Endocrine response of gilts to various common stressors: A comparison of indicators and methods of analysis

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ABSTRACT
The first aim of the present study was to determine whether various common events encountered by pigs in commercial farms or experimental units induce activation of the sympathetic and hypothalamo-pituitary-adrenal (HPA) axes. The second aim was to compare the efficiency of various indicators and methods of analysis to detect the occurrence of a stress reaction. Responses to two blood sampling methods, immobilization by snaring, brief electric shocks, loud noise, ear tagging, tattooing, biopsy, pen relocation or delayed feeding time have been evaluated. Series of blood and saliva samplings (from 10 min before to 120 min after stressor application) were collected for each stressor on a total of 8 catheterized sows. Plasma glucose, lactate, cortisol and ACTH levels as well as salivary cortisol were measured. Acute increases of cortisol or ACTH (at least at time points +5 or +15 min) were observed for intense noise, electric shocks, ear tagging, tattooing, biopsy, cava blood sampling, snaring and pen relocation. Snaring, relocation and vena cava blood sampling generated longer stress responses whereas delayed meal and tail blood sampling had no influence. Plasma lactate was also significantly increased in several time-points after stressor application contrarily to plasma glucose. Comparison of successive time points with the starting basal level and comparison with the control group were more sensitive methods to detect a stress response to moderate stressors like electric shocks and tattooing, than comparing the area under the curve. These data confirmed that salivary cortisol is a good indicator to measure the HPA response to a stressor, provided that post-treatment levels can be compared with pre-treatment levels.

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1. Introduction

In commercial piggeries as well as in experimental units, welfare problems and stress reactions might result from a number of environmental stimuli, including feeding and housing conditions, social environment but also common management practices. For example, shot biopsy, an experimental method used to study muscle tissue, has been shown to induce significant acute heart rate and cortisol increases in pigs [1]. The use of electric goads in farms and slaughter houses has been proved to be stressful for pigs [2,3]. The severity of the fear, pain or discomfort induced by manipulations such as tattooing, ear tagging or various methods of blood sampling has never been properly evaluated. The most reliable tool to evaluate animal discomfort or stress is the measurement of indicators of the activity of the sympathetic and hypothalamus-pituitary-adrenal (HPA) axes [4,5]. In response to acute aversive stimuli, catecholamines are released in a few seconds by peripheral nervous sympathetic terminations and adrenals. Because catecholamines stimulate mobilization of glycogen, which results in glucose and lactate release [6,7], plasma glucose and lactate can be used as indirect markers of sympathetic activation [8]. Simultaneously to sympathetic activation, corticotrophin is secreted by the hypothalamus, which stimulates release in the blood stream of ACTH by the pituitary. This latter hormone induces secretion of glucocorticoids by adrenals. Both plasma ACTH and cortisol (the major form of glucocorticoids in Ungulates) are commonly used as stress markers [4,5].

Due to the presence of abundant external fat tissue in pigs, blood sampling in the vena cava is difficult and is often realized in combination with immobilization by snaring that generates a significant stress response by itself [9,10]. In order to avoid stress during blood sampling, blood can be collected through a venous catheter after surgical preparation of the animals. Such procedure complicates experimental protocols, requires individual housing of pigs and is not possible in field conditions. Cortisol can also be measured in other biological fluids collected with non-invasive methods. Since cortisol is mainly excreted in urine, urinary corticosteroids provide a valuable integrated measure of their production over a period of time [5,11]. However, cortisol concentration in urine must be corrected for differences in urine production and this method is not suitable to evaluate acute HPA axis activation due to the delay between stress and urine excretion. Cortisol metabolites are also excreted in faeces but faecal samples are difficult to collect and the
delay between stress application and excretion is even longer than for urine. Saliva can be sampled without stress by allowing the animal to chew on a cotton bud, provided that it has been familiarized previously to the experimenter and to the procedure. This method allows repeated samplings over a short period of time. Salivary cortisol has been used as an indicator of HPA axis activation in numerous species (goat: [12], sheep: [13–15], cattle: [16,17], Human: [18–20]) including pigs after various stimuli such as ACTH injection [21,22], social stress [23–25], snare restraint [26] and biopsy [27]. However, salivary cortisol is blamed for its lack of sensitivity and low correlation with plasma levels [5,28].

