activity levels of mediators, eg glucocorticosteroids, by which an organism maintains the physiological stability of its internal milieu (McEwen & Wingfield 2003, 2010).

Certain environmental conditions or management systems (eg thermal extremes, mixing of unfamiliar animals, transportation and environmental changes) have the potential to activate the HPA axis in domestic animals (Minton 1994) and in laboratory animals, which may influence the outcomes of animal experiments (Baumans 2004). The New Zealand white (NZW) rabbits (*Oryctolagus cuniculus*), lagomorphs commonly used for a variety of different research-related purposes, including antibody production and as a model for orthopaedic surgery (Batchelor 1999; Castaneda *et al* 2006), are very sensitive to environmental changes and are easily frightened. Surgery presents a significant challenge to homeostasis and activates compensatory neuroendocrine systems, such as the HPA axis (Gibbison *et al* 2013), which could lead to over-stimulation for an excessive length of time and may be an additional risk factor for a successful surgery because of cortisol's immunosuppressive effect. The hair-sampling method has been already utilised to assay cortisol concentrations in rabbits (Comin *et al* 2012b; Peric *et al* 2017).

The aim of this study was to assess hair cortisol concentrations in NZW rabbits that were subjected to relocation and surgery to evaluate long-term HPA-axis activity; in addition, we used this marker of chronic cortisol secretion to evaluate the allostatic load of animals undergoing surgery.

## Materials and methods

Operative procedures and animal care were performed in compliance with national and international regulations (Italian regulation DLvo 116/1992 and European Union regulation 86/609/EC). The protocol was examined and approved prior to the start of the study by the Director's Board of the Department of Life Sciences, Animal Facility, University of Trieste, Trieste, Italy, where this study was performed. The recommendations of the ARRIVE guidelines in animal research were also consulted and considered (Kilkenny *et al* 2010).

### Study animals

The 57 NZW female rabbits included in this study were purchased from Harlan Laboratories, San Pietro al Natisone, Italy. The rabbits were brought to the animal facility of the University of Trieste at 80 days of age and kept under stable environmental conditions. The rabbits were housed singly in stainless steel cages with a plastic grid as a base (55 × 45 × 70 cm [length × width × height], Conelli Snc, Arona, Italy) in a controlled environment (21°C, relative humidity of 40–50%, room air fully recirculated 10–15 times per hour, and a light/dark cycle of 12/12 h) and fed a standard pelleted diet (2030 Teklad Global Rabbit Diet, Harlan Laboratories, Indianapolis, IN, USA) with water *ad libitum*. The rabbits were all clinically healthy.

At 120 days of age, after a period of acclimatisation (which lasted 40 days from their arrival at the enclosure), 19 rabbits were subjected to T1–T12 dorsal arthrodesis (RS) (Canavese *et al* 2007, 2008), 19 were operated on with the same protocol but did not undergo dorsal arthrodesis (sham-operated SS), and 19 were not operated on (CON). The 57 animals were allocated randomly to the three groups. This experiment was performed in collaboration with a medical-scientific team studying the consequences of selective dorsal T1-T12 arthrodesis on the thoracic spine and chest growth (Canavese *et al* 2007, 2008, 2010, 2013, 2014). As reported by Batchelor *et al* (1999) and Castaneda *et al* (2006), the rabbit is an adequate animal model for orthopaedic surgery.

### Anaesthesia protocol

Surgery of both RS and SS groups of operated rabbits was performed under general anaesthesia (GA), induced by an intramuscular injection of xylazine 5 mg $kg^{-1}$ (Virbaxil® 2%, Virbac Laboratories, Carros, France) and tiletamine-zolazepam 15 mg $kg^{-1}$ (Zoletil® 100, Carros, France). Additional skin analgesia was provided with a subcutaneous injection of 2% lidocaine hydrochloride (1 ml per animal). Pain management during the post-operative period was achieved via subcutaneous administration of Carprofen (Rymadil®, Pfizer Animal Health, West Dundee, UK; 5 mg $kg^{-1}$ twice a day over five days). Infection was prevented the week following surgery by an intramuscular injection of enrofloxacin (Baytril® 5%, Bayer Animal Health, Kiel, Germany; 5 mg $kg^{-1}$ twice daily over five days).

