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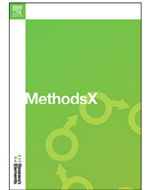

# Technical validation and a comparison of two methods to quantify individual levels of glucocorticoids in Alpine marmot hair

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Measuring individual specific concentrations of hair cortisol

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ABSTRACT

Quantification of cortisol concentration in hair has become a promising conservation tool for non-invasive monitoring of “stress” in wild populations, yet this method needs to be carefully validated for each species. The goals of the study were:

- Immunologically validate two methods (study 1 and 2 respectively) to extract and quantify cortisol in the hair of wild Alpine marmots.
- Compare the amount of cortisol extracted from hair samples using two methods i.e. cut into fine pieces (study 1) and hair samples pulverized using a ball mill (study 2).
- Determine the extent to which methods in study 2 could provide individual specific hair cortisol (HC) measures when samples were taken from the same body location. Within and between individual variations in HC levels were examined from multiple hair samples from 14 subjects in study 2. We evaluated if inter-individual variations in HC levels could be explained by sex and age.
  At least twice the amount of cortisol was obtained per g/hair when samples were pulverized in a ball mill prior to extraction compared to when cut into pieces. Our methods demonstrated intra-individual consistency in HC at a given time point: inter-individual variation in HC was three times larger than within individual variance. Sex and age did not impact HC levels.

Specifications Table

| | |
|---|---|
| Subject area: | Environmental Science |
| More specific subject area: | Conservation |
| Name of your method: | Measuring individual specific concentrations of hair cortisol |
| Name and reference of original method: | N/A |
| Resource availability: | N/A |

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## Method details

### Subjects

Hair samples were collected at the Grande Sassière Nature Reserve (French Alps, 45°29′N, 65°90′E), where a wild population of Alpine marmot has been intensively monitored since 1990 (see [17] for details). Every year the marmot pups are captured by hand, sexed and given a numbered ear-tag and a microchip inserted subcutaneously. The marmots are then recaptured once a year as part of our capture-mark-recapture program using two-door live-traps (Tomahawk Live Trap Company, Wisconsin, USA) baited with dandelion. For all animals, hair (containing follicle) was plucked from the dorsal region (between the scapula and the pelvis) and stored in envelopes at –20 °C until processing. Animals below 2 years of age were defined as sub-adults ($n = 8$) and all others were defined as adults ($n = 6$) [20]. Three samples were obtained for each individual. All procedures were approved by the ethical committee of the University of Lyon (n8BH2012–92) and the University of Chester (044/16/ED/BS).

Study 1: Hair samples were collected in May-June 2015 from 10 marmots (5 males and 5 females) ranging from 1 to 10 years of age. The age of 2 males and 1 female were unknown.

Study 2: In May-June 2017 we collected hair samples from 14 marmots (8 males and 6 females) ranging from 1 to 6 years of age.

### Experimental design for study 1 and study 2

Hair was extracted from samples in study 1 and study 2 using two different methods (detailed below) to determine whether or not the two methods yielded different quantities of cortisol. Samples only from study 2 were used to examine individual specific measures of HC and the impact of sex and age on HC levels due to a low sample number in study 1 for each category.

### Sample analysis

#### Washing protocol

Hair (180 mg) from both study 1 and 2 was mixed with 3.6 ml isopropanol in a glass tube (to remove contaminants) using a Heidolph Multi Reax test tube shaker at 800 rpm for 3 min [22]. Isopropanol was discarded and the process repeated. The hair was air dried on aluminium foil for 48 h at 23 °C covered by a fine mesh to prevent contamination.

Study 1: Washed and dried hair was cut into <1 mm pieces with a double-bladed mezzaluna chopper (Lakeland Limited, UK). 50 mg chopped hair was added to 5 ml 100 % methanol and shaken at 800 rpm for 22 h. Tubes were centrifuged at 4000 rpm for 15 min and 4 ml of the resultant supernatant was aliquoted into a tube for evaporation at 37 °C under a stream of nitrogen. The steroids were reconstituted in 3.5 ml Phosphate buffer saline (PBS, 5.42 g $NaH_2PO_4H_2O$, 8.66 g $Na_2HPO_4$ (anhydrous), 8.7 g NaCl, 1000 ml d.$H_2O$, pH 7) containing 1 % bovine serum albumin (BSA) and shaken at 1600 rpm for 20 min at room temperature before freezing at −20 °C.

