BEHAVIOURAL AND PITUITARY-ADRENAL CHARACTERISTICS OF PIGS DIFFERING BY THEIR SUSCEPTIBILITY TO THE MALIGNANT HYPERTHERMIA SYNDROME INDUCED BY HALOTHANE ANESTHESIA. 2. Pituitary-adrenal function

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2. Pituitary-adrenal function.

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Résumé.

CARACTERISTIQUES COMPORTEMENTALES ET HYPOPHYSO-SURRENALIENNES DE PORCS AYANT UNE SENSIBILITE DIFFERENTE AU SYNDROME D'HYPERTHERMIE MALIGNE INDUIT PAR L'ANESTHESIE A L'HALOTHANE. 2. FONCTION HYPOPHYSO-SURRENALIENNE. — Le fonctionnement de l'axe hypohyso-corticosurrénalien a été étudié chez deux groupes de porcs Piétrain différant par leur sensibilité à l'halothane et chez des porcs Large White (LW).

Les taux de base de glucocorticoïdes circulants, mesurés par radiocompétition protéique, et d'ACTH plasmatique, déterminés par radioimmunologie, ne diffèrent pas selon la réactivité à l'halothane mais sont plus élevés chez les porcs LW. Les trois groupes expérimentaux ne diffèrent pas par la réactivité de l'axe corticotrope à diverses stimulations (exploration d'un environnement nouveau, stress mécanique). Il en va de même en ce qui concerne l'inhibition fonctionnelle par la dexaméthasone et la réponse de la corticosurrénale à l'ACTH.

Ces résultats montrent que les porcs LW ont une sécrétion tonique d'ACTH plus élevée que les porcs Piétrain, mais une réponse phasique aux stimulations identique. Le facteur de sensibilité à l'halothane n'a pas, par lui-même, d'influence sur les critères mesurés.

Survival requires organisms to develop processus maintaining a constant internal environment in the face of changing stimuli. Superimposed on these homeostatic responses are other processes designed to meet more serious challenges. One process of particular importance is the release of corticoids by the adrenal cortex. This response has been shown to be elicited by a wide variety of psychological and physical stimuli and has therefore become, in accordance with Selye's concept (1950), the operational definition of stress in physiologically oriented stress research.

Despite extensive investigation, the adaptative value of this pituitary-adrenal response is not yet fully understood. Mason (1971), in a reevaluation of the concept of the non specificity of the stress response to various stressors, suggested that the pituitary-adrenal system was activated either when glucocorticoid secretion was necessary to the physiologic and metabolic processes leading to the maintenance of homeostatic equi-

librium (e.g. when the organism is exposed to cold) or as a response to the psychological component of stressors (e.g. the novelty of the stressfull environment), this last case being responsible for the apparent non specificity of the adreno-cortical activation. According to this last statement, the adaptative value of the adreno-cortical response cannot be understood without taking into account the relationships between the psychological reactions to aversive situations (as measured in behavioural tests) and the endocrinological status of the organism.

Although many investigators have studied the relationships between pituitary-adrenal function and stress susceptibility in pigs, defined either by muscle quality or response to such tests as halothane challenge, exposition to high temperatures or physical exercise (cf. for a recent review Cassens *et al.*, 1975), little if any work has been devoted so far to the behavioural approach. Moreover the results of the physiological studies appear far from being conclusive, since the stress-susceptible pigs have been described as suffering from adrenal insufficiency (Ludvigsen, 1957; Judge *et al.*, 1968), exhibiting adrenal hypereactivity (Marple and Cassens, 1973) or not differing from normal pigs (Aberle *et al.*, 1976).

The present study was undertaken to determine the respective influence of halothane sensitivity and breed on the pituitary-adrenal function measured by basal levels and stress response of glucocorticoids and ACTH in pigs and to explore eventual relationships between the endocrinological status and the behavioural responses (Dantzer and Mormède, 1978).

