Martin, J. E., Christensen, K., Vizzier-Thaxton, Y., and McKeegan, D. E.F. (2016) Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning. *Applied Animal Behaviour Science*, 180, pp. 157-165. (doi:10.1016/j.applanim.2016.05.007)

This is the author's final accepted version.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.
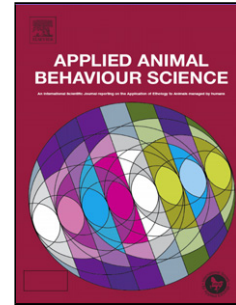
http://eprints.gla.ac.uk/119539/

Deposited on: 23 May 2016

Accepted Manuscript

Title: Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning

Author: Jessica E. Martin Karen Christensen Yvonne Vizzier-Thaxton Dorothy E.F. McKeegan

Please cite this article as: Martin, Jessica E., Christensen, Karen, Vizzier-Thaxton, Yvonne, McKeegan, Dorothy E.F., Effects of analgesic intervention on behavioural responses to Low Atmospheric Pressure Stunning.Applied Animal Behaviour Science http://dx.doi.org/10.1016/j.applanim.2016.05.007

1   **Effects of analgesic intervention on behavioural responses to Low**

2   **Atmospheric Pressure Stunning**

3

4   Jessica E Martin[a,b]*, Karen Christensen[c], Yvonne Vizzier-Thaxton[c], Dorothy E F McKeegan[b]

5

6   [a] *The Royal (Dick) School of Veterinary Studies and The Roslin Institute, Easter Bush*

7   *Campus, Edinburgh, Midlothian, EH25 9RG, United Kingdom*

8   [b] *Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical,*

9   *Veterinary & Life Sciences, University of Glasgow, G61 1QH, United Kingdom*

10   [c] *Centre of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR 72701,*

11   *United States*

12

13   * Corresponding author at: The Royal (Dick) School of Veterinary Studies and The Roslin

14   Institute, Easter Bush Campus, Edinburgh, Midlothian, EH25 9RG, United Kingdom. Tel: +44

15   131 6506062. Email address: Jessica.Martin@ed.ac.uk (J. Martin).

16

17

18

19

20

21

22

23

24

25

26

27

28 **Highlights**

29 • A novel stunning system uses hypobaric hypoxia to render poultry unconscious.

30 • We investigated whether an opioid analgesic affected behavioural responses to this

31 process.

32 • Evidence for pain was limited and observed responses relate primarily to hypoxia.

33 • This approach appears to be equivalent in welfare terms to stunning with inert gases.

34 • These findings contribute to a wider welfare assessment of low atmospheric pressure

35 stunning.

36
37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58   Short title: Effects of pain relief on hypobaric hypoxia in chickens

59

60   **Abstract**

61   Worldwide, more than 50 billion chickens are killed annually for food production so their

62   welfare at slaughter is an important concern. Low Atmospheric Pressure Stunning (LAPS) is

63   a novel approach to pre-slaughter stunning of poultry in which birds are rendered

64   unconscious by gradually reducing oxygen tension in the atmosphere to achieve a

65   progressive anoxia (hypobaric hypoxia). Advantages of this approach over electrical stunning

66   are that birds are not shackled while conscious and all birds are reliably and irreversibly

67   stunned. However, concerns remain that birds undergoing LAPS could experience

68   discomfort or pain. Here we investigated whether subjecting birds to LAPS with and without

69   administration of an opioid analgesic (butorphanol) affected behavioural responses. A

70   blocking design was used in which pairs of birds receiving either analgesic or sham

71   treatment were allocated to three types (analgesic/analgesic, analgesic/sham, or

72   sham/sham). In line with previous studies, birds showed a consistent sequence of

73   behaviours during LAPS: ataxia, loss of posture, clonic/tonic convulsions, leg paddling and

74   motionless. Overall, administration of butorphanol had no effect on the range and patterning

75   of behavioural responses during LAPS, but there were some differences in behaviour

76   latencies, counts and durations. For example, latencies to ataxia, mandibulation and deep

77   inhalation were delayed by analgesic treatment, however the duration of ataxia and other

78   behaviours related to loss of consciousness were unaffected. Fewer birds receiving

79   analgesia showed jumping and slow wing flapping behaviour compared to controls, which

80   suggests these may be pain related. These behaviours after the onset of ataxia and the

81   results may reflect a smoother induction to unconsciousness in analgised birds. Collectively,

82   the results do not provide convincing evidence that birds undergoing LAPS are experiencing

83   pain. While there were effects of analgesia on some aspects of behaviour, these could be

84 explained by potential sedative, dysphoric and physiological side effects of butorphanol. The

85 behavioural responses to LAPS appear to be primarily related to exposure to anoxia rather

86 than hypobaric conditions, and thus in terms of welfare, this stunning method may be

87 equivalent to controlled atmosphere stunning with inert gases.

88

89 **Keywords:** Hypobaric hypoxia, low atmosphere pressure stunning, pain, animal welfare,

90 humane slaughter, broiler

91

92   **1. Introduction**

93 Low Atmospheric Pressure Stunning (LAPS) is a novel approach to pre-slaughter stunning of

94 poultry in which birds are rendered unconscious by gradually reducing air pressure and thus

95 oxygen tension to achieve a progressive hypobaric hypoxia. LAPS shares many of the

96 welfare advantages of controlled atmosphere stunning (CAS) systems, which use exposure

97 to hypoxic and/or hypercapnic gas mixtures, reliably and irreversibly stunning birds in their

98 transport crates (Vizzier-Thaxton et al., 2010; Johnson, 2013).   A major benefit of CAS

99 systems and the LAPS system is that they avoid the considerable stress and pain of

100 shackling of conscious birds (Gentle and Tilston, 2000) and 100% of the chickens are

101 rendered insensible before shackling and bleeding. By contrast, electrical stunning is

102 associated with various welfare issues such as shackling of conscious birds, pre-stun shocks

103 and the risk of inadequate stunning (Raj, 2006). LAPS is in routine commercial use at a

104 poultry processing plant in Arkansas, having been given 'no objection' status by both the

105 United States Department for Agriculture (USDA) in 2010 and the Canadian Food Inspection

106 Agency in 2013. While there has been much research to determine humane gas mixtures for

107 CAS (e.g. McKeegan et al., 2007; Johnson, 2013; Joseph et al., 2013), less is known about

108 the welfare impact of LAPS.

109

110 Previous work investigating the induction of unconsciousness in hypoxic gas environments

111 (Woolley and Gentle 1988; Raj et al., 1991) suggests that the approach has promise, and the

112 gradual nature of LAPS avoids obvious concerns related to the welfare consequences of

113 rapid decompression (Close et al., 1996; AVMA 2013). Previously, Purswell et al., (2007)

114 identified process variables for a suitable decompression and some aspects of behaviour,

115 corticosterone responses, meat quality and pathology have been investigated (Battula et al.,

116 2008; Vizzier-Thaxton et al., 2010). Electroencephalogram (EEG) and electrocardiogram

117 (ECG) responses of broilers undergoing LAPS were reported by McKeegan et al. (2013),

118 where the process was associated with changes in the EEG pattern (highly significant

119 increases in total power, decreases in median frequency and progressive increases in slow

120 wave activity), indicating a gradual loss of consciousness. Recently, a detailed behavioural

121 study described the responses of broilers undergoing LAPS and reported a consistent

122 sequence of behaviours: ataxia, loss of posture, clonic and tonic convulsions and leg

123 paddling (Mackie and McKeegan, 2016). Additional responses were observed in a

124 proportion of birds such as mandibulation (repetitive and rapid opening and closing of the bill,

125 32% of birds), headshaking (76% of birds) and open bill breathing (74% of birds). Based on

126 loss of posture (on average at 84 s), the data suggest that birds are in a conscious state for

127 longer during LAPS than in controlled atmosphere stunning with inert gases (McKeegan et

128 al., 2007a; Abeyesinghe et al., 2007),other behavioural responses are equivalent. Given that

129 headshaking, mandibulation and open bill breathing are all seen during exposure to anoxic

130 gases (normobaric hypoxia) and LAPS (hypobaric hypoxia), it is difficult to conclude whether

131 they are a response to hypoxia or decompression, or both. Concerns remain that some of

132 the behavioural responses observed could be pain related, possibly resulting from painful

133 expansion of trapped air in body cavities. Vizzier-Thaxton et al. (2010) noted that the

134 anatomy and function of the avian respiratory tract with interconnecting airsacs and lungs

135 makes it unlikely that significant amounts of gas would be trapped in the abdomen, while

136 hemorrhagic lesions were found in the lungs, brain, and heart of animals undergoing rapid

137 decompression (Van Liere, 1943).

