An attention bias test to assess anxiety states in laying hens

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38

Abstract

39
40 Fear is a response to a known threat, anxiety is a response to a perceived threat. Both of these
41 affective states can be detrimental to animal welfare in modern housing environments. In
42 comparison to the well-validated tests for assessing fear in laying hens, tests for measuring
43 anxiety are less developed. Perception of a threat can result in an attention bias that may indicate
44 anxious affective states in individual hens following playback of an alarm call. In Experiment 1,
45 an attention bias test was applied to hens that differed in their range access to show that hens that
46 never ranged were more vigilant (stretching of the neck and looking around: P < 0.001) and
47 slower to feed following the second alarm call playback (P = 0.01) compared with hens that
48 ranged daily. All hens showed a reduction in comb temperature following the first alarm call (P
49 < 0.001). In Experiment 2, an open field test was used to determine an effective dose of 2mg/kg
50 for the anxiogenic drug *meta*-Chlorophenylpiperazine (*m*-CPP) in adult laying hens. Hens dosed
51 with 2mg/kg showed reduced locomotion compared with a saline solution (P < 0.05). In
52 Experiment 3, 2mg/kg *m*-CPP or saline was administered to adult hens previously habituated to
53 the open field arena to pharmacologically validate an attention bias test as a measure of anxiety.
54 Hens dosed with *m*-CPP were slower to feed (P = 0.02) and faster to vocalize following a second
55 alarm call playback (P = 0.03) but these hens did not exhibit the same vigilance behavior as
56 documented in Experiment 1. The *m*-CPP hens also spent more time stepping and vocalizing
57 (both P < 0.001) than the saline hens. An attention bias test could be used to assess anxiety.
58 However, behavioral responses of hens may vary depending on their age or test environment
59 familiarity, thus further refinement of the test is required. In these tests, 2mg/kg of *m*-CPP
60 resulted in freezing when the environment was novel, but more movement and vocalizing when
61 the environment was familiar. The extreme behavioral phenotypes exhibited by individually-
62 tested birds may both be indicators of negative states.

63

Introduction

64
65 Fear and anxiety in animals are emotional responses to a threat that stimulate adaptive behaviors
66 such as escape or defense (Steimer, 2002). While fearful and anxious states are entangled in
67 some respects and have overlap in the underlying neurological mechanisms (Davis, 1992), they
68 can be distinguished as responses to known (fear) and unknown (anxiety) dangers (Steimer,
69 2002). Fear responses are adaptive for self-preservation but an excessive or inappropriate fear
70 response can result in stress, injury, pain, or development of abnormal behavior and is thus
71 considered a negative welfare state (Barnett et al., 1994; de Haas et al., 2014a; Fraise and
72 Cockrem, 2006; Jones, 1996; Uitdehaag et al., 2008). The emotional state of anxiety may also
73 result in similar negative impacts (Ohl et al., 2008) but the assessment of this state in laying hens
74 is less defined than measurement of fear (see Forkman et al., 2007 for a critical review of fear
75 tests).
76   In laying hens, an open field test has previously been used as a measure of anxiety
77 (Nordquist et al. 2011) although it is most commonly categorized as fear assessment (Gallup and