Methods.

Subjects and housing conditions have been fully described in a previous report (Dantzer and Mormède, 1978). Forty-eight pigs including 16 Pietrain halothane positive, 16 Pietrain halothane negative and 16 Large White (LW) halothane negative pigs were used as experimental subjects. Blood was withdrawn at determined times from conscious animals, by puncture of the *vena cava*, in order to obtain plasma which was subsequently frozen until hormone assays. All blood samples were collected in vacuum tubes (Vacutainer, Becton-Dickinson) containing sodium heparine (143 U.S.P. unit per tube) or EDTA (15%, 0.048 ml) and potassium sorbate (0.34 M per tube) for glucocorticoid and ACTH respectively.

Experimental procedures.

The subjects were successively submitted to three experimental conditions, at weekly intervals.

1. EXPLORATION OF A NEW ENVIRONMENT

Pigs were individually introduced into a two-compartment cage for a 20 min. session (Dantzer and Mormède, 1978). Blood was collected before the beginning of the experimental session and 30 min. after its completion to assess besal levels of corticosteroids and response to this psychological stressor.

2. SHAKING

One week later, half the subjects were submitted, by three groups of 2 animals, to a shaking stress consisting of a 6-hour duration confinement in a $1.5 \times 1.0 \times 0.75$ m metal cage oscillating along the three dimensions (amplitude of horizontal oscillations: 115 mm; vertical oscillations: 95 mm; revolving speed: 55 cycles per min.; 30 min. clock wise rotation alternating with 30 min. anti-clock wise rotation). Controls remained in their pen during the same period. Blood was collected from control and stressed animals before (0 h.), 1 h. after the beginning (1 h.) and at the end (6 h.) of the test, in order to measure basal ACTH and glucocorticoid levels (0 h.), ACTH and glucocorticoid response to shaking (1 h.) and evolution of the glucorcorticosteroid response (6 h.).

3. FUNCTIONAL INVESTIGATIONS OF THE PITUITARY-ADRENAL AXIS

To assess the ability of a synthetic glucocorticoid, dexamethasone (DX) to block pituitary ACTH release, and to test the adrenocortical reactivity to a standard dose of ACTH, dexamethasone acetate (0.2 mg/kg) was injected by the intramuscular route at 10.00 a.m. (Dectan, SOVETAL); four hours later, ACTH 1-24 (5 μg/kg or 0.5 I.U. per kg) was administered by the same route (Synac-

thène, CIBA-GEIGY). Blood was taken immediately before DX administration, before ACTH injection and 1 hour later. These doses and time intervals were selected according to the results from previous experiments (Favre and Moatti, 1977).

Hormonal assays.

1. Plasma *corticosteroids* were estimated by a protein binding assay (Murphy, 1967) using pregnant woman plasma for preparation of cortisol-binding globulin (Favre and Moatti, 1977). Corticosteroids were extracted from 100 µl plasma with 4 ml dichloromethane. Two 1 ml fractions were evaporated to dryness under a light air stream. They were added 200 µl of 1% pregnant woman plasma (stripped from endogenous steroids with charcoal) in 0.1 M phosphate buffer (pH 7.4) and 5 nCi of ^{3}H-cortisol (C.I.S., 45-55 mCi/mM). The assay tubes were incubated at 40°C during 20 min. and then equilibrated at 0°C in crushed ice during at least 1 hour. Free and protein bound radioactivity were separated using 1 ml of a coated charcoal suspension (1 ml of gelatin powder MERCK, 2.5 mg of Norit A charcoal SERLABO and 0.25 mg of Dextran T 70 PHARMACIA per ml of 0.1 M phosphate buffer). After a 45-min. incubation period, assay tubes were centrifugated. The whole supernatant was added to 3.6 ml of scintillation liquid (Istagel PACKARD) and radioactivity was counted in a spectrometer (Intertechnique, SL 33). The reference binding curve was made with 0, 0.3125, 0.625, 1.25, 2.5 and 5 ng of cortisol and non specific binding was measured with cortisol excess (25 ng). Results were calculated from the standard curve after logit-log transformation.