The first aim of the present study was to determine whether various common events encountered by pigs in commercial farms or experimental units differed in sympathetic and HPA axis activation. Therefore, responses to various blood sampling methods, immobilization by snaring, brief electric shocks, loud noise, ear tagging, tattooing, biopsy, pen relocation or delayed feeding time have been evaluated. The second aim was to compare the efficiency of various indicators and methods of analysis to detect the occurrence of a stress reaction.

2. Materials and methods

2.1. Animals, experimental procedure and sample collection

A total of 8 nulliparous non-pregnant Large White × Landrace females were used for the experiment in two replicates of 4 females each. Gilts aged 8–9 months of age and weighing 150–170 kg live weight were catheterized surgically into one jugular vein, under general anaesthesia using halothane, 8–10 days before the start of the experiment. After surgery and before sampling, females were trained to be handled. Gilts received a daily Regumate® treatment (Roussel-Uclaf, 92235 Romainville, France) starting two days before the beginning of the experiment in order to prevent cyclic-related variations in hormonal patterns. They were housed in the same room in individual pens (6 m² each) and could see and hear each other. They had free access to water. They received 2.6 kg of a standard feed formulated for gilts equally divided into two meals of at 0900 h and 1300 h in order to supply 170–185% of the metabolic energy requirements and to limit the food frustration that is commonly observed at this physiological stage. This food regimen was maintained all over the experiment except on the day of “delayed meal”. Gilts were euthanized at the end of the experiment in the local slaughterhouse.

Over a 3-week period, gilts were exposed successively to twelve potential stressful manipulations frequently encountered in commercial pig farms or experimental units, in the order described in Table 1. Gilts were exposed to these manipulations between 1300 h and 1345 h in order to be free of diurnal cortisol variations which are sharper in the morning, and to avoid any stress related to potential food frustration. The possible effect of the meal itself on endocrine variations was controlled on day 1 when gilts were normally fed without any stressful event afterward. The possible chronic frustration related to food, was evaluated by measuring the response to a delayed meal stress, which consisted in feeding at 1300 h the three neighbouring gilts but not the experimental one, who received her meal at 1600 h. Snaring consisted in restraining the gilts with a nose ring and keeping manually the lasso under tension for 5 min. For noise stress, a speaker was placed at a height of 1.2 m above the floor at one end of the corridor and a white noise recorded on a digital audio tape recorder was emitted for 5 min at 110 db. For tail blood sampling, the upper inner side of the tail was cleaned with 70% ethanol solution and a scalpel was used to make a quick and small incision (around 0.5 cm) through the skin until the caudal vena at a site located between two vertebrae. Three to four millilitres of blood were collected and bleeding was stopped by a bandage. The total procedure lasted less than 2 min. Four electric shocks (4.5 V) were applied at 5-s intervals with a commercial stock-moving prod. Ear tagging consisted in applying a plastic tag at 4–5 cm from the border, on the second upper third of the ear using a pair of pliers. Tattooing on the back was performed on both sides with a conventional farm inking pad. Vena cava blood sampling was performed in less than 2 min by venipuncture under snaring restraint. Shot biopsy is a method commonly used on living animals to sample muscle or adipose tissues for biological studies or genetic selection. A biopsy of 1 cm depth and diameter was performed at the level of the shoulder with a spring-loaded shot-biopsy device (Biotech, Slovakia). It lasted less than 1 min. Transportation and relocation stress consisted of an individual transport of gilts in a lorry for a couple of min to a new building, a short walk and entrance in individual farrowing crates where they were maintained. The overall procedure took about 5 min. The crates in the new building restrained movements and did not allow animals to turn round.

Pre-treatment blood samples were drawn at 12:50. Post-treatment blood samples were drawn 5, 15, 30, 60, 90 and 120 min after starting application of each stressor. Sample at 5 min was therefore collected 5 min after food delivery for the control meal group and, 30 to 50 min after food delivery for other stressors except the delayed meal. Blood was collected in EDTA containing tubes and aliquots of plasma were kept at −20 °C until assayed for cortisol, ACTH, glucose and lactate. Salivary samples were collected at the same times, except at 5 min, by allowing pigs to chew on cotton buds until they were moistened. Cotton buds were rapidly centrifuged at 3000 g for 15 min at 4 °C. Saliva samples were stored at −20 °C until cortisol measurement. Samples for the last stressor of one gilt were discarded since she became lame for a reason independent of the experiment. A few blood or saliva samples were missing due to problems of blood collection. However, each parameter was measured on at least 6 animals per stressor except glucose. This parameter was measured only in animals of the first replicate since preliminary analyses indicated no effect of any stressor.