78 Suarez, 1980; Suarez and Gallup, 1982). Typically an open field test in chickens is interpreted as
79 representing a balance between anti-predatory behavior manifested as freezing, and a desire for
80 social contact, manifested as vocalizations (Gallup and Suarez, 1980; Vallortigara, 1988;
81 Vallortigara and Zanforlin, 1988). A chicken's desire for social reinstatement when placed into a
82 novel and isolated environment has also been used as an indication of anxiety (de Haas et al.,
83 2014b). Attention bias towards a threat is a further potential measure of anxiety (reviewed in
84 Crump et al., 2018). When presented with a threat, anxious individuals will show increased or
85 biased attention towards that threat including increased vigilance behavior and a reduced
86 willingness to feed (Bethell et al., 2012; Bradley et al., 1997; Eilam et al., 2011; Lee et al., 2016,
87 2018; Monk et al., 2018a,b). This attention and vigilance behavior can be assessed as an
88 indicator of the anxious state of the animal. Brilot and Bateson (2012) first demonstrated the
89 importance of attention bias towards threats as a measure of welfare in starlings. Birds that were
90 denied access to a water bath, which is important for maintaining feather condition, were more
91 vigilant towards conspecific alarm calls than those birds that were provided a water bath. This
92 study used threat perception to indicate that birds denied bathing opportunities were in a more
93 anxious state and that water baths were important for starling welfare.
94   Attention bias towards threats has recently been pharmacologically validated as a
95 measure of anxiety in sheep (Lee et al., 2016; Monk et al., 2018a,b) and cattle (Lee et al., 2018)
96 using the anxiogenic drug *meta*-Chlorophenylpiperazine (*m*-CPP). In the recent validation tests,
97 sheep or cattle that were briefly presented a dog while dosed with *m*-CPP showed increased
98 vigilance and attention towards the previous location of the dog relative to control animals (Lee
99 et al., 2016, 2018; Monk et al., 2018a,b). Internal temperature loggers were also used in these
100 studies to demonstrate stress-induced hyperthermia as a measurable physiological temperature
101 response to the threat while under pharmacological influence (Lee et al., 2018; Monk et al.,
102 2018a,b). In laying hens, observations of vigilance have been made in individual or group
103 settings to assess the fear or stress status of birds in response to different environmental
104 conditions (e.g. group size during perching: Newberry et al., 2001; perch height: Brendler et al.,
105 2014; presence of cockerels: Odén et al., 2005; a novel arena: Rutherford et al., 2003; presence
106 of predator odors: Zidar and Løvlie, 2012), including differences in vigilance in response to an
107 acoustical disturbance (Brendler et al., 2014). Favati and colleagues (2014) also measured
108 vigilance following playback of an alarm call and correlated this with dominance in male
109 domestic fowl. Moriarty (1995) gave an anxiogenic β-carboline to layer chicks and found longer
110 durations of tonic immobility and reduced movement and vocalizations in an open field test
111 compared to control chicks which was interpreted as increased fear and anxiety. However, to the
112 best of the authors' knowledge, there is no research on pharmacologically validating an
113 individual-bird test of attention bias following perception of a threat (alarm call) in layers that
114 could be used as a standard welfare assessment test for domestic hens. The drug *m*-CPP was
115 selected for pharmacological manipulation of anxiety based on the previous success with cattle
116 (Lee et al., 2018) and sheep (Lee et al., 2016; Monk et al., 2018a,b). Physiologically, peripheral
117 temperatures on a hen's head can also be measured as an indicator of a stress-induced