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### Surgical procedure

Nineteen rabbits were operated on following a modified 'Wisconsin' technique or extra-canal dorsal T1–T12 vertebral arthrodesis, corresponding to posterior vertebral arthrodesis in bipeds (Resina & Ferriera-Alves 1977; Drummond 1988). Access to the operating area was achieved on the midline of the back, between the first thoracic and first lumbar vertebrae, with the *point de repere* represented by the spinous processes. Once the muscular plane was reached, the *musculus trapezius*, *latissimus dorsi*, *spinalis thoracis*, and *longissimus lumborum et thoracis* were retracted symmetrically to allow a wide exposure of the vertebral laminae, spinous and transverse processes, and cranial and caudal facies articulares of the thoracic vertebrae (Barone 1996–2004). Two specifically designed C-shaped stainless-steel bars, approximately 120 mm in length and 1.5 mm in diameter, were positioned laterally at the base of the spinous processes of the thoracic vertebrae and fixed with multiple 2/0 non-absorbable wire ligatures. In the 19 sham-operated subjects, the surgical procedure was performed in the same manner as for the operated subjects (manually performed, as described above) except for the use and attachment of the C-shaped stainless steel bars. Each operation lasted, on average, approximately 50 min in the operated rabbits and 25 min in the sham-operated rabbits.

### Hair samples

Hair samples (1–2 g) were carefully obtained from the left thigh of the rabbits using clippers (clipping provides a sample with only the keratinised part of the hair shaft) as described in Comin *et al* (2012a). Hair samples were collected at the time of arrival (ST1) at the animal breeding facility. At this time, the single hairs collected were at different physiological phases (anagen, catagen and telogen). Hair samples (ST2, ST3, ST4, ST5, ST6, ST7, ST8) were collected at 40-day intervals from the same area of skin for a period of up to 240 days as re-shaved hair (anagen phase): ST2 samples were collected immediately before surgery, and ST3, ST4, ST5, ST6, ST7, and ST8 samples were collected after the surgery. We hypothesised a lag-time of 15 days because hair requires two weeks to emerge (Rony *et al* 1953). The 456 hair samples were stored in dry tubes at room temperature until analysis. To avoid stress and to comply with the current legislation on animal welfare, the samples were collected during routine activities.

### Hair cortisol assay

The hair cortisol analysis was carried out using the RIA method described by Comin *et al* (2012b). Briefly, the hair strands were washed in 5 ml isopropanol and approximately 60 mg of hair trimmed with scissors into smaller segments was extracted in a glass vial with 3 ml of methanol. The vials were incubated at 37°C for 18 h. Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.6 ml of phosphate-buffered saline (PBS), 0.05 M, pH 7.5 (RIA buffer). The hair cortisol was measured using a solid-phase microtitre RIA procedure. In brief, a 96-well microtitre plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum, diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.4, and incubated overnight at 4°C with 200 µl of the anti-cortisol serum diluted 1:12,000. The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). The cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8% and aldosterone < 0.02%. After washing the plate with RIA buffer, standards (5–300 pg per well), a quality control extract, the test extracts and tracer (Hydrocortisone [Cortisol, (1,2,6,7-3H [N])-], Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting the extract and washing the wells in RIA buffer. After the addition of 200 µl scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). The intra- and inter-assay coefficients of variation were 3.6 and 9.8%, respectively. The assay sensitivity (defined as the hormone concentration resulting in a displacement of the labelled hormone at least 2 standard deviations from maximal binding) was 1.23 pg per well.

### Statistical analysis

Statistical analysis was performed using SPSS for Windows, v 7.5.21, 1989-1997. The normality of the data distribution was tested using the Shapiro-Wilk test. Hair cortisol concentrations were analysed before and after surgery using repeated ANOVA measures, considering the sampling time (ST1 and ST2 for the analysis before surgery, and ST3, ST4, ST5, ST6, ST7, and ST8 for the analysis after surgery) as the within-subject factor and the treatment (RS, SS, CON) as the between-subject factor. The Holm-Bonferroni *post hoc* test was computed to assess the mean differences between treatments for the specific time-points as suggested by Park *et al* (2009).

## Results

The Shapiro-Wilk test showed that the residuals were normally distributed ($P > 0.05$). As shown in Figure 1, the hair cortisol concentrations increased significantly in all the groups from ST1 to ST2 ($F_{1,54} = 107.83$; $P < 0.01$). Interaction between sampling time and treatment was not found ($F_{2,54} = 0.94$; $P > 0.05$). *Post hoc* tests revealed that, at the time of arrival at the animal breeding facility (ST1), the cortisol concentrations were similar between the three experimental groups ($P > 0.05$), with a mean (± SEM) value of 1.10 (± 0.142) pg mg$^{-1}$, and that, before surgery (ST2), the hair cortisol concentration was 2.82 (± 0.142) pg mg$^{-1}$, with no significant differences between the groups ($P > 0.05$).