138

139   Pain is difficult to assess as it cannot be measured directly, but behaviour is the parameter

140   most often used to assess animal pain (Rutherford 2002) and signs of stress during stunning

141   in poultry include head shaking (Erhardt et al., 1996; Raj, 1996), gasping (Raj and Gregory,

142   1990), yawning (Erhardt et al., 1996), vocalisation (Zeller et al., 1988), sneezing

143   (Hoenderken et al., 1994) and defecating (Morton et al., 1998). Some of these signs may

144   also indicate pain or varying degrees of discomfort, or may reflect physiological responses.

145   Quantitative differences may be significant from a welfare point of view, as well as the time at

146   which they occur during the stunning process.

147

148   Analgesic intervention has been widely used in a range of contexts in animal welfare

149   research, for example to examine pain associated with lameness (e.g. Hocking et al., 1997).

150   It is widely recognised that the abolition of suspected pain related behaviour with analgesic is

151   circumstantial evidence of pain (Rutherford, 2002; Walker et al., 2014). However, analgesic

152   drugs may have behavioural effects unrelated to pain and nociception, and some also have

153   general sedative or side effects. Thus, care must be taken with the choice of agent and the

154   dose applied. The primary objective of this study was to investigate whether subjecting birds

155   to LAPS with and without administration of an opioid analgesic would affect their behavioural

156   responses, especially those suspected to relate to pain and discomfort. Butorphanol was

157   chosen for this trial, as it is a Kappa opioid receptor agonist and a mu opioid receptor

158   antagonist with characterised pharmacokinetics (Guzman et al., 2014) and is the currently

159   recommended opioid for use in birds (Paul-Murphy and Fialkowski, 2001; Paul-Murphy,

160   2013). We used a low-moderate dose (Paul-Murphy, 2013) to minimise sedation and side

161   effects. Broilers were exposed to LAPS in pairs to maximise visibility of their reactions to the

162   process while eliminating isolation stress. A blocking design was used in which birds

163   receiving analgesic or sham treatments were randomly allocated to three types of pairs

164   (analgesic/analgesic, analgesic/sham, or sham/sham)). This robust design, random

165   allocation and blinding of behavioural observers to pair type allowed us to reliably determine

166 the effects of analgesic intervention on behaviour during LAPS, and thus contribute to a

167 thorough welfare assessment of the process.

168

169   **2. Material and methods**

170 *2.1 Animals and housing*

171 Ninety Cobb 500 male broiler chickens (Gallus gallus domesticus) from the female breeder

172 line were used in this study. They were sourced from a commercial hatchery and were wing

173 tagged at 4 weeks of age. The birds were housed at the University of Arkansas poultry

174 facilities within a larger single flock split into three groups, reared in three identical

175 environmental chambers (measuring 3.05 X 3.05 m, approximately 100 birds per pen

176 resulted in a stocking density of ~30 kg/m2). Clean pine shavings were used for litter. Single-

177 pass ventilation was maintained at a constant rate of 6 m3/min in all chambers. The

178 photoperiod was 23L:1D for d 1 to 4, and 16L:8D thereafter. Chambers were equipped with 2

179 rows of nipple waterers, and 2 hanging feeders and birds had ad libitum access to feed

180 (standard commercial starter and grower diet) and water. Environmental controls for climate

181 were maintained to follow recommended management practices (Cobb, 2012). Birds and

182 environmental controls were monitored twice daily by trained staff. The trials were

183 undertaken in Arkansas, USA, and therefore were not subject to UK legal requirements

184 through DEFRA or Home Office regulations. The experimental design and animal husbandry

185 was performed following the EU Directive on the Protection of Animals used for Scientific

186 Purposes (EU 2010/63) for guidance. The experiments were specifically authorized by the

187 University of Arkansas Institutional Animal Care and Use Committee (Protocol 15031).

188

189 *2.2 LAPS process*

190 The LAPS chamber was developed by Technocatch LLC in Mississippi, USA the system and

191 the pressure curves applied by the process are patented (Cheek & Cattarazzi, 2010). The

192 chamber, it's monitoring and control systems used in the current study is a scaled down

193 research unit, but is otherwise identical to those used commercially except for manual door

194   operation. The chamber is cylindrical (2.2 m in length and 1.8 m in diameter) and is designed

195   to accommodate a reduced scale transport module (153 cm x 121 cm x 102 cm, three tiers

196   each 23 cm height). The required decompression curve is automatically applied and

197   controlled by a computer and once started, can only be stopped in the case of an

198   emergency. An infra-red camera (130° camera with (2.1mm lens) 18 infra-red illuminators,

199   Model #RVS-507, RVS Systems) was fitted into the chamber to observe the birds (fixed

200   centrally on the front wall of the chamber allowing full view of the relevant tier). A manually

201   operated door is present that allows the entry of the transport module and seals them into the

202   chamber to begin the process. The LAPS cycle takes exactly 280 s and consists of two

203   phases, in the first of which the vacuum chamber pressure is reduced from atmospheric

204   pressure to an absolute vacuum pressure of ~250Torr (~33 kPa) in ~67 s. In the second

205   phase a sliding gate valve is partially closed gradually reducing the effective pumping speed

206   by 'choke flow', to a minimum chamber pressure of ~150Torr (~20 kPa). The rate of

207   reduction of chamber pressure in the second phase is varied in relation to starting ambient

208   temperature and barometric pressure. The reduction in total pressure results in a reduced

209   oxygen partial pressure. At the end of the second phase at 280 s the chamber is returned to

210   atmospheric pressure using a baffled air inlet, prior to the door opening and the exit of the

211   transport module. Because cold air is denser and therefore contains more oxygen than

212   warm air and birds have been shown to respond differently to LAPS at different temperatures

213   (Mackie and McKeegan 2016), slightly different pressure reduction curves must be applied to

214   achieve the same hypobaric effect under different ambient conditions. As discussed by

215   Holloway (unpublished results), water in the LAPS chamber may also lead to modification of

216   the rate of decompression based on temperature. Ambient temperature and humidity were

217   recorded for each LAPS cycle and means were 13.5 ± 0.5 °C and 76.3 ± 0.6%, respectively.

218   In this study, all 45 LAPS runs were carried out within a single temperature setting.

219

220   *2.3 Experimental procedure*

221   The experimental birds were randomly selected from the flock by a random number

222   generator (Microsoft Excel 2010) based on wing tag number. They were systematically and

223   equally allocated via a Latin-Square design across two treatments (analgesic - A, sham - S)

224   and then allocated by individual wing tag number into three types of blocked pairs

225   (analgesic/analgesic (AA), analgesic/sham (AS), and sham/sham (SS)) and pair kill order

226   following a Graeco Latin-Square design (Martin & Bateson 2007). There were 15

227   replications of each block (AA, AS, and SS), each containing a pair of birds. The birds

228   underwent LAPS in 45 consecutive pairs over two days (day 1 = 23 pairs; day 2 = 22 pairs)

229   at 36-37 days of age (mean bodyweight 2.30 ± 0.12 kg). To mimic commercial transport and

230   lairage conditions, experimental birds for each day were removed from the flock and

231   transported and held in poultry transport crates (97 x 58 x 27 cm, maximum 8 birds per crate)

232   prior to LAPS. Thus birds had food and water withdrawn for between 2-6 hrs before LAPS,

233   dependent on the pair kill order.