118 hyperthermic response where elevated core temperature correspondingly results in a decrease in
119 peripheral temperatures (Edgar et al., 2011, 2013; Herborn et al., 2015).
120   Radio-frequency identification technology has demonstrated that individual free-range
121 laying hens, both in commercial and experimental settings, will vary greatly in their range use
122 (Campbell et al., 2017; Gebhardt-Henrich et al., 2014, Larsen et al., 2018). Although the
123 majority of hens will visit the range on most days, there are small percentages of hens that will
124 rarely or never go outside (low ranging). External health assessments have, to date, not identified
125 specific illness or injury in these low-ranging birds, suggesting they may vary in behavioral traits
126 which impacts their choice to not go outdoors (Campbell et al., 2016; Larsen et al., 2018).
127 Correlations of range use with individual responses in a compilation of tests (tonic immobility,
128 open field tests, manual restraint tests, novel object tests) have shown that indoor-preferring hens
129 present higher levels of fear and greater stress responses compared to birds that regularly use the
130 range (Campbell et al., 2016; Hartcher et al., 2016; Hernandez et al., 2014a; Larsen et al., 2018),
131 although not consistently across every behavioral test in every study. Within a free-range system,
132 the indoor barn is a more controlled and predictable environment than the outdoor range where
133 birds can be exposed to variable and changing conditions, including predators. Indoor birds may
134 choose to stay indoors because they are also more anxious than hens that regularly go outside.
135 Previous research on personality assessment in domestic fowl has confirmed repeatable
136 personality differences in fowl that are also correlated with other behavioral profiles such as
137 coping style or some aspects of learning (Zidar et al., 2017, 2018).
138   The objectives of the current study were three-fold, presented as they occurred
139 chronologically. The first objective was to use an attention bias test with an alarm call playback
140 to assess differences in behavioral and peripheral temperature responses between free-range hens
141 that had used the range every day (outdoor) compared to hens that did not visit the range
142 (indoor). The alarm call playback was proposed to elicit fear when it was played, but anxiety
143 once it stopped. It was predicted the indoor birds would pay greater attention towards the
144 surrounding environment manifested as greater vigilance behavior and delayed willingness to
145 feed indicating higher anxiety than indoor birds. These indoor birds would also exhibit a stress-
146 induced hyperthermic response with a drop in peripheral temperatures. The second objective was
147 to determine an appropriate dose rate of the anxiogenic drug meta-Chlorophenylpiperazine (*m*-
148 CPP) for young-adult laying hens using an open field test. This drug was predicted to induce
149 anxiety in chickens resulting in reduced locomotion but more vocalizations. The third objective
150 was to compare the responses of birds given *m*-CPP or saline solution in an attention bias test to
151 pharmacologically validate this test as a measure of anxiety to be used in welfare assessment of
152 domestic poultry. It was predicted that the *m*-CPP dosed birds would show greater vigilance,
153 reduced locomotion, more vocalizations and a longer latency to feed.
154
155

156 **Materials & Methods Experiment 1: Indoor and outdoor free-range**
157 **hens**

158
159 **Animals and housing**
160 Research was approved by the CSIRO FD McMaster Laboratory Chiswick Animal Ethics
161 Committee (ARA 12-13) in compliance with the Australian Code for the Care and Use of
162 Animals for Scientific Purposes (National Health and Medical Research Council, 2004).
163 Two hundred ISA Brown pullets were obtained from a commercial supplier in 2012 at 18 weeks
164 of age and housed at CSIRO's Chiswick Poultry Facility, Armidale, NSW. The birds had been
165 floor-reared until 8 weeks of age, then housed in cages until 18 weeks of age. Birds were housed
166 indoors in an experimental tunnel-ventilated shed with straw litter area of 0.5m2/hen, access to
167 water nipples, suspended pan feeders, nest boxes and perches. All of the provided resources per
168 bird exceeded the current Model Code of Practice for Domestic Poultry (Primary Industries
169 Standing Committee, 2002). The birds were fed commercial layer pellets ad libitum. Lighting
170 duration in the shed gradually increased up to a schedule of 15h L: 9h D. Starting at 22 weeks of
171 age, birds were given daily access to a range area of 12.5m2 per bird or 800 birds/hectare (at
172 maximum occupancy) from 09:30 until 16:00 (pop holes required manual operation). Range
173 access was available via 4 pop hole passageways (36 cm H x 18 cm W). The range area was
174 grassed but devoid of trees, shelter or shade structures. All birds were visually inspected daily.
175 All birds were leg-banded upon arrival with numbered adjustable bands (Roxan Developments
176 Ltd) containing a Trovan® Unique ID 100 (FDX-A) microchip: operating frequency 128 kHz.
177 Individual range use was tracked for 51 days from first pop hole opening using radio-frequency
178 identification (RFID) technology. The RFID systems were purchased from Microchips Australia
179 Pty Ltd (Keysborough, VIC) with equipment developed and built by Dorset Identification B.V.
180 (Aalten, Netherlands) using Trovan® technology. Antennas situated within the pop hole
181 passageways registered the date, time and microchip of the individual bird as it went out onto the
182 range, and again when the bird came back inside. The functionality of all microchips were
183 confirmed prior to placement on birds and at the end of the experiment. Further details on the
184 specifications and validation of the RFID system are available in Campbell et al. (2017)
185
186 **Experimental protocols**
187 At 30 weeks of age, 36 hens were selected based on their range use patterns. 'Outdoor' birds
188 were defined as birds that ranged daily with 20 hens selected that also comparatively ranged for
189 the longest period of time daily, and 16 'indoor' birds that were never registered to go outside
190 (this was the total sample size of indoor birds). These birds were then tested in a manual restraint
191 test, tonic immobility test and an open field test (carried out in the same arena as the attention
192 bias test) as part of a separate dataset (Hernandez et al. 2014b, unpublished data). Each
193 behavioral test was conducted once per bird. At 33 to 35 weeks of age the birds were also trained
194 for tests of judgement bias as part of a separate dataset which involved several weeks of handling
195 (Hernandez et al., 2014b, unpublished data). The birds had daily range access on days when tests
196 did not occur, but range use was not further tracked past 30 weeks of age when the
197 indoor/outdoor birds were identified. At 51 weeks of age, birds were tested in an attention bias