Study 2: Washed hair was milled to a fine powder using a ball mill (Retsch, PM100) in 12 ml stainless steel jars with 3 × 20 mm stainless steel grinding balls at a speed of 425 rpm for 5 min. A volume of 5 ml 100 % methanol was added to 150 mg sample of powder and shaken at 800 rpm for 22 h. Tubes were centrifuged at 4000 rpm for 15 min and 4 ml of the resultant supernatant was aliquoted into a tube for evaporation at 37 °C under a stream of nitrogen. The steroids were reconstituted in 3.5 ml PBS containing 1 % BSA and shaken at 1600 rpm for 20 min at room temperature before freezing at −20 °C.

#### GC enzyme-immunoassay

We used a modified version of an enzyme-immunoassay (EIA) protocol described by Smith and French [54] to quantify HC in Alpine marmots in both studies. Briefly, antibody [R4866, raised in rabbits against cortisol-3-carboxymethyloxime: BSA [43]] was diluted to 1:8500 in 0.05 M carbonate buffer (1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$, 1000 ml d.$H_2O$, pH 9.6), loaded 50 μl/well onto a 96-well Maxisorp Nunc-Immuno microtitre plate (Thermo-Fisher Scientific, UK) and incubated overnight (4 °C). Plates were washed four times with 1:5 diluted ELISA wash buffer (40 g NaCl, 1 g KCl, 1.2 g $KH_2PO_4$, 7.2 g $Na_2HPO_4$, 2.5 ml Tween 20, 1000 ml d.$H_2O$, pH 7). The plate was loaded with 50 μl/well PBS buffer, followed by either 50 μl/well cortisol standard (Cat No. C-106 1Ml, Merck UK) or 50 μl/well sample and 50 μl/well of horseradish peroxidase conjugate (diluted 1:94,000 in PBS buffer) and left to incubate for 3 h in darkness. In study 1, a single hair sample from the dorsal region, was analysed from each individual in duplicate. In study 2, for each individual we analysed three separate hair samples (each assayed in duplicate) taken from the same body location (the dorsal region). Following incubation, the plate was washed as before and 100 μl/well EIA substrate was added (12.5 ml Citrate buffer, 125 μl EIA 2,2'-azinodi(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 40 μl $H_2O_2$. Citrate buffer – 9.61 g citric acid (anhydrous), 1000 ml d.$H_2O$, pH 4. EIA ABTS – 0.329 g ABTS, 15 ml d.$H_2O$, pH 6. $H_2O_2$ – 2 % w/v, 500 μl $H_2O_2$, 7.5 ml, d.$H_2O$). The plate was left to incubate in darkness until the blank wells reached an optical density of 1.0. The plate was read at 405 nm using a microplate reader (MRX II, Dynex Technologies; Revelation, Version 4.22). Samples were re-run if any coefficients of variance (CV) were above 5 %. See Ziegler et al. [61] for cross-reactivity data.

#### Validation methods and statistical analysis

All statistical analyses have been conducted in the statistical environment R version 3.5.3 [48]. The assay was immunologically validated for each study using a sample pool containing aliquots taken from all ten subjects for study 1, and all 14 subjects for study 2. To assess specificity, halved dilutions of a pool of marmot hair extracts (10 serial dilutions running from neat to 1:512) were run

in conjunction with serial dilutions of cortisol standards ($n$ = 10 dilutions; 39 pg/ml to 20,000 pg/ml) and the extent to which the lines were parallel assessed using ANCOVA. Accuracy was evaluated by quantifying percentage recovery of increasing amounts of synthetic cortisol (10,000 pg, 5000 pg and 2500 pg), added to a known quantity of cortisol in the pooled marmot hair extract (which contained around 36 ng/g for study 1 and 48 ng/g cortisol for study 2). Observed and expected GC levels were compared using linear regression. All samples in study 1 were run on a single plate and the average coefficient of variation (CV) for each sample duplicate was used to compute a mean intra-assay CV. Coefficients of variation (CV) of low and high concentration quality controls (QC), aliquoted from the marmot sample pool, were assessed within and between $n$ = 4 plates for study 2. Sensitivity was determined as the lowest concentration of cortisol in the working range of the assay. Results were considered statistically significant if $p < 0.05$.