2. Plasma levels of *ACTH* were determined with a commercially available heterologous radioimmunoassay kit (ACTH-K, CEASORIN), using synthetic human ACTH as a standard (range 25-800 pg/ml), iodine labelled porcine ACTH as a tracer and a rabbit anti-human ACTH serum. Blood was collected in refrigerated Vacutainers containing EDTA, which were immediately centrifugated to separate the plasma. The plasma samples were stored in polystyrene tubes at —20°C until analyzed. There was no prior extraction; 0.1 ml plasma was added to the incubation medium containing a barbitone buffer (pH 8.4), mercaptoethanol and the appropriate reagents (0.1 ml labelled ACTH and/or 0.1 ml antiserum). The incubation volume was 1 ml. After 48 h. incubation at 4°C, free ACTH was separated from bound ACTH using charcoal. After centrifugation, the supernatants were decanted into separate tubes and counted in an Intertechnique liquid scintillation counter, using gamma-vials (KOCH-LIGHT). The concentration of ACTH in plasma samples was obtained by reference to the calibration curve which was plotted as the logit of the percent of zero radioactive antigen bound value (logit B/B_0) *versus* log concentration of unlabelled antigen.

Parallelism between samples and standards was assessed using serial dilutions of a high ACTH plasma sample, obtained from a pig stressed by chasing with a goad, by a low ACTH plasma sample, obtained from a pig treated twice at 4-hour interval with 0.2 mg/kg dexamethasone acetate. The results are illustrated in Fig. 1.

Only 12 out of the 16 plasma samples in each experimental group were used for ACTH determination.

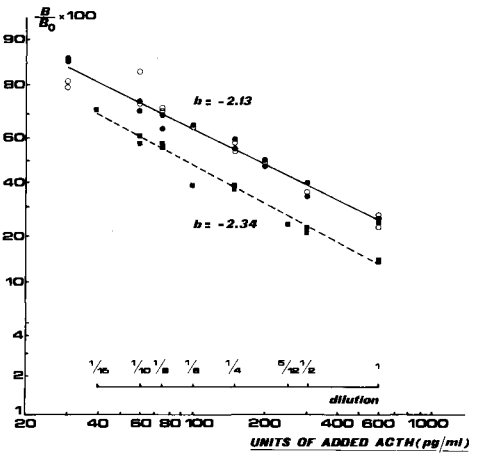

Fig. 1.—Parallelism between the capacity of synthetic human ACTH (upper line) and porcine immunoreactive ACTH to displace labelled porcine ACTH from a rabbit anti-human ACTH serum. The data have been pooled from two different assays, using the same batch of reagents.

Table 1.—Basal levels of glucocorticoids and ACTH in pigs.

Experimental group	Number of subjects	Log scale (mean+S.E.M.)	Arithmetic scale [a]	
			Mean	5% confidence interval
I. *Glucocorticoids*				
Pietrain negative	16	0.443±0.033	2.77	1.00-7.71
Pietrain positive	16	0.498±0.037	3.15	1.00-9.90
LW negative	16	0.568±0.028	3.70	1.52-8.97
II. *ACTH*				
Pietrain negative	12	1.673±0.096	47.1	5.0-443
Pietrain positive	12	1.728±0.093	53.4	6.2-460
LW negative	12	1.957±0.057	90.6	24.2-339

a) glucocorticoids: μg/100 ml; ACTH: pg/ml.

Results.

1. DISTRIBUTION OF THE RESULTS

The distribution of plasma hormonal levels in the population was analyzed by the W test of Shapiro and Wilk (1965). Apart from basal corticosteroid levels in experiment I, populations were log normally distributed. We therefore used the $\log_{10}$ transformate of the results for statistical analysis and in figures.