198 test. Range use may have changed during this period but other studies carried out with RFID
199 tracking indicate outdoor birds are consistent in daily access across time (Campbell et al., 2017)
200 and birds that remained indoors for the first 8 weeks of range access are distinct from birds that
201 ventured outside immediately.
202
Attention bias test
203
204 The arena used for an attention bias test was a wooden square open field arena of 1.25 m L x
205 1.22 m H and elevated 0.24 m off the ground with a clear frontal Perspex sliding panel. This
206 arena was located in a separate enclosed room adjacent to the main poultry shed. Birds that were
207 not being tested (i.e. within their home pens) could not hear the alarm call being played. A small
208 pile of mixed grain was present in the back center of the arena. Birds were randomly selected
209 from the home pen, held for thermal images (see following section for further details), carried to
210 the test arena, placed in the front center and the test began. A conspecific alarm call was played
211 immediately from portable speakers (Altec Lansing Technologies, InMotion, Milford, PA, USA
212 MODEL #42820 ALUK0037708). Alarm calls had been previously opportunistically recorded
213 using a different group of 100 ISA Brown hens that were disturbed by a wild bird making noise
214 outside the shed. As the alarm calls were produced by hens, they were likely signaling a 'ground
215 predator' (Wilson and Evans, 2012) which should elicit a 'vigilant, erect posture' (Evans et al.,
216 1993). Recordings were made using a Roland R-05 MP3 recording device (Roland Corporation
217 US, Los Angeles, CA) with a sampling rate of 48 kHz, and a bit rate of 128kbps. The audio clips
218 were edited into short playback files using the software Audacity 2.0.3
219 (http://audacity.sourceforge.net/). The same alarm call (8 sec length) was played for all birds at a
220 volume perceived to be similar to an alarm call volume of hens but precise details on the dB are
221 unavailable. A video camera recorded all bird behavior and a second camera was connected to a
222 screen to allow monitoring of the bird by an experimenter that remained out of sight. Once the
223 bird reached the grain pile, it was permitted 5 seconds to consume grain, then the alarm call was
224 played a second time to gain a measure of their latency to resume feeding. Once the bird resumed
225 eating the grains the test concluded, with a maximum test time of 10 min allotted.
226
Behavior analysis
227
228 All videos were coded by a single experimenter using The Observer XT 12.0 (Noldus
229 Information Technology) who was blind to the ranging status of each hen and trained in the
230 behavioral observations. Behavior was quantified to provide latency to first step, first vocalize,
231 first eat, and the latency to eat following the second alarm call. Vigilance behavior was observed
232 for the first 30 seconds following the first alarm call. Vigilance was quantified as the bird visibly
233 stretching their neck while looking around, turning of the head may/may not also occur.
234 Definitions of vigilance vary between laying hen studies (Newberry et al., 2001; Odén et al.,
235 2005; Zidar and Løvlie, 2012) with this definition chosen as these clearly definable behaviors
236 were observed. The terms of this behavior were agreed upon by three different observers prior to
237 video observations but subsequently coded by a single observer only. Behavior was also