As shown in Figure 2, after surgery, statistical analysis revealed an interaction between sampling time and treatment ($F_{10,100} = 7.133$; $P < 0.01$), *post hoc* tests showed that the RS group had higher cortisol concentrations than the SS group (4.16 [± 0.340] vs 1.66 [± 0.180] pg mg$^{-1}$; $P < 0.01$) and the CON group (4.16 [± 0.340] vs 1.26 [± 0.132] pg mg$^{-1}$; $P < 0.01$) at ST3; at ST4, the experimental groups showed similar values with an average of 1.63 (± 0.151) pg mg$^{-1}$ ($P > 0.05$). These values remained constant until ST8.



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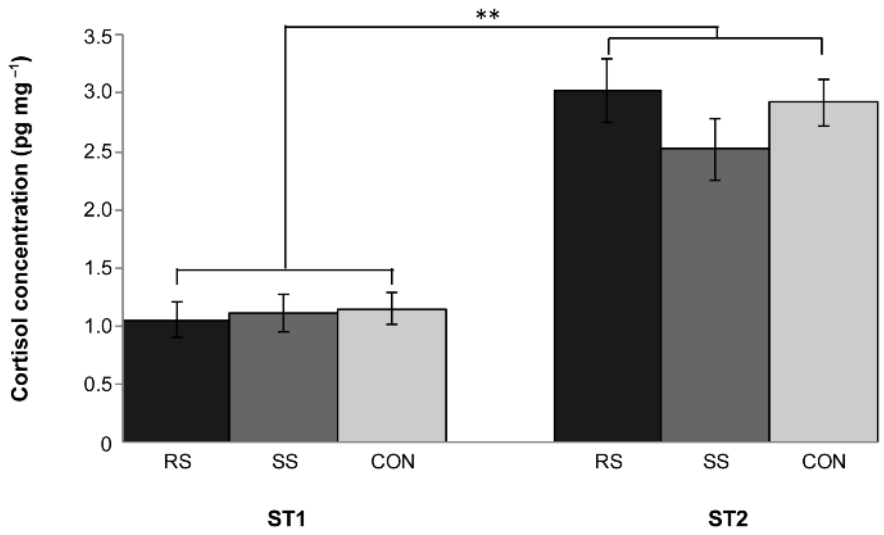
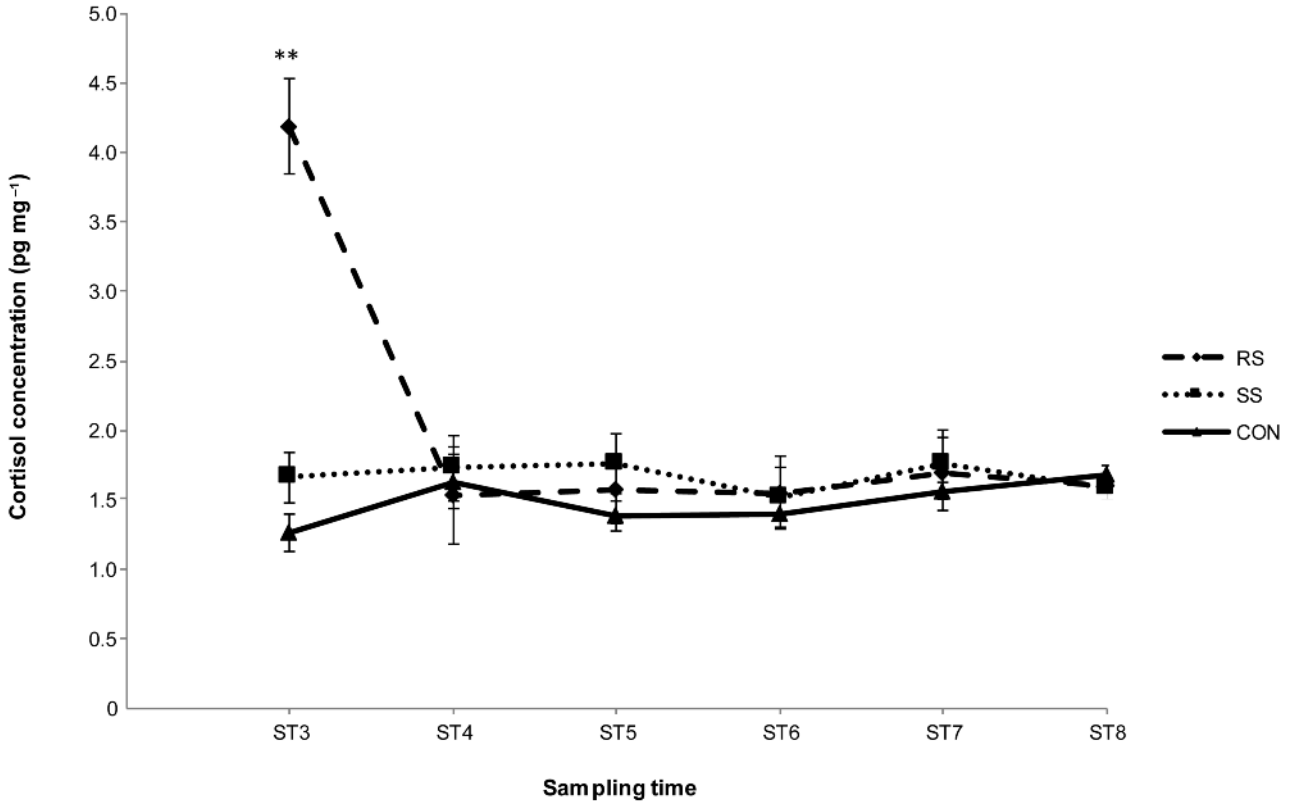
**Figure 1**