2.2. Assays

Plasma concentrations of glucose and lactate were measured by automated enzymatic methods (Bio-Mérieux kits, Marcy l'Etoile, France) with a Cobas Mira multichannel analyzer (Hoffmann-Laroche, Basel, Switzerland). ACTH was measured in 200 μl plasma using a two-site [25] immunoradiometric assay (Nichols Institute Diagnostic, San Juan Capitano, CA, USA). The quantification limit of the assay was 5 pg/ml plasma, and the intra- and interassay CV were 2.8% and 6.8% at 206 pg/ml, respectively. Total plasma cortisol was measured in 50 μl samples using a competitive [26] RIA kit (Immunocheck, 95942 Roissy, France). The quantification limit of the assay was 2 ng/ml plasma and the intra- and interassays CV were 7.0% and 8.7% at 73 ng/ml, respectively. Salivary cortisol was measured in 200 μl saliva using a gamma coat cortisol [27] RIA kit (Diasorin, Antony, France). The detection limit

<table>
<thead>
<tr>
<th>Day</th>
<th>Stressor</th>
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<tbody>
<tr>
<td>1</td>
<td>Control meal at 1300 h (gilts 1–4)</td>
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<tr>
<td>2</td>
<td>First 5-min snaring (gilts 1–4)</td>
</tr>
<tr>
<td>3</td>
<td>5-min loud (110 db) white noise (gilts 1–4)</td>
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<td>4</td>
<td>Tail blood sampling (gilts 1–4)</td>
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<tr>
<td>7</td>
<td>Electric shocks (gilts 1–3) or delayed meal (gilt 4)</td>
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<tr>
<td>8</td>
<td>Ear tagging (gilt 2–4) or delayed meal (gilt 1)</td>
</tr>
<tr>
<td>9</td>
<td>Tattooing on the back (gilts 1, 3, 4) or delayed meal (gilt 2)</td>
</tr>
<tr>
<td>10</td>
<td>Vena cava blood sampling (gilts 1, 2, 4) or delayed meal (gilt 3)</td>
</tr>
<tr>
<td>11</td>
<td>Ear tagging (gilt 1), tattooing (gilt 2), vena cava blood sampling (gilt 3) or electric shocks (gilt 4)</td>
</tr>
<tr>
<td>14</td>
<td>Back fat shot biopsy</td>
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<tr>
<td>17</td>
<td>Second 5-min snaring</td>
</tr>
<tr>
<td>18</td>
<td>10-min lorry transportation and transfer to farrowing crates</td>
</tr>
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of the assay was 0.3 ng/ml cortisol and the intra and inter-CV were 7.7% and 9.8% at 6.1 ng/ml.

2.3. Calculations

Area under the curve was calculated for each parameter and day of sampling starting at 5 (plasma measures) or 15 min (salivary cortisol) after stressor application. For that purpose, the shape of the curve between two successive samples was estimated to be linear. Area was calculated from the “ground” as described by Pruessner [29].

2.4. Statistical analyses

Analyses of variance were performed using the mixed procedure of SAS® software (SAS, 2004). A square-root-transformation was performed for salivary cortisol and a log-transformation for concentrations of plasma cortisol, ACTH and lactate and for areas under the curve in order to normalize data. A first repeated-in-time model included stressor and gilt as main effects, the stressor×time interaction and the stressor×gilt as subunits. Since the stressor×time interaction was significant for plasma cortisol, ACTH and lactate as well as for salivary cortisol, subsequent analyses were performed within each stressor on one hand and within each time of sampling on the other hand. Areas under the curve were analyzed in a similar way. All these models included the animal as a random effect. In order to test whether baseline levels varied across days of sampling, ANOVA were performed that included the effects of the day of sampling (main effect) and animal (random effect). When a F-statistic was significant (P<0.05), comparisons between means were performed by using pre-planned contrasts. A paired Student-t test was used to compare the ratio salivary cortisol/plasma cortisol measured at 5 min before stressor application to the control meal at 5 and 15 min for electric shocks, vena cava sampling and both snarings, from 5 to 60 min for relocation (P<0.05, Table 2, Fig. 2). Between snaring 1 and 2, lactate did not differ at any time (P>0.1). The area under the curve was increased for electric shocks, vena cava blood sampling, both snarings and relocation (P<0.05, Table 2).