To compare the HC values obtained in study 1 ($n$ = 10) and study 2 ($n$ = 14), the single HC value from each individual in study 1 was used together with a mean HC value obtained for each individual from their three samples in study 2. These values were log transformed and compared using a non-paired t-test.

Within and between individual variability, as well the effect of age and sex on individual HC levels from samples in Study 2 (log transformed), was estimated using linear mixed effects models (LME) with Individual as a random effect. Models with differing fixed effects (age and sex) were compared using the Akaike Information Criterion adjusted for small sample sizes (AICc [3]). LMEs were implemented using the 'lmer' function in the 'lme4' R package [5]. Intra-class correlation (ICC) and coefficient of variation (CV) were extracted from the best fitting model using respectively the 'icc' and 'cv' functions in the 'sjstats' R package [34]. Estimates of individual random effects ± 95 % confidence intervals have been simulated from the distribution of each of the random effects obtained for the best fitting LME model using the 'REsim' function in the 'merTools' R Package [30].

## Results

### *Assay validation*

We immunologically validated two methods to reliably measure HC in marmots. Displacement curves of serial dilutions of pooled marmot hair extracts and the commercial standards were parallel over the 10–90 % binding range (ANCOVA study 1: $F_{1,6} = 0.268$, NS, study 2: $F_{1,22} = 0.645$, NS). Recovery of the commercial standards added to the marmot pool was mean ± s.e.m., for study 1: 94.5 ± 2.3 % ($R^2 = 0.999$, $P < 0.001$) and for study 2: 112.0 ± 1.7 % ($R^2 = 0.999$, $P < 0.001$). Intra-assay CV for study 1 was 4.85 %, $n$ = 1 plate. In study 2 the intra-assay CVs for low and high concentration QCs were 2.3 % and 1.7 % respectively. Inter-assay CVs for low and high concentration QCs were 13.7 % and 17.9 % respectively ($n$ = 4 plates) for study 2. Sensitivity, of the assay was 18 ng/g hair for both studies.

### *Comparison of HC measured in study 1 and 2*

Significantly higher hair cortisol values were obtained from samples in study 2, in which the hair was pulverized using a ball mill compared to samples in study 1 which were cut into fine pieces ($t$ = 7.395, df = 22, $P < 0.0001$).

### *Within and between individual variability and effects of age and sex*

Mean ± s.e.m. concentrations of HC for males and females and juveniles and adults from both studies are shown in Table 1. Analyses assessing individual variation were conducted on samples from study 2. The best fitting LME model included only the intercept and the Individual random effect factor while models including sex and/or age did not improve the model fit (Table 2). The LME model taking into account repeated measures within individuals as random effects had a much better fit than a linear model ignoring the random effects structure (ΔAIC = 33.9). Between individual variance ($\sigma^2 = 0.096$) was about 3 times larger than within individual variance ($\sigma^2 = 0.033$). The ICC coefficients was 0.744 representing the proportion of the variance explained by the individual random effect. In Fig. 1 we plot the individual random effects (± 95 % CI).

### *More HC is extracted from samples pulverized using a ball mill compared to samples cut into pieces*

The HC values measured in study 1 approximated those measured for Alpine marmots by Jewgenow et al. [28] i.e. mean ± SD 60.6 ± 18.6 ng/g and those of Zenth et al. [60] i.e. approximately 100 ng/g and 75 ng/g for females and males respectively. The latter three studies used the same method to break up the hair matrix prior to extraction i.e. manually cut the hair shaft into small

**Table 1**
Mean ± s.e.m. concentrations ng/g hair of HC for males and females and juveniles and adults from study 1 ($n$ = 10) and study 2 ($n$ = 14).