Mean (± SEM) hair cortisol levels in rabbits undergoing real surgery (RS) or sham surgery (SS) and in the control (CON) group, recorded at the beginning of the trial (ST1) and after 40 (ST2) days immediately before surgery. ** Statistically significant difference ($P < 0.01$) between ST1 and ST2.

**Figure 2**




Mean (± SEM) changes in hair cortisol levels in rabbits undergoing real surgery (RS) or sham surgery (SS) and in the control (CON) group, recorded 40 (ST3), 80 (ST4), 120 (ST5), 160 (ST6), 200 (ST7) and 240 (ST8) days after surgery. ** Statistically significant difference ($P < 0.01$) between the RS and the other groups at ST3.

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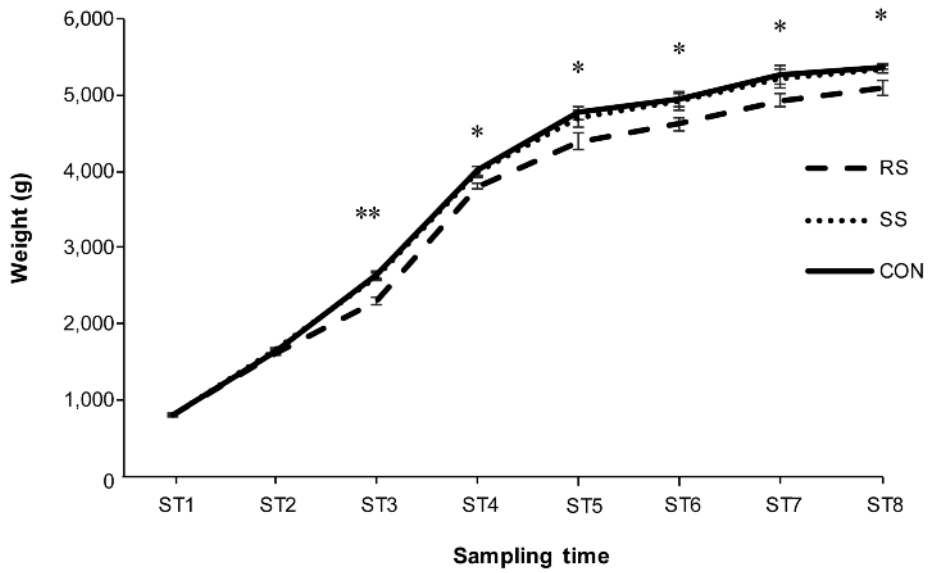
**Figure 3**




Mean (± SEM) bodyweight in rabbits undergoing real surgery (RS) or sham surgery (SS) and in the control (CON) group, recorded at the beginning of the trial (ST1), immediately before surgery (ST2) and 40 (ST3), 80 (ST4), 120 (ST5), 160 (ST6), 200 (ST7) and 240 (ST8) days after surgery. * Statistically significant difference ($P < 0.05$) between the RS and the other groups at ST4, ST5, ST6, ST7 and ST8. ** Statistically significant difference ($P < 0.01$) between the RS and the other groups at ST3.

## Discussion

Hair cortisol concentrations have been hypothesised to be a retrospective reflection of the integrated cortisol secretion (Kirschbaum *et al* 2009; D'Anna-Hernandez *et al* 2011; Pereg *et al* 2011; Russell *et al* 2012; Stalder & Kirschbaum 2012).