2. BASAL HORMONE LEVELS (Table I).

Basal glucocorticoid levels were measured three times at weekly intervals. Results were submitted to a 3-way analysis of variance (3 measurements×3 groups×2 sexes). The only significant variation resulted from the group factor ($F=3.59$; d.f.=2,126; $P<0.05$), with LW pigs differing from Pietrain negative animals (Newmann-Keuls test: $P<0.05$).

Basal ACTH levels were measured twice (experiment II and III). The group factor was only marginally significant ($F=3.04$; d.f.=2,60; $P<0.10$). Other factors and interactions were not significant.

Basal ACTH and glucocorticoid levels measured in the same plasma samples were highly correlated (experiment II: $r=0.514$; d.f.=34; $P<0.01$; experiment III: $r=0.548$; d.f.=34; $P<0.01$).

3. ADRENOCORTICAL RESPONSE TO THE EXPLORATION OF A NEW ENVIRONMENT

Glucocorticoid measurements were submitted to a 3-way analysis of variance (before *vs.* after exploration × 3 groups × 2 sexes). The only significant variation resulted from the time factor ($F=32.34$; d.f.=1,84; $P<0.01$). Basal levels were 3.57 μg/100 ml (1.23-10.7 μg/100 ml) [1]; 30 min. after the experimental session the plasma glucocorticoid levels reached 6.44 μg/100 ml (2.77-14.9 μg/100 ml) [1]. Neither interaction reached statistical significance: the adrenocortical response to the exploration session was therefore the same whatever the experimental group.

4. PITUITARY-ADRENOCORTICAL RESPONSE TO SHAKING (Fig. 2).

Plasma glucocorticoid levels were submitted to a 3-way analysis of variance (2 conditions×3 periods×3 groups). The experimental condition (shaking *vs.* control) ($F=8.45$; d.f.=1,126; $P<0.01$) and the time factor ($F=21.37$; d.f.=2.126; $P<0.01$) were both significant. The group factor was only marginally significant ($F=3.01$; d.f.=2,126; $P<0.10$). Concerning the interaction terms,

(1) Confidence interval (P=0.05).

only the experimental condition × time interaction reached significance ($F=20.14$; d.f.$=2,126$; $P<0.01$). Comparisons between means revealed that the only significant difference was between stressed animals at the 1 hour time and the other means ($P<0.01$). These results indicate that shaking is an effective stressor in pigs, but that the adrenocortical axis habituates quickly to this stimulus. Adrenocortical activation and habituation of the response are the same whatever the experimental group.

Plasma ACTH levels of pigs subjected to shaking increased significantly after 1 hour of such a treatment ($F=94.8$; d.f.$=1,30$; $P<0.01$): LW pigs had higher levels of ACTH than Pietrain positive or Pietrain negative pigs ($F=18.4$; d.f.$=2,30$; $P<0.01$) but did not differ in response to shaking (interaction experimental condition × group: $F=2.39$; d.f.$=2,30$). During the same period the plasma ACTH concentrations of controls did not change ($F=0.07$; d.f.$=1,30$).

5. FUNCTIONAL TESTS

Pituitary-adrenocortical blockade by dexamethasone: Four hours after the administration of 0.2 mg/kg dexamethasone acetate, plasma glucocorticoid levels were under 0.5 μg/100 ml in every animal. This level being the sensitivity limit of our dosage, no further analysis was performed on these results. ACTH concentrations were clearly distributed in two subgroups, the first under 10 pg/ml (the sensitivity limit of the radioimmunoassay) and the second above this limit (respectively 8 pigs and 4 pigs in Pietrain negative animals, 6 and 6 in Pietrain positive pigs 5 and 7 in LW pigs). The repartition between these two subgroups was not significant ($\chi^2=1.48$; d.f.$=2$).