238   observed during the alarm call to quantify if birds were responding to the playback as an
239   indication that they were perceiving it as a threat. A 'yes' response was categorized as exhibiting
240   vigilance, a 'maybe' response was categorized as the bird standing still but moving their head to
241   look around (without neck stretching) and a 'no' was quantified as the bird walking or eating
242   with no apparent alteration in behavior during the playback. Intra-observer reliability for
243   vigilance behavior across 8 birds was 91% as assessed by correlation analysis in Microsoft
244   Excel. Intra-observer reliability for latency measures across 8 birds was 95 to 97%. A second
245   observer watched the behavior during the alarm call to confirm 100% agreement with the
246   assigned categories.
247

248   **Peripheral temperature assessment**
249   To measure bird's temperature responses to the alarm call, a FLIR® ThermaCAM S60 was used
250   (with emissivity set at 0.98) to take thermal images of the bird's head approximately every 10
251   seconds for the first 2 min of the test when possible. Because the test concluded following the
252   second time eating, the total test length varied between birds but the first 2 mins included the
253   majority of tested birds. The camera was placed within a cut-out on the side of the open field
254   arena. To obtain baseline temperature values, images were first taken in the home pen (bird was
255   unrestrained), and again with the bird held by the experimenter to allow clear images to be
256   obtained. The bird was placed in the attention bias arena following collection of these images,
257   which occurred up to approximately 2 mins before the test began. Ambient temperature (°C) and
258   relative humidity (%) inside the sampling area were recorded every 30 min and entered into the
259   infrared camera to calibrate it for atmospheric conditions. The maximum temperatures of the
260   eye, comb, and head (as defined in Edgar et al., 2013) were recorded from the thermal images
261   using image analysis software (ThermaCam Researcher 2.7, FLIR Systems AB, Danderyd,
262   Sweden) and averaged per bird across the baseline images, then 1 min periods for the first 2 mins
263   of the test. Hens that finished the test before 2 mins and hens that did not have clear images at
264   each time point were excluded (n = 12 outdoor, n = 12 indoor birds remained for analyses).
265

266   **Data and statistical analyses**
267   All data were checked for homoscedasticity of the residuals to proceed with parametric tests. The
268   time spent vigilant was analyzed using a two-tailed t-test to compare range use groups. The
269   latencies to first step, vocalize and eat (after first and second alarm call) were analyzed using
270   separate Kaplan-Meier estimates with a log-rank test for differences between range use groups.
271   Birds which failed to perform each behavior within 600 s were censored results as per methods
272   for adjusting survival data to account for individuals which did not reach a specific point within
273   the allotted timeframe (Leung et al., 1997). A total of 18 outdoor and 15 indoor birds were
274   included in the analysis of the response to the second alarm call. The average max temperatures
275   for the bird's head, comb and eye from baseline up to 2 mins post alarm call were compared
276   using a General Linear Mixed Model with range use groups (indoor/outdoor) and time (baseline,
277   0-1 and 1-2 min post alarm call) as fixed effects including their interaction, bird ID was included

278 as a random effect, nested within range use group. Restricted maximum likelihood estimation
279 methods were applied. The interactions were not significant (all P ≥ 0.29) and thus were
280 removed from the final model. Post-hoc Students t-tests were conducted on the least squares
281 means. All analyses were conducted in JMP 13.0.0 (SAS Institute, Cary, NC) with α set at 0.05.