| Catagory | Study 1 (hair samples cut into pieces) | Study 2 (hair samples pulverized using ball mill) |
|---|---|---|
| Males | 53.6 ± 3.63 | 195.8 ± 18.9 |
| Females | 83.9 ± 16.47 | 215.2 ± 38.5 |
| Young | 41.8 ($n$ = 1) | 211.1 ± 28.6 |
| Adults | 72.8 ± 13.14 | 194.4 ± 25.5 |

**Table 2**
Model selection using the AICc criterion between LME models including various Fixed and Random effect structures. A '+' symbol indicates if a particular effect was included in the model. The last model, with no random effects, was fitted as a simple linear model. Age:Sex is the interaction term between Age and Sex. Log transformed cortisol levels measured in Alpine marmot hair samples was the dependant variable in all tested models.

| Random(Individual) | Age | Sex | Age:Sex | df | AICc | ΔAICc |
|---|---|---|---|---|---|---|
| + | – | – | – | 3 | 8.275 | 0.000 |
| + | – | + | – | 4 | 9.405 | 1.130 |
| + | + | – | – | 4 | 9.725 | 1.450 |
| + | + | + | – | 5 | 11.162 | 2.886 |
| + | + | + | + | 6 | 13.333 | 5.058 |
| – | – | – | – | 2 | 44.204 | 35.929 |

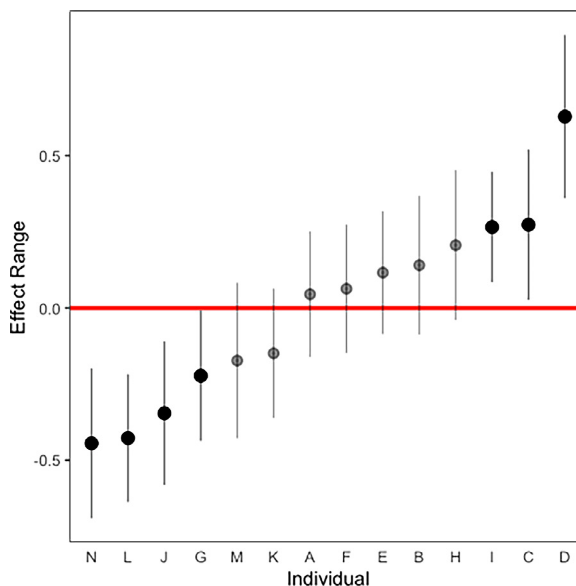


**Fig. 1.** Plot of the individual random effects and 95 % confidence intervals simulated from the distribution of each of the random effects obtained for the best fitting LME model with log(Cortisol) as dependant variable, intercept only as fixed effect and Individual as random effect. Individual estimates in grey are not distinguishable from 0 (i.e. the red line representing the population median).

pieces. However, when we pulverized the hair to a powder prior to extraction in study 2, we measured roughly twice to three times as much HC than samples in study 1 taken from a similar cohort of animals from the same population. Similarly, fine particles of hair from chimpanzees (*Pan troglodytes*) yielded higher concentrations of HC when extracted compared to larger, coarser particles from comparable hair [59]. In cannot be excluded that the higher HC concentrations obtained in study 2 could be attributed to differing subject characteristics or differing initial weights of hair being extracted. Practical difficulties of manipulating the cut hair segments in study 1 hair meant only 50 mg hair could be extracted in the 5 mL methanol solvent although as all HC values are presented as ng/g hair extracted the initial weight of hair extracted should not impact the final amount measured. Pulverizing the hair sample to a powder (study 2) provides a uniform and large surface area from which hormones can be extracted which may explain the increased HC yield. This is a useful methodological consideration for studies with small hair sample sizes as often obtained in the field. However, using a ball mill (study 2) is costly due to the need for specialized equipment and energy usage plus it will produce noise and chemical pollutants. Even though cutting the hair into small pieces in study 1 is time consuming, this method is cheap, environmentally friendly, does not require the costly equipment and energy use required by methods in study 2 yet it still yields levels of HC that can be detected by commercial and our in-house EIA of standard sensitivity. Future studies quantifying HC should weigh up the costs and benefits of the two methods used here in light of available resources, environmental policy, the quantity of cortisol in the hair and the sensitivity of the assay.