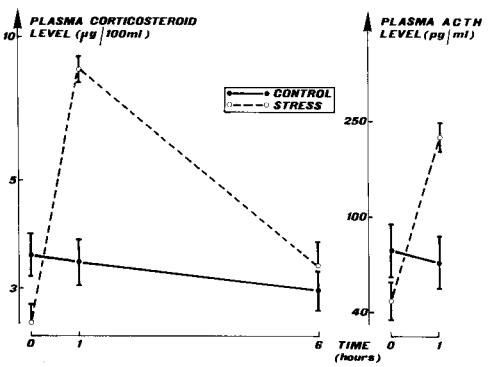

Fig. 2.—Evolution of the plasma glucocorticoid (N=24) and ACTH (N=18) concentrations (mean ± S.E.M., log scale) of pigs subjected to the shaking stress, compared with control animals remaining in their pen during the same period.

Adrenocortical response to ACTH stimulation: One hour after ACTH 1-24 (0.5 I.U. per kg) plasma glucocorticoid levels were not different between groups and sexes. Overall mean was 10.8 μg/100 ml (6.7-17.5 μg/100 ml) [2].

Discussion.

Taken as a whole, the results of the present experiments compare favourably with current data available from the literature about pituitary-adrenal function in pigs. When measured by competitive protein binding methods, basal levels of glucocorticoids usually lay between 2 and 6 μg/100 ml (Whipp *et al.*, 1970; Killian *et al.*, 1972; Stith and Bottoms, 1972; Topel *et al.*, 1973; Weiss and Scherzinger, 1973; Marple *et al.*, 1974; Aberle *et al.*, 1976; Favre and Moatti, 1977). Concerning plasma ACTH concentrations, the range of variation found in the literature appears extremely high since such levels as 40 pg/ml or less (Donald *et al.*, 1968) or 790 pg/ml (Marple *et al.*, 1972a), with various intermediate values (Marple *et al.*, 1972b; Sebranek *et al.* 1973; Blatchford *et al.*, 1974) have been described. The ACTH levels found here have been expressed with reference to a human ACTH standard curve; therefore their relative variation is of major importance than their absolute value.

The contrast between the higher basal levels of corticosteroids and ACTH found in the present study and the absence of difference in resting cortisol levels of 60 kg Pietrain and Large White pigs (Lister *et al.*, 1972) is difficult to explain and could be due to such factors as age variations, environmental factors, or strain differences within

(2) Confidence interval ($P.=0.05$).

the same breed. In any case, the existence of breed differences in basal hormone levels has been recognized (e.g. Marple *et al.*, 1972a; Aberle *et al.*, 1976); however the biological adaptative value of such differences has not been questioned. In physiologically oriented stress research, the criterion of adrenocortical activation has become the operational definition of stress. In pigs, investigators have concentrated on the relationship between pituitary-adrenocortical activity and stress susceptibility defined by *post mortem* muscles changes or *ante mortem* reaction to standardized "stessors" (eg. exposition to high temperatures, forced exercise or halothane challenge). First studies, in 1956-1968, have suggested that pigs exhibiting the pale, soft, exsudative condition were suffering from adrenal insufficiency (Ludvigsen, 1957; Henry *et al.*, 1958; Judge *et al.*, 1966, 1968). However it has subsequently been reported that stress-susceptible pigs have elevated concentrations of plasma ACTH, without corresponding increases in plasma cortisol concentration, due to a higher metabolic clearance rate of cortisol (Marple *et al.*, 1972b; Marple and Cassens, 1973; Sebranek *et al.*, 1973). But in the above mentioned studies, stress-susceptible pigs were also from different breeds and/or strains. Aberle *et al.* (1976) found no differences in plasma cortisol level between littermate pigs which were either negative or positive to the halothane screening test. Identical conclusions were reached here, with extension to plasma ACTH levels, the only significant differences being between Pietrain and Large White pigs. The same was true for the response to stressors: exposure of animals to a new environment or to shaking had pronounced effects on the pituitary-adrenal axis, but the variation was not related to the stress-susceptibility status. Moreover the adrenal reactivity to ACTH was the same whatever the experimental group. Sebranek *et al.* (1973) reported that ACTH release was not blocked by dexamethasone in stress-susceptible pigs, although glucocorticoid levels were near zero; we were unable to find the same result: some pigs had high ACTH levels (with a maximum value of 420 pg/ml) with near zero plasma glucocorticoid concentrations, but this was clearly unrelated to halothane susceptibility.