282

283

284 **Results Experiment 1: Indoor and outdoor free-range hens**

285

286 The majority of birds showed clear vigilant responses to the alarm call playback with the
287 exception of two birds that were categorized as 'maybe' showing a response, and two birds were
288 categorized as a 'no' response. Two outdoor and one indoor bird never received a second alarm
289 call and reached the maximum test time.
290   The indoor birds spent more time vigilant in the first 30 seconds following the first alarm
291 call than the outdoor birds (t = 4.74, df = 41.20, p < 0.001; mean ± SEM outdoor birds: 11.78 ±
292 1.82 seconds, indoor birds: 24.56 ± 1.99 seconds). The outdoor birds were faster to eat following
293 the second alarm call than the indoor birds (χ2 = 6.76, df = 1, p = 0.009, Fig. 1). However, there
294 were no differences between range use groups in latency to eat following the first alarm call (χ2
295 = 0.60, df = 1, p = 0.44; mean ± SEM outdoor birds: 176.23 ± 33.80 seconds, indoor birds:
296 227.54 ± 34.50 seconds), the latency to first step (χ2 = 0.05, df = 1, p = 0.82; mean ± SEM
297 outdoor birds: 141.37 ± 26.98 seconds, indoor birds: 151.56 ± 30.20 seconds), or the latency to
298 first vocalize (χ2 = 0.93, df = 1, p = 0.33; mean ± SEM outdoor birds: 49.16 ± 13.13 seconds,
299 indoor birds: 62.21 ± 11.28 seconds).
300   There was a significant difference across time for the comb temperature ($F_{(2,46)}$ = 12.39, p
301 < 0.001) with the highest temperature at baseline (mean ± SEM baseline: 32.59 ± 0.58 ° C, 0-1
302 min: 31.13 ± 0.58 ° C, 1-2 min: 31.43 ± 0.58 ° C). The indoor birds also showed significantly
303 lower comb temperatures overall ($F_{(1,22)}$ = 4.79, p = 0.04; mean ± SEM indoor birds: 30.50 ±
304 0.48 °C, outdoor birds: 32.93 ± 0.48 °C). There were no significant differences across time for
305 the head ($F_{(2,46)}$ = 3.09, p = 0.06; mean ± SEM baseline: 38.21 ± 0.11, 0-1 min: 37.93 ± 0.13, 1-2
306 min: 38.06 ± 0.13), or eye ($F_{(2,46)}$ = 0.71, p = 0.50; mean ± SEM baseline: 36.62 ± 0.17, 0-1 min:
307 36.36 ± 0.11, 1-2 min: 36.60 ± 0.13) temperatures nor differences between range use groups in
308 the overall head ($F_{(2,46)}$ = 3.11, P = 0.09; mean ± SEM outdoor birds: 38.24 ± 0.09, indoor birds:
309 37.89 ± 0.10), or eye ($F_{(1,22)}$ = 0.78, p = 0.39; mean ± SEM outdoor birds: 36.65 ± 0.12, indoor
310 birds: 36.47 ± 0.11) temperatures.