Within stressor, analyses indicated that plasma lactate varied significantly with time for all stressors except control meal and ear tagging (Table 2). However, comparisons with the pre-treatment value indicated a significant increase within 30 min only for electric shocks, tattooing, vena cava sampling, and relocation. After 30 min, a significant increase was observed for tail blood sampling and a significant decrease for delayed meal and biopsy.

3.2. Plasma ACTH

Pre-treatment concentrations of plasma ACTH varied significantly across sampling days (P<0.01) but no clear trend of variation could be observed. Overall, mean pre-treatment concentration was 12.4 pg/ml with a coefficient of variation of 86% (n=93). ACTH was elevated in comparison to the control meal at 5 and 15 min for electric shocks and tattooing, from 5 until at least 30 min for biopsy, vena cava blood sampling, both snarings and relocation (P<0.05, Table 2, Fig. 2). A significant increase of the area under the curve was depicted for biopsy, vena cava blood sampling, both snarings and relocation. Between snaring 1 and 2, ACTH did not differ at any time (P>0.1) except before and 90 min after stressor application when concentrations were higher for the second snaring (P<0.05).

Within stressor, analyses indicated that plasma ACTH varied significantly with time for all stressors except control meal, tail
blood sampling, delayed meal and ear tagging (Table 2). Comparisons with the pre-treatment value indicated a significant increase at 5 min after noise, at 5 and 15 min for electric shocks and biopsy, from 5 until at least 30 min for tattooing, vena cava blood sampling, both snarings and relocation.

### 3.3. Plasma cortisol

Pre-treatment concentrations of plasma cortisol varied significantly across sampling days (P<0.05) but no clear trend of variation could be observed. Overall, mean pre-treatment concentration was 1.4 ng/ml with a coefficient of variation of 62% (n = 91). Plasma cortisol was elevated in comparison to the control meal at 15 min for vena cava blood sampling, and at 15 and 30 min for both snarings and relocation (P<0.05, Table 2, Fig. 2). A significant decrease was observed at 60 min after stressor application only for noise. A significant increase in the area under the curve was depicted for both snarings and relocation. Contrarily a significant decrease was observed for delayed meal and noise. Between snaring 1 and 2, salivary cortisol did not differ at any time (P>0.1).

Time had a significant influence on salivary cortisol for all stressors (Table 2). A significant increase relatively to pre-treatment concentration was observed at 15 min for tattooing and vena cava blood sampling, at 15 and 30 min for electric shocks, biopsy and relocation, from 15 to 60 min for both snarings. A significant decrease was observed at 60 min or later for control and delayed meals, and for noise.

The average ratio between salivary and plasma concentrations was 0.15 ± 0.02 (mean ± s.d., n = 531). Interestingly, it was higher before stressor application for intensive stressors (snaring, jugular vein sampling, relocation). Salivary cortisol increased significantly with plasma cortisol levels (P<0.001, Fig. 3) but the slope of the regression varied between gilts as indicated by a significant gilts × plasma cortisol interaction (P<0.001). For individual gilts, the correlation between plasma and salivary cortisol varied between 0.75 and 0.86.

### 3.4. Salivary cortisol

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4. Discussion

The present experiment allowed classifying stressors according to the endocrine response they elicited in gilts but it should be emphasized that these responses were variable among animals especially for mild stressors like biopsy or electric shocks. Moreover, these data showed that lactate can be used as an indicator of stress reaction contrarily to glucose. They also allowed classifying indicators according to their ability to detect a physiological stress response.