234

235   In sequential order, bird pairs were removed from the transport crates and weighed.

236   Dependent on their pre-determined treatment, birds were injected with either butorphanol

237   ('Dolorex', butorphanol tartrate 10mg/ml, Merk) delivered IM in the right thigh at 1mg/kg or

238   saline (veterinary 0.9% Sodium Chloride Injection, Hospira Inc.) delivered IM in the right thigh

239   at equivalent volume to the analgesic treatment based on bird weight. Treatments were

240   staggered to provide a consistent 30 minute interval between injection and LAPS. At the time

241   of injection one bird per pair had its wing tip feathers marked by a black permanent marker

242   (Sharpie® Magnum chisel tip); this marking was to allow better visualisation of individuals

243   during behavioural observations and was randomly allocated by wing tag number,

244   irrespective of treatment. Birds were then housed within their pairs in separate cardboard pet

245   carriers (28 x 35 x 46 cm) until transferred into the LAPS chamber by hand. Each pair of

246   birds was placed in the top right tier (1.53 x 1.21 x 0.23 m) of the US poultry container within

247   the LAPS chamber. Soft polystyrene dividers were used to position the two birds at the front

248   of the tier (available space 0.76 x 1.21 x 0.23 m, resulting in a stocking density of 5.0 Kg/m2

249   based on average bird weight of 2.3 kg), in order to minimise damage to the birds when

250   convulsing and reduce the risk of birds from disappearing from camera view during the LAPS

251   cycle. Once the birds had been placed in the tier, the chamber door was closed and sealed

252   and the LAPS cycle started. During the trials, the birds were watched in real time on a

253   monitor to check for unexpected behaviour. Video footage was recorded on a digital video

254   recorder (Datavideo M# DN300) to allow behavioural observations to be conducted later,

255   continuous recordings from 5 s prior to the start of LAPS to 5 s after the end of the cycle

256   were obtained for each pair of birds. On completion of the LAPS cycle, the birds were

257   removed from the chamber and reflexes were immediately assessed (e.g. presence of

258   rhythmic breathing, nictitating membrane) to confirm death.

259

260   *2.4 Behavioural Observations*

261   An ethogram was developed based on previous behavioural work on LAPS (Mackie &

262   McKeegan, 2016) as well as CAS research (Lambooij et al., 1999; Coenen et al., 2009)

263   (Table 1). Behaviours for both birds in each pair were recorded using The Noldus Observer

264   XT 11.0 programme by a single observer who was blinded to pair number, block type and

265   individual bird treatment. Behavioural variables measured included latencies, counts, total

266   durations, bout durations and bout counts; see Table 1 for specific measures for each

267   behaviour. Birds which went out of sight for more than 10% of the total observation time

268   (280 s) were excluded from the data set. Reasons for birds going out of sight were that they

269   moved behind the other bird or to the far end of the chamber. Data was exported from

270   Observer to Microsoft Excel 2010.

271

272   *2.5 Statistical analysis*

273   All data were summarised in Microsoft Excel (2010) spread sheets and analysed using

274   Genstat (14th Edition). Statistical significance was based on F statistics and $P < 0.05$

275   threshold level. Summary graphs and statistics were produced at bird level. Statistical

276   comparisons of behavioural variables were conducted via Generalised Linear Mixed Models

277 (GLMM) (Poisson distribution) or Linear Mixed Models (LLM) (normal distribution) dependent

278 on the data distributions for each variable. Data transformations were attempted when

279 necessary via Logarithm function. All models included bird ID, companion bird ID and pair

280 block type as random effects. All fixed effects were treated as factors and all interactions

281 between factors were included in maximal models. All models included treatment, pair order,

282 and marked bird as fixed effects and bird weight, ambient temperature, ambient humidity as

283 covariates. Correlations between variables and fixed effects were performed as Pearson's

284 Correlations for parametric data, and Spearman's Rank Correlations for non-transformable

285 non parametric data. For behaviours which were not exhibited by all birds, the effect of

286 treatment on the proportions of birds showing the behaviour was compared with Chi Square

287 tests using two by two contingency tables.

288

289 **3. Results**

290 No birds showed any signs of life at the end of the LAPS cycle (absence of rhythmic

291 breathing, absence of corneal or palpebral reflex (EFSA 2013). A total of 17/90 birds went

292 out of sight at some point during observations (by treatment: A = 9; S = 8), with mean total

293 out of sight durations of 29.4 ± 10.9 s for analgesic birds (10.5 ± 3.9% of total observation

294 time) and 90.8 ± 33.1 s for sham birds (33.1 ± 11.6% of total observation time). Based on

295 exclusion criteria (>50% of observation time out of sight), 3 sham birds were removed from

296 analysis to avoid bias. The birds showed a consistent sequence of behaviours during LAPS:

297 ataxia, loss of posture, clonic/tonic convulsions, leg paddling and motionless. Clonic

298 convulsions, sitting, lying, ataxia, loss of posture, loss of jaw tone and motionless were

299 observed in all birds as they underwent LAPS. No birds were observed performing escape

300 behaviour, pecking or panting.

301

302 Almost all birds (83/90) exhibited vigilance behaviour at the onset of LAPS, and the total

303 duration, bout duration and number of bouts this behaviour was increased in birds receiving

304 analgesic (total mean duration 37.1 s compared to 30.5 s in controls, Table 2). The mean

305   latency to show mandibulation was delayed by analgesic treatment (18.8 s vs. 25.5 s, Table

306   3), while saline treated birds exhibited more counts of mandibulation than analgesic treated

307   birds (mandibulations per bird ranged from 1-12, mean count 2.7 vs. 2.1, Table 4). Mean

308   counts of headshaking were higher in analgesic treated birds compared to saline treated

309   birds (headshakes per bird ranged from 1-7, mean counts 2.4 with analgesic compared to

310   1.7 in controls, Table 4). Total duration of standing was higher in analgesic treated birds

311   (16.0 s compared to 12.3 s in saline birds, Table 2) there were also longer standing bout

312   durations with analgesic (13.1 s compared to 7.5 s, Table 2).

313

314   Analgesic treatment affected the latency to ataxia but the effect in terms of time difference

315   was small (44.5 s for birds receiving analgesia compared to 41.8 s for sham treated birds,

316   overall range 21.4 – 65.2 s, Table 3). Analgesic treatment had no effect on the duration of

317   ataxia (Table 2). Analgesic treatment also had no effect on other latencies related to the

318   onset of unconsciousness (loss of posture or loss of jaw tone; Table 3). Figure 1 shows the

319   patterning of key behaviours relating to loss of consciousness in the first 100 s of LAPS

320   according to treatment, indicating the sequence of behaviour and showing that analgesia

321   treatment was associated with a delay in the latency of some behaviours, but had no effect

322   on latency to loss of consciousness, as indicated by loss of jaw tone and loss of posture.