311

312 **Materials & Methods Experiment 2: Drug dose determination**

313

314 **Animals and housing**

315 All research was approved by the University of New England Animal Ethics Committee (AEC
316 15-129) in compliance with the Australian Code for the Care and Use of Animals for Scientific
317 Purposes (National Health and Medical Research Council, 2004).
318 Commercial floor-reared ISA Brown pullets ($n = 102$) were obtained at 15 weeks of age in 2017
319 and housed at the University of New England's Laureldale Poultry Facility. All birds were leg-
320 banded upon arrival and equally divided between two adjacent floor pens (4.8 m L x 3.6 m W) at
321 an approximate stocking density of 3 birds per m2. Each pen contained perches, nest boxes, a
322 single round feeder, a water nipple line and rice hulls as floor litter. All of the provided resources
323 per bird exceeded the current Model Code of Practice (Primary Industries Standing Committee,
324 2002). The birds were fed ad libitum a pullet grower feed, followed by a commercial layer mash
325 from 18 weeks of age. The fluorescent lights in the shed were on a 16h L: 8h D schedule with
326 fan ventilation but no temperature or humidity control. Birds were visually inspected daily. A
327 third adjacent pen of identical resources and configuration was available to temporarily house
328 birds during testing periods. There were no visual barriers between the adjacent pens.
329
330 **Drug treatments**
331 Four different treatments were used to determine the optimal dose rate of *meta*-
332 Chlorophenylpiperazine (*m*-CPP). These included a saline solution (control), 0.5mg/kg, 1mg/kg
333 and 2mg/kg of *m*-CPP mixed into a saline solution at a dosing volume of 1:1. These drug
334 dosages were selected based on prior studies using *m*-CPP with rats and sheep (Bilkei-Gorzó et
335 al., 1998; Lee et al., 2016). The drug was mixed a maximum of 24 h prior to use and kept
336 refrigerated (4ºC) while not in use. Sixty hens were randomly allocated to the four treatment
337 groups (n = 15 birds/treatment). The majority of the birds (n = 51) came from within a single
338 pen, with remaining birds taken from the second pen (n = 9) and distributed across the treatment
339 groups. We acknowledge that birds from separate home pens may behave differently but we
340 determined the impact of home pen would be minimal as the birds were from the same rearing
341 flock and the home pens were directly adjacent allowing both visual and acoustic contact
342 between neighboring birds. There were also no direct statistical comparisons between groups of
343 birds from the separate pens (i.e. between Experiment 2 and Experiment 3) and where
344 applicable, birds from the two home pens were balanced across a statistical treatment
345 comparison. Birds were individually weighed (Bat1, VEIT Electronics, Moravany, Czech
346 Republic) one day prior to the first day of testing to provide body weights for calculating
347 individual dosages.
348   The open field test was used to behaviorally assess the impacts of the drug as this test is
349 known to measure fear and desire for social reinstatement in chickens (Forkman et al., 2007);
350 social reinstatement has been used as an indicator of anxiety (de Haas et al., 2014b). On the three
351 testing days, individual birds, 19 weeks of age, were captured in their home pen and carried to a
352 table located within the same shed. The bird was placed on its side with two handlers securing
353 the bird gently (including covering their head) while a third person administered a subcutaneous
354 injection of the drug or saline at the shoulder site. After each bird was injected, it was placed into

355   the temporary holding pen. The first bird of the day was in visual contact with the neighboring
356   pen of birds but physically isolated. Subsequent birds were not physically isolated as both newly
357   dosed and tested birds were placed into the same pen to either wait for the drug to take effect or
358   to observe for any longer-term drug effects across the day. A 20-min waiting interval for the
359   drug to take effect was determined based on rat studies with *m*-CPP (Bilkei-Gorzó et al., 1998).
360   Previous drug studies with young chicks have applied a 15-min waiting interval (Sufka et al.,
361   2009; Hymel and Sufka, 2012) but there has been no previous application of *m*-CPP on adult
362   hens. The same waiting interval was applied to birds injected with saline. During this time
363   experimenters were able to observe the birds from a distance to ensure no observable adverse
364   effects of the drug. On the first day of testing, the first hen received a dose of *m*-CPP at
365   0.5mg/kg, followed by a dose of 1mg/kg on the second hen, and a dose of 2mg/kg on the third
366   hen. Almost no visible effects were observed with the hens in their pen (and specifically no
367   observable adverse effects) and thus the drug treatment doses henceforth were administered in a
368   random order.
369
370   **Open field testing** Individual birds were re-caught from the temporary pen just prior to the
371   conclusion of the 20-min interval following drug administration and carried to the open field test
372   arena located in a separate room. At exactly 20 min following dosing, the bird was placed into
373   the front center of the open field arena with the room lights turned off. The wooden open field
374   arena was a square of 1.25 m L x 1.22 m H and elevated 0.24 m off the ground with a clear
375   frontal Perspex sliding panel (the same as per used in Experiment 1). Lights were then switched
376   on and an open field test began. One video camera recorded all bird behavior for later analysis
377   and a second camera was connected to a screen so all birds could be visually monitored during
378   the test by an experimenter who remained within the room but out of sight. The open field test
379   was 10 min in duration. At the conclusion of the test the lights were turned off and the bird was
380   carried back to the temporary holding pen to allow further visual monitoring for any adverse
381   effects of the drug.
382
383   **Video observations**
384   The videos were watched by a single observer blind to the dosages for each hen who was first
385   trained in all types of behavioral observations that were made. The observer recorded the latency
386   to first move (when the hen moved more than just their head but did not take a step), latency to
387   first step, and latency to first vocalize. Measuring the latency to first move differentiated the
388   birds that were motionless in position upon placement in the arena versus those that were moving
389   their body but cautious to step. The number of steps and number of vocalizations were also
390   recorded within 1-min intervals. Any individual noise made by the hen was recorded as a single
391   vocalization. The vocalizations were also re-classified by a separate trained observer (blind to
392   treatments) into comparatively 'short' and 'long' calls. No bird made alarm calls while in the
393   arena. A random selection of 12 videos were looked at by a second observer with the inter-