The hair samples collected at ST1 were at different physiological phases (anagen, catagen and telogen), consequently, this represented the time prior to relocating the rabbits from the rabbitry to the animal breeding facility. Though hairs sampled at ST1 were much longer/older, whereas the ST2 hair sample was short and newly grown, there is a low probability of a leaching effect in these rabbits at ST1, as found by Kirschbaum *et al* (2009) in humans, because the rabbits were kept under stable environmental conditions and not subjected to hair treatments. The transfer of the animals that occurred at ST1 may have induced an increase in HPA-axis activity in response to the environmental change, which was reflected in the same increased hair cortisol concentrations at ST2 in the RS, SS and CON groups. A similar increase in hair cortisol concentrations was found following relocation to a new environment in rhesus monkeys (*Macaca mulatta*) (Davenport *et al* 2006, 2008) and in vervet monkeys (*Chlorocebus pygerythrus*) (Fairbanks *et al* 2011; Dettmer *et al* 2012). Taking into account the lag-time, the hair sample taken at ST2 does not allow us to determine whether, at surgery, the cortisol concentration decreased to baseline values. To overcome this problem, it would be necessary to obtain hair samples at shorter intervals (eg every 15 days).

In the RS group, hair cortisol concentrations were higher at ST3 than those recorded in the SS group and in the CON group. The effects of dorsal arthrodesis on the HPA axis, which were more profound and longer lasting in terms of intensity and duration than those of sham surgery, resulted in different hair cortisol concentrations between the SS and RS groups. The higher hair cortisol concentrations could be linked to an interaction of the effects of surgery with the endocrine system and an increase in pain even though the animals did not show significant differences in resuming normal species-specific behavioural patterns and regular feeding after surgery as observed by the technical staff at the animal breeding facility. The pattern of body mass gain in the 57 animals followed that described for the animals in a study by Canavese *et al* (2013). Higher cortisol concentrations could be linked to the lower body mass gain showed by RS rabbits (Figure 3), given that these animals were subjected to a higher allostatic load. It is well known that surgery induces a release of cortisol in plasma (Rees *et al* 1983). Ilçöl *et al* (2002) describe an associated increase in circulating cortisol in humans and in dogs (Ilçöl *et al* 2003) undergoing surgery. Surgery is physiologically stressful and produces a variety of neuroendocrine and metabolic changes (Dubois *et al* 1981; Haxholdt *et al* 1981; Deuss *et al* 1994; Kehlet 1999) related to surgical stress; this induces an increase in HPA-axis activation and an increase in hormones, such as cortisol, in the blood circulation. After surgery, the RS group showed a reduction in hair cortisol concentrations 40 days later than the others, most likely due to post-operative HPA-axis activation.

From ST4, all three groups maintained low HPA-axis activity until the end of the trial (ST8). This effect may be due to the stable environmental conditions under which the rabbits were kept up to the end of their stay at the animal breeding facility.



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From the results obtained, it is evident that in spite of the protocol and the rules related to animal welfare (which were strictly adhered to), the transition from the rabbitry to the animal breeding facility performed before surgery, was associated with a long-term increased activity within the HPA axis, likely due to the particular sensitivity of the rabbits to environmental changes, such as moving to the animal facility or the change of personnel. This stimulation may be an additional risk factor for a successful surgery because of cortisol's immunosuppressive effect. The HPA axis interferes with physiological mechanisms and plays a role in the allostatic process, the active process of maintaining and/or re-establishing homeostasis, which helps an animal adapt to a new situation and/or challenge (McEwen 1998); furthermore, the HPA axis influences the resilience that characterises every individual. However, it is known that hormones, such as glucocorticoids, secreted in response to generalised stress can increase an animal's susceptibility to disease, often by initiating immunosuppression (Sevi 2009).

In conclusion, the management of rabbits undergoing surgery should be evaluated very carefully, and hair cortisol concentrations may provide a means of avoiding the dangerous, cumulative effects of additional stressors close to surgery.

### Animal welfare implications

The collection of hair is not invasive, does not cause cumulative stress in animals, and does not affect the animals' welfare. Hair cortisol analysis is a potentially powerful tool for evaluating adrenal function and chronic increases in this hormone. Thus, the allostatic load or 'stress' may be measured without resorting to invasive and stressful techniques, such as blood sampling. Hair cortisol can be an important marker to reduce risks of detrimental effects on health. When an investigative animal model must be used, it is important to ensure the highest level of animal health by any means necessary.

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