323   Jumping was not seen until birds started to show ataxia and loss of posture (mean latency

324   55.4 ± 1.4 s); this was seen in fewer birds receiving analgesic compared to those receiving

325   saline (46.5% vs. 67.5%; Table 5). Saline treated birds also exhibited more jumps than

326   analgesic treated birds (jumps per bird ranged from 1-3, mean count 1.0 compared to 0.5,

327   Table 4).

328

329   Slow wing flapping was seen in significantly more birds receiving saline (74%) than analgesic

330   (47%, Table 5), but longer bout durations were observed with analgesic (Table 2). Longer

331   and more bouts of tonic convulsions were also observed in birds received analgesic, but

332   latencies and overall durations were unaffected (Table 3, Table 2). There were no effects of

333   analgesic on clonic convulsions. Frequency of bouts and bout durations of lying were

334   increased in birds receiving analgesic (75.2 s) compared to controls (72.7 s, Table 2). The

335   total duration of leg paddling was affected by treatment, with analgised birds exhibiting longer

336   durations (9.1 s compared to 6.8 s in saline birds). Latency, bout duration and bout

337   frequency of leg paddling was unaffected by treatment, as was latency to become

338   motionless.

339

340   The latency to the first deep inhalation behaviour was 82.5 s in saline treated birds, greater

341   compared to 101.8 s analgesic treated birds (Table 3), but counts of this behaviour (counts

342   per bird ranged from 1-8) were not affected by treatment (Table 2). The duration of open bill

343   breathing bouts was shorter in analgesic treated birds (8.1 s compared to 6.8 s in saline

344   treated birds, Table 2). Only four birds vocalised; three of the vocalisations occurred during

345   clonic convulsions suggesting that they may have been involuntary. The fourth bird vocalised

346   once at 14 s into LAPS.

347

348   Fixed effects had minimal influence on behaviour latencies; however some factors affected

349   certain behaviours. Bird weight affected latency to ataxia ($F_{1,84}$ = 7.77, p = 0.021) and

350   mandibulation ($F_{1,41}$ = 17.7, p <0.001) and was negatively correlated with both, but not

351   significantly (r = -0.109, p = 0.322 and r = -0.123, p = 0.428, respectively). The onset of

352   open-bill breathing ($F_{1,67}$ = 8.63, p = 0.005) and deep inhalation ($F_{1,48}$ = 9.41, p = 0.002) were

353   positively related to bodyweight, with significant positive correlation with first deep inhalation

354   (r = 0.354, p = 0.014). Ambient temperature had no effect on the majority of behavioural

355   latencies except for time to become motionless ($F_{1,84}$ = 5.51, p = 0.022) which was non-

356   significantly negatively correlated (r = -0.098, p = 0.373). Latency to slow wing flapping was

357   also related to ambient temperature ($F_{1,51}$ = 2.33, p <0.001) with a non-significant positive

358   correlation (r = 0.075, p = 0.600). In terms of behaviour durations, fixed effects did not

359   explain a significant proportion of the data except for ambient temperature ($F_{1,64}$ = 5.00, p =

360   0.028) and humidity ($F_{1,64}$ = 4.26, p = 0.042) which were related to the durations of tonic

361 convulsions, although neither had significant correlations ($r = 0.178$, $p = 0.159$; $r = -0.138$, p

362 $= 0.278$ respectively).

363

364 The majority of fixed effects and interactions had no significant effect on the total counts of

365 behaviour including jumping, mandibulation, head shaking or deep inhalation behaviours.

366 The only significant effects were between mandibulation and bird weight ($F_{1,44} = 3.11$, $p =$

367 0.008), ambient humidity ($F_{1,44} = 7.68$, $p = 0.007$) and ambient temperature ($F_{1,44} = 6.42$, $p =$

368 0.011), as well as between headshaking and ambient humidity ($F_{1,51} = 5.22$, $p = 0.025$).

369

370 **4. Discussion**

371 A consistent series of behavioural responses were seen during LAPS, similar to previous

372 reports (Vizzier-Thaxton et al., 2010; Mackie and McKeegan 2016). The responses also

373 closely resembled those observed during exposure to controlled atmosphere stunning with

374 inert gases such as Argon and Nitrogen (Raj et al., 1991; Gerritzen et al., 2000; McKeegan

375 et al., 2007). Previously, EFSA (2004) opined that "anoxia is not aversive to poultry and

376 does not induce any signs of respiratory distress prior to loss of consciousness". Mackie and

377 McKeegan (2016) discussed the welfare implications of behavioural responses to LAPS but

378 noted that further work would be required to determine if any of them are specifically pain

379 related. Our expectation was that the most likely pain related behaviours would be

380 headshaking, vocalisation and escape behaviour. In general, administration of butorphanol

381 had no effect on the type and patterning of behavioural responses during LAPS compared to

382 control birds, but there were differences in behaviour latencies, counts and durations. While

383 bout durations and frequencies of some behaviours were affected by analgesic, total

384 durations were generally unaffected except for vigilance, standing and leg paddling.

385

386 Pain related behaviour in birds has been previously identified in a variety of contexts, and

387 includes active escape/withdrawal, guarding, sick bird posture, freezing and vocalisation

388 (Gentle, 2011; Paul-Murphy, 2013). Since these responses were not seen during LAPS, this

389   study presents an opportunity to use analgesic intervention to identify potential pain related

390   behaviour. There is a danger that using the effects of analgesic treatment on behaviour to

391   recognise pain becomes a circular argument (i.e. pain is something removed by an

392   analgesic; an analgesic is something which removes pain; Bateson, 1991). It is also

393   important to note that analgesic drugs may have behavioural effects unrelated to pain and

394   nociception. The analgesic applied in this study was potentially optimal, systemic and

395   centrally acting with proven effectiveness in clinical contexts (Paul-Murphy, 2013).

396   Butorphanol has been shown to have high bioavailability following IM administration in

397   psittacines and raptors (Guzman et al., 2011; Gustaven et al., 2014), though Paul-Murphy

398   (2013) notes that dosage of butorphanol for effective analgesia needs to be balanced with

399   sedation and respiratory depression, which may vary between avian species.

400

401   Latencies to ataxia, mandibulation and deep inhalation were slightly delayed by analgesic

402   treatment, however the duration of ataxia and other behaviours related to loss of

403   consciousness were unaffected. These delayed initial responses raise the question of

404   whether butorphanol had a sedative effect. Previous work administering butorphanol IM to

405   Kestrels at 1, 3 or 6 mg/kg did not change mean sedation-agitation scores, except in at

406   6mg/kg 1.5 hours after injection (Guzman et al., 2014), but responses to this compound are

407   likely to be species specific (Paul-Murphy, 2013). Possible sedation effects of the analgesic

408   are not supported by results showing that analgised birds spent more time vigilant at the start

409   of the LAPS cycle, and the latency to become vigilant was unaffected by treatment. In some

410   species such as dogs (Hofmeister et al., 2006) butorphanol can produce side effects such as

411   dysphoria where the animals appear agitated and disorientated. This could provide an

412   explanation for some of the differences in behaviour seen, but such dysphoric effects have

413   not been reported in birds (Hawkins, 2006).One of the most obvious candidates for pain

414   related behaviour during LAPS is headshaking, which has been previously associated with

415   disorientation, discomfort, respiratory distress (Webster and Fletcher, 2001) or arousal

416   (Hughes, 1983). Nicol et al., (2011) found that head shaking may be a valid indicator of a

417   less preferred environment and high rates of head shaking may indicate poor welfare. Only

418   around half of birds showed this behaviour (as reported previously, Mackie and McKeegan,

419   2016) and the proportion of birds exhibiting the behaviour was unaffected by treatment; in

420   fact its frequency was increased in birds receiving analgesia. This does not fit with it being

421   pain related behaviour abolished by analgesia, and it is possible that the observed increase

422   may be related to dysphoria and/or a sensation of disorientation. Headshaking is also a

423   behaviour that is routinely seen in controlled atmosphere stunning of chickens with both inert

424   and hypercapnic gas mixtures (McKeegan et al 2007; Abeyesinghe et al 2007). Interestingly,

425   birds receiving analgesia spent more time standing at the start of the LAPS cycle. While

426   none of the birds was obviously lame, several sources of leg pain may be present and these

427   may have been relieved by butorphanol in treated birds.