Present data showed that submitting pigs to stressors at 1 to 3 day intervals had no effect on baseline levels of cortisol and ACTH since, although pre-treatment concentrations varied across sampling days, no clear trend of variation could be observed. This is in agreement with previous experiments (space restriction: [30]; unpredictable electric shocks: [31]). Our data indicated that control meal was not followed by modifications in HPA axis activity as previously reported [32]. This indicates that the hormonal response to the meal was weak and thus did not significantly interfere with the responses to the experimental stressors which were applied 30 to 45 min later. The lack of activation of the HPA axis to delayed meal can be explained by the fact that gilts received a first meal 4 hours before with amounts of food covering their metabolic requirements. Therefore, they were probably not hungry enough to feel frustration.

Tail blood sampling did not elicit any significant increase of any physiological indicator suggesting a lack of stressful effect. Ear tagging and noise were followed by occasional increases suggesting a low stressful effect. The lack of clear stress reaction after acute food frustration, ear tagging and loud noise is in accordance with the few studies that investigated the response to these stressors [33–35]. We are not aware of any other experiment testing tail blood sampling. Although the tail is innervated until the tip [36], nociceptive stimuli induced by disruption of the tissues were probably not intense enough to induce an adrenal and sympathetic stimulation. Electric shocks, tattooing, biopsy, vena cava blood sampling, snaring and relocation induced ACTH and cortisol increases that were repeatedly detected suggesting a deeper stressful effect. However, amplitude and duration of increases varied among this last group of stressors. The highest amplitude and longest duration were observed for vena cava blood sampling, the two snarings and relocation, whereas tattooing the animals in the back as well as fat biopsy were followed by shorter increases in plasma cortisol and ACTH as well as less clear salivary cortisol increases, especially when comparisons were done with the
control group. Performing a deeper biopsy, which disrupted muscle tissue in addition to the subcutaneous fat tissue, Geverink et al. [37] reported also an increase in salivary cortisol which was significant when the comparison was done with the pre-treatment level but not with the control group. Overall, our data clearly indicated that electric shocks, vena cava blood sampling, snaring for 5 min as well as transport followed by transfer to a new environment elicited a stimulation of the adrenal (depicted by cortisol and ACTH) and sympathetic (depicted by lactate) axes in agreement with previous studies [electric shocks: [23], snaring for 3.5 to 15 min: [9,10,38,39], transport and transfer to new environment: [40–43]]. However, contrarily to Desautes et al. [43], our data did not show any sign of glucose increase after relocation. Based on these observations, for the following discussion, vena cava blood sampling, both snarings and relocation will be qualified as intense stressors, electric shocks, tattooing and biopsy as moderate stressors, ear tagging and noise as weak stressors.

Differences in the response to these various stressors can be explained by intrinsic characteristics of the stressors that are known to influence the behavioural and physiologic responses like the nature, intensity and duration of the sensorial/psychological stimulus, and the controllability of the situation [4,44–46]. For example, tail blood sampling did not induce any hormonal response but also no flight attempt (observations not shown), suggesting that the intensity of the nociceptive stimuli was not sufficient to induce a neuro-endocrine reaction and hence to be perceived as painful by the animals. Electric shocks and tattooing were followed by attempts to escape of the pigs (observations not shown) and were sufficient to induce a neuro-endocrine reaction but of relatively low amplitude and duration probably in relation to the intensity and duration of the stimuli. Finally, long, intense and inescapable stressors like vena cava blood sampling, snaring and relocation induced a sustained neuro-endocrine response. Furthermore, comparison of the response to snaring at a 2-week interval confirmed that the HPA response of pigs to a sensorial inescapable stressor does not decrease, which is in agreement with previous studies [electric shocks: [3]; snaring: [38]; biopsy: [27]]. Contrarily, the cortisol response to a stress with a high emotional component such as social stress seems to decrease when it is repeated [47,48]. However, it should be pointed out that the experimental design of the present study does not allow excluding with certainty that the endocrine responsiveness was not subjected to some kind of interactions between successive stressors, like sensitization or desensitization. However, if it existed, this phenomenon was not strong since pre-stress blood and salivary cortisol levels did not increase nor decrease along the successive days and the response to a repeated stressor (snaring) was stable. Finally, it is interesting to mention that, although there was a high variability of responses among gilts, a classification analysis revealed that it was not possible to classify gilts as high or low responders (data not shown).