It is worth nothing that the study of Jewgenow et al. [28] used samples that had been clipped hence the follicle would have been absent (although this is not stated in the methods) where-as the study of Zenth et al. [60] and both study 1 and 2 in this current manuscript used plucked samples which most likely included the follicle. This may have lead to reduced HC values being measured by Jewgenow et el. [28] compared to the other studies due to exclusion of cortisol from localised skin/sebaceous sources. As suggested by Yamanashi et al. [59], standardization of hair processing methods across protocols would better facilitate cross study comparisons.

*Reliable determination of individual specific HC concentration*

Our methods reliably detect intra-individual consistency in HC levels at a given time point when hair is sampled from the same body region. Variation in HC levels is significantly greater between individuals than within individuals and hence our methods can usefully provide individual specific measures of stress in Alpine marmots.

Much attention has been paid in the literature to the differences in HC levels measured from different body locations within an individual (e.g., [10]), including one study showing marked inter-site differences in HC in the related species, Vancouver Island marmot *Marmota vancouverensis* [1]. To avoid these confounds of location, our study used hair collected from a single body region and observed a CV within individual HC levels of 18 % which was almost half that observed in GC levels measured from intact faecal pellets from individual snowshoe hares (35 %) ([32], see also [40]). Our methods point to reliably low intra-individual variation in HC samples inferring it as a repeatable, robust indicator of individual HPA function in Alpine marmots when hair is sampled from the dorsal region only. The latter goes some way to providing the empirical methodological evidence required for standardizing hair sampling location as called for by several authors (e.g., [33,56]).

Between individual variation in HC in our study was not due to age or sex. Comparable HC levels across ages are observed in some species e.g. orangutans *Pongo* spp. [9] but not others e.g. baboons *Papio* spp. [35]. Several studies demonstrate equal HC levels across the sexes e.g. grey wolves *Canis lupus* [7] whereas other studies reveal higher HCG levels in one sex e.g. vampire bats *Desmodus rotundus* [51]. Inter-specific variation in factors modifying HC levels may be attributed to inherent physiological differences across the species, or, alternatively confounding impacts of study design such as extraction methods [24].

A recent study on Alpine marmots similarly measured comparable HC across different age classes (using a single sample from each individual, [60]) although in contrast to our results, they report significant differences between the sexes with higher HC in females (in our study there was a tendency for HC levels to be higher in females than in males, but these differences were not significant). Samples from our study and those of Zenth et al. [60] were collected at the same time points in the females' reproductive cycle ruling out confounding influences on HC levels from reproductive hormones [14]. The different methods used in the study of Zenth et al. [60] and ourselves may explain the different results since the former used an assay developed and validated for use in humans whereas our study used assay methods validated specifically for Alpine marmots. It cannot be excluded that the absence of a significant gender effect in our study 2 is related to a lack of statistical power in view of the relatively small sample size ($n$ = 14 individuals).

Inter-individual variation in HC measured in our study, while not attributable to age or sex, would have reflected differences in factors known to modulate HC in other species such as social environment [11], human intervention [12], social rank [47], amount of aggression received [58], temperament [38], body condition [51], health [46] or reproductive status [23] all of which warrant further investigation in our species.

There are still multiple challenges and unknowns regarding the methods for quantifying HC such as the relative contributions of systemic versus localized cortisol entering the hair shaft [53], the impact of hair colour [6], location on the body [10], ultraviolet light [57], skin blood flow and rain [10] and hair type [36]. Whether these confounding factors apply to Alpine marmots is currently not known but requires further research.