428

429   Administration of butorphanol has been shown to cause lowering of the heart rate, tidal

430   volume, and inspiratory and expiratory times in psittacines (Curro et al., 1994), but such

431   opioid side effects appear to be less pronounced in chickens (Concannon et al., 1995). In

432   contrast to previous work describing behavioural responses to LAPS (Mackie and

433   McKeegan, 2016), in this study we attempted to distinguish between deep inhalation and

434   open bill breathing. Analgesic treatment was associated with a delayed latency to deep

435   inhalation and increased duration of open bill breathing bouts (but not total duration). These

436   differences suggest that there were some physiological side effects of the drug which

437   affected the response to hypobaric hypoxia, possibly due to respiratory depression. While

438   53% of birds performed deep inhalation behaviours, 77 to 83% of birds exhibited open bill

439   breathing and similar responses have been seen in response to controlled atmosphere

440   stunning using hypoxic gas mixtures (e.g. McKeegan et al., 2007) suggesting they probably

441   relate to anoxia.

442

443   A wide range of behavioural responses were seen in all birds, with a few (e.g. standing)

444   exhibited only in a small proportion of birds and were generally unaffected by treatment.

445   Exceptions to this were slow wing flapping and jumping, both behaviours associated with

446   ataxia and loss of posture. Fewer birds receiving analgesia showed jumping (20 compared

447   to 27) and slow wing flapping behaviour (20 compared to 31) compared to controls, which

448   suggests these may be pain related. The latencies of these behaviours show that they

449   occurred, on average, after the onset of ataxia and they did not appear to be escape

450   behaviours. The results may reflect a smoother induction to unconsciousness in analgised

451   birds, with butorphanol possibly having an effect similar to a premedication.

452

453   No panting behaviour was shown and only 4 birds vocalised, although it was apparent that

454   the three of the vocalisations may have been unconscious forced exhalation by the birds due

455   to simultaneous vigorous wing flapping and clonic convulsions as all but one vocalisation

456   was observed after loss of jaw tone, ataxia and loss of posture had occurred, suggesting the

457   birds were no longer conscious (McKeegan *et al.*, 2013; Sandercock *et al.*, 2014; Martin,

458   2015).

459

460   There were some effects of temperature and humidity but many of the underlying

461   correlations were not significant. The LAPS system operates a series of decompression

462   curves according to ambient temperature, and these have been previously shown to affect

463   some behaviour latencies and durations (Mackie and McKeegan, 2016). In this study, only

464   one curve was applied so determining the effects of ambient temperature and humidly was

465   not our aim. Bird weight effects on ataxia, mandibulation, open bill breathing and deep

466   inhalation were apparent in the current study, but a more powerful factorial study would be

467   needed to investigate these relationships further.

468

469   **5. Conclusion**

470   There are few studies on the side effects of butorphanol in chickens, which limits our ability

471   to draw firm conclusions from this study. Another obvious limitation is the lack of a positive

472   control and thus any conclusions depend on acceptance of the fact that butorphanol is an

473   effective analgesic in chickens. Apart from the ethical concerns raised by deliberate

474   induction of pain, it is not clear what sort of pain model would be relevant to this study. With

475   these limitations in mind, it may still be argued that the results do not provide convincing

476   evidence that birds undergoing LAPS are experiencing pain. While there were effects of

477   analgesia on some aspects of behaviour, and jumping and slow wing flapping was reduced,

478   these effects may be explained by the potential sedative, dysphoric and physiological side

479   effects of butorphanol. In particular, obvious pain related behaviours such as

480   escape/withdrawal and freezing were not seen at all, while others such as head-shaking and

481   vocalisation were not reduced with analgesic intervention during LAPS.   EEG data

482   (McKeegan *et al.*, 2013; Martin *et al.*, submitted) demonstrates the maintenance of slow

483   wave EEG patterns induced by darkness in the early part of LAPS (while birds are still

484   conscious); desynchronisation of the EEG resembling 'waking' from sleep would be expected

485   during aversive or painful stimulation (Gentle, 1975). These findings support the notion that

486   during the period of the gradual reduction of pressure in LAPS the behavioural responses

487   seen are primarily related to exposure to hypoxia rather than hypobaric conditions. The

488   patterns of behaviour are also similar to those seen in normobaric hypoxia using inert gases,

489   and thus in terms of welfare, this stunning method could be considered to be equivalent to

490   controlled atmosphere stunning with inert gases.

491

496

497   **References**

498   Abeyesinghe, S.M., McKeegan, D.E.F., McLeman, M.A., Lowe, J.C., Demmers, T.G.M.,

499   White, R.P., Kranen, R.W., van Bemmel, H., Lankhaar, J.A.C., and Wathes, C.M.,

500   2007. Controlled atmosphere stunning of broiler chickens. I. Effects on behaviour,

501 physiology and meat quality in a scale system at a processing plant. Br. Poult. Sci., 48,

502 406 – 423.

503 AVMA, 2013. Guidelines for the Euthanasia of Animals: 2013 Edition American Veterinary

504 Medical Association ISBN 978-1-882691-21-0

505 https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

506 Bateson, P., 1991. Assessment of pain in animals. Anim. Behav., 42, 827–839.

507 Battula, V., Schilling, M.W., Vizzier-Thaxton, Y., Behrends, J.B., Williams, J.B., and Schmidt,

508 T.B., 2008. The effects of low atmosphere stunning and deboning time on broiler breast

509 meat quality. Poult. Sci., 87, 1202–1210.

510 Concannon, K.T., Dodam, J.R., and Hellyer, P.W., 1995. Influence of a mu- and kappa-

511 opioid agonists on isoflurane minimal anaesthetic concentration in chickens. Am. J.

512 Vet. Res., 56, 806-811.

513 Curro, T.G., Brunson, D.B., and Paul-Murphy, J., 1994. Determination of the ED50 of

514 isoflurane and evaluation of the isoflurane-sparing effect of butorphanol in cockatoos

515 (Cacatua spp.). Vet Surg., 23, 429-433.

516 Cheek, H., and Cattarazzi, B., (inventors and assignees), 2010. United States Process

517 Patent 7662030. Method for humanely stunning and slaughtering poultry using

518 controlled low atmospheric pressure. Feb. 16, 2010.

519 Close, B., Banister, K., Baumans, V., Bernoth, E., Bromage, N., Bunyan, B., Erhardt, W.,

520 Flecknell, P., Gregory, N., Hackbarth, H., Morton, D., and Warwick, C., 1996.

521 Recommendations for euthanasia of experimental animals: Part 1.

522 Lab. Anim., 30, 293-316

523 Cobb-Vantress, 2012. Cobb 500TM Performance and nutrition supplement manual. 2nd

524 edition.

525 Coenen, A.M.L., Lankhaar, J., Lowe, J.C., and McKeegan, D.E.F., 2009. Remote monitoring

526 of electroencephalogram, electrocardiogram, and behavior during controlled

527 atmosphere stunning in broilers: implications for welfare. Poult. Sci., 88, 10-19.

528   European Food Safety Authority, 2004. Welfare aspects of the main systems of stunning and

529   killing the main commercial species of animals. EFSA J., 45, 1–29.

530   European Food Safety Authority, 2013. AHAW Panel (EFSA Panel on Animal Health and

531   Welfare), Guidance on the assessment criteria for studies evaluating the effectiveness

532   of stunning interventions regarding animal protection at the time of killing. EFSA J.,

533   2013, 11, 3486, 41 pp. doi: 10.2903/j.efsa.2013.3486.

534   Erhardt, W.H., Gehra, M., Schaefer, M., Brill, T., and Henke, J., 1996. CO_2-Betaeubung zur

535   Schlachtung von Puten. Dtsch Tierarztl Wochenschr., 103, 62-64.

536   Gentle, M., 1975. Using arousal changes in the electroencephalogram to measure taste

537   sensitivity in the chicken. J Physiol., 244, 9-10.