Our data confirmed that there was a very good correlation between salivary and plasma concentrations of cortisol and that measuring cortisol in saliva is a suitable technique to detect an HPA activation as already shown in pigs. Nevertheless, comparing the results of statistical analyses for salivary and plasma cortisol, our data showed that significant increases after stressor application were more frequent in plasma than in saliva, especially when comparisons were done between a stressor and the control group. A lower increment in saliva than in blood of cortisol levels may explain why it was more difficult to detect a stressor effect. Comparing the results of statistical analyses for plasma cortisol and ACTH, our data indicated that the increase is usually detected earlier for ACTH (usually at 5 min) than for cortisol (usually at 15 min). In most cases, the time lapse for normalization of cortisol and ACTH concentrations was similar. However, after application of stressors of moderate intensity (e.g. ear tagging and noise), the increase was significant only at 5 min for ACTH and at 15 min for cortisol. Overall, the efficacy to depict a physiological reaction after a stressor application was very similar, provided that the sampling is performed 15 min after the application of stressor. Plasma lactate was slightly less efficient since statistical increments after ear tagging, noise and biopsy could not be detected. It should be reminded that lactate increases very rapidly but for a short duration after a stressor application [8] under the influence of adrenalin that stimulates breakdown of glycogen and production of lactate in muscles [6,7]. Therefore, the increase observed at 30 min and later after tail blood sampling should not be interpreted as a sign of stress. This increase could be related to a slow increase of plasma lactate after the meal as previously observed [49,50]. This meal effect was probably detectable only after tail blood sampling because baseline levels were very low on that day. Contrarily, plasma glucose was not altered by any of the stressors in agreement with previous observations from our laboratory [8]. This is in contradiction with data from other laboratories showing an increase in plasma glucose after a stressor application [43,51,52]. Since in our experiment the various stressors were applied 30 to 45 min after the meal, when insulin was probably high [53], it can be hypothesized that the postprandial increase in insulin had prevented the mobilization of glycogen from the liver. In other experiments, a lack of glucose increase after stress was related to a fasted state when glycogen stores from the liver were probably depleted [51]. Taken together, these results suggest that glucose variation is a poor indicator of a stress response.

In the present study, the levels of salivary cortisol were approximately 15% of those measured in plasma, which is comparable to the ratio reported previously [22,39]. In addition, our data show that the slope of the regression between salivary and plasma concentrations vary between gilts. Inter-individual variations may be related to variations in binding capacity of the plasma towards cortisol [54] or to variations in the activity of 11 beta-hydroxysteroid dehydrogenase type II that is expressed in salivary glands and inactivates cortisol into cortisone [55]. Interestingly, the salivary/ plasma cortisol ratio decreased shortly after stressor application in agreement with previous data [21,39,56]. This could be interpreted as a delay for cortisol to increase in saliva relatively to plasma. In accordance with this hypothesis, it was found that the correlation between salivary and plasma cortisol was stronger in situations where they induced stable long term secretion of cortisol than when they induced an acute cortisol rise [56].

The area under the curve calculated for an hormone after a stressor application is thought to be an integrated measure of the animal’s response [17,29]. For intense stressors, an increase in the area under the curve was always depicted except for salivary cortisol after vena cava blood sampling where only a tendency was observed ($P = 0.1$). For moderate stressors, significant increases of the area under the curve could be detected only for plasma lactate after electric shocks and for plasma ACTH after biopsy. Limiting the calculation of the area to the first 60 min after stressor application gave the same results except for plasma cortisol after electric shocks whose area became significantly higher than for the control meal (data not shown). Therefore, area under the curve is a good indicator of a stress response for stressors that elicit a sustained response but is not efficient after a moderate stressor.

5. Conclusion

Present data confirmed that measuring cortisol in saliva is a good method to measure the HPA response to a stressor. Although it is less sensitive than measuring cortisol in blood, saliva has the advantage to be easily collected without inducing any physiological reaction. However in order to improve sensitivity, post-treatment levels should be compared with pre-treatment levels. Taking into account that increased levels of cortisol are often measured for only 30 min, any perturbation should be carefully avoided during that period. In addition to these guidelines, present experiment demonstrated or confirmed that some
techniques commonly encountered in commercial farms or in experiments stimulate the HPA and SNA axes. Among them, snaring, relocation and vena cava blood sampling have a strong influence whereas delayed meal, tail blood sampling and noise have a weak influence.

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