Most of the previous cited work bears on slaughter pigs, i.e. 5-6 months old pigs, weighing 90-100 kg. In the present study, juvenile subjects were used. These age differences could have consequences on such factors as pituitary-adrenal function or stress susceptibility. Concerning the former point, there is no evidence of large variations in pituitary-adrenal function between two and six months of age, the major changes being observed during the first days of life (Dvorak, 1972). It is known that the incidence of the porcine stress syndrome is higher in slaughter pigs, and also that halothane susceptibility can be detected more easily, but also with more risk, in older pigs, but, to our knowledge, no systematic study bears on this subject.

From our results, it is apparent that breed differences in plasma cortisol levels have to be related to variations in plasma ACTH concentrations rather than to differential sensitivity of the adrenal to ACTH stimulation. These data suggest that LW pigs have a higher central tonus of ACTH secretion than Pietrain pigs, but with a similar phasic response to stress. In a previous report (Dantzer and Mormède, 1978), we have established that behavioural differences are more pronounced between breeds than according to halothane susceptibility status: Pietrain pigs are characterized by a better ability to initiate active responding in the face of aversive events than LW pigs. Considering both the behavioural differences and the endocrinological status, it is tempting to question whether they are dependent on a common brain state related for instance to the predominance of a given neurochemical system, the nature of which is still open to speculation.[3]

(3) ACTH secretion has been shown to be regulated by neurochemical mechanisms (Wilson, 1974). Although the exact neurotransmitters involved in these processes are still unclear, noradrenaline appears to play the role of an inhibitory transmitter. In a similar vein, it is of interest to note that in pigs neuroleptics such as azaperone, with potent central antiadrenergic activity, increase the plasma level of corticosteroids (Blatchford *et al.*, 1978). According to these data, it might be speculated that LW pigs are characterized by a lower central noradrenergic activity, an hypothesis already formulated on the basis of behavioural measures in the same animals (Dantzer and Mormède, 1978).

In general, it appears that a negative or a positive reaction to the halothane screening test is of little if any help to understand the mechanisms and physiological significance of responses to aversive situations. This test undoubtly reveals a genetic taint, with lethal consequences, but to reduce all the stress problems in pig husbandry to such a test is just a miscalculation. More has to be gained from a more general approach, emphasizing the relationships between the organism and its socio-physical environment and acknowledging the complexity of the stress response rather than attempting to bring it down to a convenient and *ad hoc* formulation.

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Summary.

Pituitary-adrenal function was investigated in two groups of Pietrain pigs differing by their susceptibility to the malignant hyperthermia syndrome induced by halothane, and in Large White pigs (LW).

Plasma basal levels of glucocorticoids, measured by a protein binding technique, and plasma ACTH concentrations, determined by radioimmunoassay, did not differ according to halothane susceptibility but were higher in LW pigs compared with Pietrain pigs. The reactivity of the pituitary-adrenal axis to such stressors as exposition to a novel environment or shaking was the same for all experimental groups. The same was true concerning plasma levels of glucocorticoids and ACTH after injection of dexamethasone and response of the adrenal cortex to a standard dose of ACTH.

These results demonstrate that LW pigs have a higher tonic secretion of ACTH than Pietrain pigs, but with identical phasic response to stimulations. Halothane susceptibility by itself appears to have no influence on pituitary-adrenal function.

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