538   Gentle, M.J., and Tilston, V.L., 2000. Nociceptors in the legs of poultry: implications for

539   potential pain in preslaughter shackling. Anim. Welf., 9, 227-236.

540   Gentle, M.J., 2011. Pain issues in poultry. Appl. Anim. Behav. Sci., 135, 232-258.

541   Gerritzen, M.A., Lambooij, E., Hillebrand, S.J.W., Lankhaar, J.A., and Pieterse, C., 2000.

542   Behavioral responses of broilers to different gaseous atmospheres. Poult. Sci., 79,

543   928–933

544   Gustavsen, K.A., Guzman, D.S., Knych, H.K., Petritz, O.A., Olsen, G.H., and Paul-Murphy,

545   J.R., 2014. Pharmacokinetics of buprenorphine hydrochloride following intramuscular

546   and intravenous administration to American kestrels (Falco sparverius). Am. J. Vet.

547   Res., 75, 711-5. doi: 10.2460/ajvr.75.8.711.

548   Guzman, D.S., Flammer, K., Paul-Murphy, J.R., Barker, S.A., and Tully, T.N., 2011.

549   Pharmacokinetics of butorphanol after intravenous, intramuscular, and oral

550   administration in Hispaniolan Amazon parrots (Amazona ventralis). J. Avian Med.

551   Surg., 25,185-91.

552   Guzman, D.S., Drazenovich, T.L., KuKanich, B., Olsen, G.H., Willits, N.H., and Paul-Murphy,

553   J.R., 2014. Evaluation of thermal antinociceptive effects and pharmacokinetics after

554   intramuscular administration of butorphanol tartrate to American kestrels (Falco

555   sparverius). Am. J. Vet. Res., 75,11-8. doi: 10.2460/ajvr.75.1.11.

556   Hawkins, M.G., 2006. The Use of Analgesics in Birds, Reptiles, and Small Exotic Mammals.

557   J. Exot. Pet Med., 15, 177-192.

558   Hawkins, M.G., Paul-Murphy, J.R., 2011. Avian analgesia. Vet. Clin. North. Am. Exot. Anim.

559   Pract., 14, 61-80. doi: 10.1016/j.cvex.2010.09.011.

560   Hoenderken, R.B., Lambooij, B., van den Bogaard, A.E.J.M., and Hillenbrand, S.J.W., 1994.

561   Tierschutzgerechte Gasbetaeubung von Gefluegel. Fleischwirtschaft.,74, 497-500.

562   Hofmeister, E.H., Herrington, J.L., and Mazzaferro, E.M., 2006. Opioid dysphoria in three

563   dogs. J. Vet. Emerg. Crit. Care (San Antonio), 16, 44-49. doi:10.1111/j.1476-

564   4431.2005.04022.x

565   Hocking, P., Gentle, M., Bernard, R., and Dunn, L., 1997. Evaluation of a protocol for

566   determining the effectiveness of pretreatment with local analgesics for reducing

567   experimentally induced articular pain in domestic fowl. Res. Vet. Sci., 63, 263-267.

568   Hughes, B.O., 1983. Head shaking in fowls: the effect of environmental stimuli. Appl. Anim.

569   Ethol., 11, 45-53.

570   Johnson, C.L., 2013. A review of bird welfare during controlled atmosphere and electrical

571   water-bath stunning. J. Am. Vet. Med. Assoc., 245, 60-68.

572   Joseph, P., Schilling, M.W., Williams, J.B., Radhkishnan, V., Battula, V., Christensen, K.,

573   Vizzier-Thaxton, Y., and Schmidt, T.B., 2013. Broiler stunning methods and their

574   effects on welfare, rigor mortis, and meat quality. World's Poult. Sci. J., 69, 99-112,

575   http://dx.doi.org/10.1017/S0043933913000093.

576   Lambooij, B., Gerritzen, M.A., Engel, B., Hillebrand, S.J.W., Lankhaar, J., and Pieterse, C.,

577   1999. Behavioural responses during exposure of broiler chickens to different gas

578   mixtures. Appl. Anim. Behav. Sci., 62, 255-265. http://dx.doi.org/10.1016/S0168-

579   1591(98)00214-7.

580   Mackie, N., and McKeegan, D.E.F., 2016. Behavioural responses of broiler chickens during

581   Low Atmospheric Pressure, Appl. Anim. Behav. Sci., 174, 90-98.

582   Martin, J.E., 2015. Humane mechanical methods to kill poultry on-farm. PhD Thesis,

583   University of Glasgow, United Kingdom.

584 Martin, J.E., Christensen, K., Vizzier-Thaxton, Y., Mitchell, M.A., and McKeegan, D.E.F.,

585   2016. Behavioural, brain and cardiac responses to hypobaric hypoxia in chickens.

586   Physiol. Behav., (in press).

587 Martin, P., and Bateson, P., 2007. Measuring Behaviour: An Introductory Guide, third ed.

588   Cambridge University Press, Cambridge, United Kingdom.

589 McKeegan, D.E.F., McIntyre, J.A., Demmers, T.G.M., Lowe, J.C., Wathes, C.M., Broek,

590   P.L.C., Coenen, A.M.L., and Gentle, M.J., 2007. Physiological and behavioural

591   responses of broilers to controlled atmosphere stunning: implications for welfare. Anim.

592   Welf.,16, 409-426.

593 McKeegan, D.E.F., Sandercock, D.A., and Gerritzen, M.A., 2013. Physiological responses to

594   low atmospheric pressure stunning and the implications for welfare. Poult. Sci., 92,

595   858-868.

596 Nicol, C.J., Caplen, G., Statham, P., and Browne, W.J., 2011. Decisions about foraging and

597   risk trade-offs in chickens are associated with individual somatic response profiles.

598   Anim. Behav., 82, 255–262.

599 Paul-Murphy, J., and Fialkowski, J., 2001. Injectable Anaesthesia and Analgesia of Birds. In:

600   Recent Advances in Veterinary Anaesthesia and Analgesia: Companion Animals,

601   R.D. Gleed and J.W. Ludders (Eds.) Publisher: International Veterinary Information

602   Service (www.ivis.org), Ithaca, New York, USA.

603 Paul-Murphy, J., 2013. Pain Management. Chapter 8 In: Clinical Avian Medicine Volume 1

604   http://avianmedicine.net/content/uploads/2013/03/08_pain_management.pdf

605 Purswell, J.L., Thaxton, J.P., and Branton, S.L., 2007. Identifying process variables for a low

606   atmospheric pressure stunning-killing system. J. Appl. Poult. Res., 16, 509-513. doi:

607   10.3382/japr.2007-00026

608 Raj, A.B.M., Gregory, N.G., and Wotton, S.B., 1991. Changes in the somatosensory evoked

609   potentials and spontaneous electroencephalogram of hens during stunning in argon-

610   induced hypoxia. Br. Vet. J., 147, 322-330

611   Raj, A.B.M., and Gregory, N.G., 1990. Investigation into the batch stunning/killing of chickens

612   using carbon dioxide or argon-induced hypoxia. Res. Vet. Sci., 49, 364-366

613   Raj, A.B.M., 2006. Recent developments in stunning and slaughter of poultry. World's Poult.

614   Sci. J., 62, 467-48. DOI: http://dx.doi.org/10.1017/S0043933906001097.

615   Rutherford, K.M.D., 2002. Assessing Pain in Animals. Anim. Welf., 11(1): 31-53.

616   Morton. D,, Barton-Gade, D., Fernandez, X., Lambooij, B., Raj, M., and Troeger, D., 1998.

617   The use of Mixtures of the Gases CO2, O2, and N2 for Stunning or Killing Poultry -

618   Report of the Scientific Committee on Animal Health and Animal Welfare adopted 23rd

619   June 1998 available at http://ec.europa.eu/food/fs/sc/scah/out08_en.html

620   Sandercock, D.A., Auckburally, A., Flaherty, D., Sandilands, V., and McKeegan, D.E.F.,

621   2014. Avian reflex and electroencephalogram responses in different states of

622   consciousness. Physiol. Behav., 153, 252-289.