## Background information

The ability to non-invasively monitor hypothalamic-pituitary-adrenal (HPA) function as a proxy of stress in wild populations by quantifying levels of glucocorticoids (GCs), is a powerful conservation tool [18]. Non-invasive estimation of GCs is used in the wild to monitor animal welfare [55], assess response to anthropomorphic factors [29], monitor the 'health' of a population [21], evaluate the impact of conservation management strategies [37] and make predictions pertaining to survival of wild populations facing environmental changes [49]. Although GCs can be quantified in numerous matrices the predominant media used for wild populations has been faeces [45]. Faecal matrices are particularly relevant for representing individual GC levels over a short time window (~ up to 24 h) [39]. However, there is a strong need to non-invasively quantify individual stress level over longer time periods to investigate animal response to chronic stress such as ongoing anthropogenic pressures [8]. Measuring GC from hair has been validated as a valuable biomarker of chronic HPA activity in numerous species [25,26]. There are several advantages of measuring GC in hair: Hair glucocorticoid (HC) levels are not impacted by recent events or circadian variations in GC levels [42], the sampling procedure is minimally invasive, hair samples are easy to store and remain stable for extended periods at room temperature [24], hair can be collected non-invasively at a distance without capture [31] and cortisol levels can be determined for specific time frames by selecting hair segments to analyse [50]. Hair samples are already being used to extract biological information such as DNA [44], toxins [27] and reproductive hormones [13] and are often already available research media. However, extraction and measurement methods to quantify HC need to be specifically validated for each target species

The importance of understanding intraspecific variation amongst individual characteristics, as opposed to mean population values is becoming increasingly recognized in conservation biology [41]. Individual differences in temperament [as determined in large by HPA reactivity and concomitant glucocorticoid levels [15]] determine how animals respond to environmental disturbances [16]. Populations with high inter-individual HPA reactivity (as reflected in high inter-individual variation in GC levels) are likely to be better able to cope with environmental changes than populations with low inter-individual variation [16]. Validated methods, such as those described here, that understand baseline GC concentrations at the level of the individual and degrees of variation between individuals, are thus essential if we want to understand the response of populations to environmental changes [19].

Our study builds on that of Zenth et al. [60] which explored the correlates of HC in Alpine marmots using methods validated for measuring HC in humans. The goal of the current study was to immunologically validate extraction and assay methods for extracting

and measuring HCs *specifically* for the Alpine marmot (*Marmota marmota*). Second, we wanted to compare the amount of HC yielded by two commonly used extraction methods. A variety of extraction and biochemical methods are used to quantify HC across the taxon, possibly since the study of HC as an index of stress is relatively new [24,28]. The lack of method standardization across HC studies hinders data interpretation and cross species and treatment comparisons [59]. There is a need for method standardization in the analysis of HC [52] but this requires systematic methodological comparisons to ascertain the most appropriate methods to use. For example there are cross study methodological differences in how the hair matrix is broken prior to extraction i.e. cutting the hair into small pieces using a sharp blade (e.g. [60]) or grinding the hair to a powder using a ball mill (e.g. [13]). Our second goal was to systematically compare the amount of HC yielded by these two methods i.e. cutting the hair using a sharp blade or grinding the hair to a powder using a ball mill. We predicted the latter would yield higher contractions of cortisol per unit of hair than the former due to the increased disintegration of the hair matrix, releasing HC and the larger surface area provided for extraction.

Finally, we examined the extent to which these customized methods could yield an individual specific GC level by examining within and between individual variations based on multiple hair samples from 14 individuals. Several studies point to high variation in HCs measured from different body regions of a single animal complicating the formation of individual specific HC levels e.g. Vancouver Island marmot [1], caribou *Rangifer tarandus granti* and reindeer *R. t. tarandus* [2], common marmosets *Callithrix jacchus* [4], grizzly bears *Ursus arctos* [10], horse *Equus ferus caballus* [33] and Canada lynx *Lynx canadensis* [56]. For this reason, our method specifically targeted hair samples taken from the same body region to see if these could yield individual specific values.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Elina Marielle Doss:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original draft. **Mathilde Jouffroy:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original draft. **Benjamin Rey:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original draft. **Aurélie Cohas:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – review & editing. **Achaz von Hardenberg:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – review & editing. **Tessa Ellen Smith:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Data curation, Writing – original draft.

## Data availability

Data will be made available on request.

## Ethics statements

All procedures were approved by the ethical committee of the University of Lyon (n8BH2012–92) and the University of Chester (044/16/ED/BS). The research was conducted in accordance with the EU Directive 2010/63/EU for animal experiments.

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