623   Sparrey, J.M., and Kettlewell, P.J., 1994. Shackling of poultry: is it a welfare problem?

624   World's Poult. Sci. J., 50, 167-176.

625   Vizzier-Thaxton, Y., Christensen, K.D., Schilling, M.W., Buhr, R.J., and Thaxton, J.P., 2010.

626   A new humane method of stunning broilers using low atmospheric pressure. J Appl

627   Poult Res., 19, 341-348. doi: 10.3382/japr.2010-00184

628   Walker, K.A., Duffield, K.A., Weary, D.M., 2013. Identifying and preventing pain during and

629   after surgery in farm animals. Appl. Anim. Behav. Sci., 135, 259– 265.

630   Webster, A.B., and Fletcher, D.L., 2001. Reactions of laying hens and broilers to different

631   gases used for stunning poultry. Poult. Sci., 80, 1371-1377.

632   Woolley, S.C., and Gentle, M.J., 1988. Physiological and behavioural responses of the

633   domestic hen to hypoxia. Res. Vet. Sci., 45, 377-382.

634   Van Liere, E.J., 1942. Anoxia. Its Effect on the Body. Chicago, University of Chicago Press,

635   United States, 1-269

636   *Zeller, W., Mettler, D., and Schatzmann, U., 1988. Untersuchungen zur Betaeubung des*
637   *Schlachtgefluegels mit Kohlendioxid. Fleischwirtschaft., 68, 1308-1312.*
638
639   **Figure captions**

640

641 **Figure 1** Mean latencies and durations (ataxia only) and the relationship in time of key

642 behaviours related to loss of consciousness during LAPS in saline and analgesic

643 treated birds.
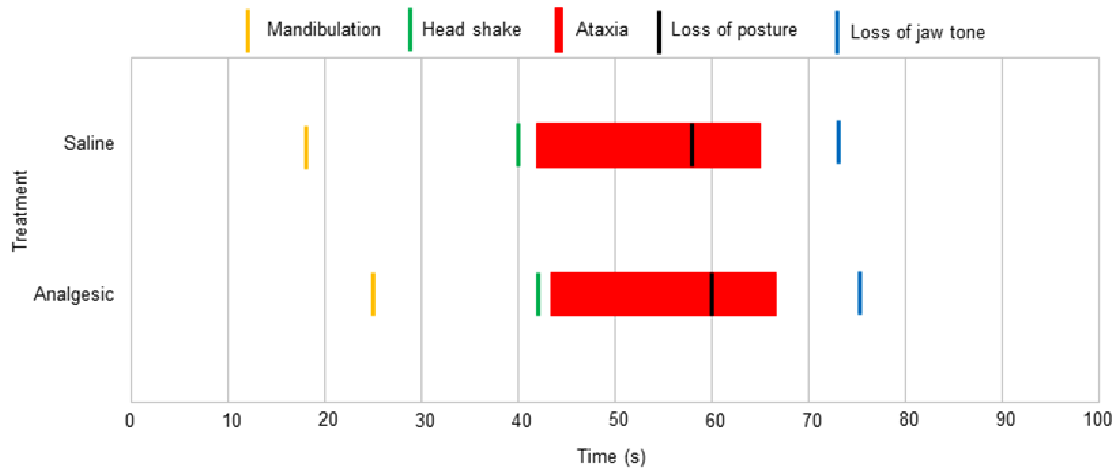
644

645

646

647 Tables

648 **Table 1** Ethogram of bird behaviours during LAPS cycle.

Behaviour	Description	Measures
Vigilance	Alert movements of the head, including 'Notice' as defined by Mackie and McKeegan (2016).	Latency duration
Mandibulation	Repetitive and rapid opening and closing of the bill, not associated with inspiration or exhalation.	Counts Latency
Headshake	Rapid lateral head movement.	Counts Latency
Open bill breathing	Gentle rhythmic breathing with bill open, with or without neck extension.	Latency durations
Panting	Rapid rhythmic breathing with bill open with tongue extended	Latency durations
Deep inhalation	Deep non-rhythmic inspiration from the mouth may be accompanied by extension of the neck	Counts Latency
Ataxia	Apparent dizziness, staggering, swaying of body and/or head, attempts to stand/sit or flaps wings to try and regain balance.	Duration Latency
Loss of posture	Unable to regain/maintain a controlled posture.	Latency
Clonic convulsion	Rapid/vigorous movement of the wings, a new bout was defined as following a pause of at least one	Duration Latency

	second.	
Tonic convulsion	Uncontrolled twitching (visible muscular spasms within the body). A new bout was defined as following a pause of at least one second.	Duration Latency
Slow wing flapping	One short burst or prolonged slow/moderate movement of the wings, occurring without any twitching of the body. A new bout was defined by a pause of one second.	Duration Latency
Leg paddling	Involuntary, usually alternating, leg movements in the air or towards the ground depending on the body position of the bird. Leg paddling can also be determined by an alternating upwards and downwards movement of the body if bird is lying sternal. A new bout was defined by a pause of one second.	Duration Latency
Loss of jaw tone	Bill open for more than 2s without deep breathing and/or neck extension.	Latency
Jump	Explosive upwards movement from a sitting/lying position during ataxia.	Counts
Escape	Rapid locomotor behaviours in an apparently conscious attempt to exit the situation	Counts
Peck	Moving head backwards and forwards in a pecking motion.	Counts
Vocalising	Any audible vocal produced by the focal bird (e.g. alarm call or peeping).	Counts Latency
Motionless	No discernible body or breathing movements.	Latency
Sitting	Legs underneath the body cavity and wings relaxed against body wall.	Duration
Standing	Standing with the body fully or partly lifted off of the ground.	Duration
Lying	Lying once posture is lost and not perceived to be purposefully controlling posture.	Duration
Out of sight	Bird was completely out of view.	Duration

649

650 **Table 2** Summary statistics (mean, SE, minimum and maximum) of behavioural total
651 durations of bouts and individual bouts during LAPS and statistical differences (F statistic
652 and P value) dependent of A/S treatment. Values within a row with different superscripts
653 differ significantly at *p<0.05.*

Measure	Behaviour	N	Analgesic (A)				Saline (S)				F	P
			Mean	SE	Min	Max	Mean	SE	Min	Max		
Total duration (combined bouts) (s)	Ataxia*	84	23.7	1.7	4.4	65.2	23.5	1.6	3.7	53.0	0.00	0.872
	Leg paddling	65	9.1[a]	1.2	0.3	33.1	6.8[b]	0.8	1.1	17.5	4.04	0.048
	Clonic convulsions	84	23.9	1.7	5.3	65.2	24.2	1.6	3.6	55.7	0.03	0.866
	Tonic convulsions	64	12.6	4.7	2.9	152.9	7.8	0.9	0.1	19.8	1.87	0.175
	Slow wing-flapping	51	2.7	0.3	0.5	6.8	2.8	0.5	0.2	10.8	0.29	0.590
	Sitting	84	60.4	3.7	15.6	168.9	59.0	2.3	37.4	135.9	0.25	0.618
	Standing	22	16.0[a]	4.2	0.9	51.9	12.3[b]	2.9	0.6	20.6	19.39	<0.001

		N	Analgesic (A)				Saline (S)				F	P
			Mean	SE	Min	Max	Mean	SE	Min	Max		
Individual bout duration (s)	Lying	82	76.4	3.0	23.4	120.2	79.1	3.4	23.2	129.6	0.19	0.661
	Open-bill breathing	63	11.0	1.3	3.0	36.4	10.8	1.2	1.8	40.5	0.10	0.716
	Vigilance	83	37.1[a]	1.6	9.6	57.9	30.5[b]	2.0	6.5	50.1	14.50	<0.001
	Leg paddling	65	6.5	0.7	0.3	17.8	4.8	0.6	1.1	17.5	0.15	0.700
	Clonic convulsions	84	7.8	0.4	3.4	15.5	8.8	0.7	1.6	23.5	0.07	0.789
	Tonic convulsions	64	10.9[a]	4.8	3.0	152.9	6.3[b]	0.7	0.1	16.1	7.59	0.007
	Slow wing-flapping	51	3.6[a]	0.4	0.5	6.7	2.1[b]	0.2	0.2	6.3	6.09	0.016
	Sitting	84	51.0	4.1	7.8	168.9	53.2	3.2	18.6	135.9	2.23	0.139
	Standing	22	13.1[a]	3.8	0.9	51.9	7.5[b]	3.0	0.6	20.6	15.65	<0.001
	Lying	82	72.7[a]	3.9	11.7	120.2	75.2[b]	4.2	11.6	129.6	18.53	<0.001
	Open-bill breathing	63	6.8[a]	0.6	3.0	18.2	8.2[b]	0.7	1.0	18.1	7.85	0.006
	Vigilance	83	32.7[a]	2.2	3.2	57.9	26.2[b]	2.5	1.7	50.1	5.62	0.020
Frequency of bouts	Leg paddling	65	1.4	0.1	1.0	4.0	1.2	0.1	1.0	2.0	2.55	0.114
	Clonic convulsions	84	1.1	0.1	1.0	2.0	1.1	0.1	1.0	2.0	0.02	0.890
	Tonic convulsions	64	1.1[a]	0.1	1.0	2.0	1.0[b]	0.0	1.0	1.0	6.10	0.016
	Slow wing-flapping	51	1.3	0.1	1.0	2.0	1.5	0.1	1.0	4.0	1.50	0.225
	Sitting	84	3.1	0.2	1.0	7.0	3.1	0.2	1.0	7.0	0.02	0.881
	Standing	22	1.4	0.1	1.0	4.0	1.3	0.1	1.0	3.0	0.81	0.370
	Lying	82	1.2[a]	0.1	1.0	3.0	1.3[b]	0.1	1.0	3.0	4.41	0.039
	Open-bill breathing	63	1.4	0.1	1.0	3.0	1.6	0.2	1.0	5.0	2.95	0.090
	Vigilance	83	1.6[a]	0.1	1.0	3.0	1.3[b]	0.1	1.0	3.0	5.60	0.020

* No individual bout duration for ataxia, as ataxia only occurred in one single bout, therefore descriptive statistics listed under total duration.

Table 3 Summary statistics (mean, SE, minimum and maximum) of behavioural latencies during LAPS and statistical differences (F statistic and P value) dependent of treatment. Values within a row with different superscripts differ significantly at *P*<0.05.

Latency to behaviours (s)	N	Analgesic (A)				Saline (S)				*F*	*P*
		Mean	SE	Min	Max	Mean	SE	Min	Max		

Behaviour	N	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	F statistic	P value
Ataxia	84	44.5[a]	1.3	21.4	65.2	41.8[b]	1.0	28.4	53.0	4.76	0.032
Loss of jaw tone	65	75.1	1.7	61.6	105.1	72.5	1.9	42.1	98.0	0.80	0.375
Motionless	84	144.7	2.5	105.9	180.3	144	2.9	86.1	185.3	0.03	0.870
Leg paddling	65	98.9	3.4	63.4	143.0	92.6	4.3	43.8	155.0	3.93	0.051
Clonic convulsions	85	76.1	2.6	57.2	137.4	78.2	3.3	44.8	147.6	0.49	0.486
Tonic convulsions	64	121.7	3.3	88.5	160.5	119.1	3.5	71.3	163.8	0.23	0.630
Slow wing-flapping	51	64.7	2.5	42.4	91.6	60.8	2.5	6.1	87.5	0.49	0.145
Loss of posture	83	59.6	1.1	46.8	73.8	57.2	0.9	44.0	70.0	3.09	0.083
Mandibulation	41	25.0[a]	3.2	3.6	72.4	18.8[b]	3.7	2.8	62.3	7.40	0.008
Head shake	51	42.1	5.4	7.3	107.1	40.5	3.4	6.5	72.4	0.03	0.869
Open-bill breathing	67	64.2	1.7	43.3	84.0	65.3	1.6	51.9	92.1	0.58	0.448
Deep inhalation	48	101.8[a]	5.1	55.2	141.4	82.5[b]	7.1	4.7	126.1	15.62	0.001
Vigilance	83	2.3	0.5	0.3	17.8	2.6	0.7	0.1	20.1	0.70	0.406
Vocalisations*	4	62.7	7.1	14.6	94.5	103.9	0.0	103.9	103.9	-	-

660 * No modelling possible for latencies for vocalisations (too few observations (N=4)).

661

662 **Table 4** Summary statistics (mean, SE, minimum and maximum) of behavioural total counts

663 during LAPS and statistical differences (F statistic and P value) dependent of A/S treatment.

664 Values within a row with different superscripts differ significantly at *p*<0.05.

Behaviour[1]	N[2]	Analgesic (A)[3]				Saline (S)[3]				F statistic	P value
		Mean	SE	Min.	Max.	Mean	SE	Min.	Max.		
Jump	83	0.5[a]	0.1	0.0	2.0	1.0[b]	0.1	0.0	3.0	10.93	0.001
Mandibulation	44	2.1[a]	0.4	1.0	12.0	2.7[b]	0.5	1.0	8.0	32.33	<0.001
Vocalisation	4	2.0	1.0	1.0	4.0	1.0	0.0	1.0	1.0	-	-
Head shake	51	2.4[a]	0.3	1.0	7.0	1.7[b]	0.2	1.0	5.0	8.69	0.004
Deep inhalation	48	2.0	0.3	1.0	8.0	1.9	0.2	1.0	5.0	1.39	0.241

665
666 **Table 5** Frequency table demonstrating the proportions of birds which were observed

667 performing (yes), or were not recorded (missing data) due to being out of sight, total number

668 of birds (total) and the percentage of birds which performed the behaviour %).

Behaviour	Analgesic				Saline			
	Yes	Missing data	Total	%	Yes	Missing data	Total	%
Standing	14	2	43	33	8	3	40	20
Leg paddling	32	2	43	74	33	3	42	79
Clonic convulsions	43	2	43	100	42	3	42	100
Tonic convulsions	31	2	43	72	33	3	42	79
Slow-wing flapping	20	2	43	47	31	3	42	74
Notice	42	2	43	98	41	4	41	100
Mandibulation	23	2	43	53	18	4	41	44
Head shaking	23	2	43	53	28	4	41	68
Open-bill breathing	33	2	43	77	34	4	41	83

Deep inhalation	22	3	42	52	26	4	41	63
Jump	20	2	43	47	27	5	40	68
Vocals	3	2	43	7	1	4	41	2
Sitting	43	2	43	100	41	4	41	100
Lying	42	3	42	100	41	4	41	100
Motionless	43	2	43	100	41	4	41	100
loss of jaw tone	30	15	30	100	35	10	35	100
ataxia	43	2	43	100	41	4	31	100
LOP	43	2	43	100	40	5	40	100
Escape	42	3	42	100	42	3	42	100
Peck	42	3	42	100	42	3	42	100
Panting	42	3	42	100	42	3	42	100

669

670

671

672

673

674

675

676

677

678