394   observer reliability correlation at 95 – 98% across all variables as assessed by correlation
395   analysis in Microsoft Excel.
396
397   **Data and statistical analyses**
398   All latency data (first move, step, and vocalize) and counts of steps and vocalizations per 1 min
399   intervals were collated for each dosing treatment group. The latency to first move, step, and
400   vocalization data were analyzed using Kaplan-Meier estimates with a log-rank test for
401   differences between treatment groups. Birds which failed to perform each behavior within 600 s
402   were censored results. The count data were square-root transformed and analyzed using General
403   Linear Mixed Models that included the effects of treatment, time, and their interaction. Non-
404   significant interactions were removed from the final model. Bird ID nested within treatment was
405   included as a random effect. Restricted maximum likelihood estimation methods were applied.
406   All analyses were conducted in JMP 13.0.0 (SAS Institute, Cary, NC) with α set at 0.05.
407

Results Experiment 2: Drug dose determination

409
410   The Kaplan-Meier estimates indicated an effect of drug dosing treatment in the latency to first
411   move ($\chi 2 = 15.67$, df = 3, $p = 0.001$, Fig. 2), but no differences between dosing treatment groups
412   in the latency to first vocalize ($\chi 2 = 6.02$, df = 3, $p = 0.11$; mean ± SEM saline: 114 ± 54,
413   0.5mg/kg: 156 ± 61, 1mg/kg: 147 ± 53, 2mg/kg: 317 ± 71), or the latency to first step ($\chi 2 = 3.27$,
414    $p = 0.35$; mean ± SEM saline: 211 ± 66, 0.5mg/kg: 271 ± 63, 1mg/kg: 335 ± 62, 2mg/kg: 355 ±
415   66).
416   There was no overall effect of dosing treatment on the number of steps birds made across the 10
417   min test duration ($F_{(3,56)} = 2.09$ $p = 0.11$; mean ± SEM saline: 8.41 ± 0.97, 0.5mg/kg: 6.4 ± 0.90,
418   1mg/kg: 4.41 ± 0.70, 2mg/kg: 1.82 ± 0.40). However, visually the data were distinct and thus
419   more focused post hoc tests were applied (Hsu, 1997) which did show significantly fewer steps
420   were made by the 2mg/kg dosed birds compared to the saline dosed birds. There was also an
421   effect of time ($F_{(9,531)} = 5.11$, $p < 0.001$) with comparatively fewer steps made during the first 2
422   min of the test (Table 1). There was no interaction between treatment and time ($p = 0.63$) and
423   this was removed from the final model. When step data from the first 2 min of the test only were
424   analyzed, there was a significant effect of dosing treatment ($F_{(3,56)} = 3.34$, $p = 0.03$) with birds
425   given both 1mg/kg and 2mg/kg *m*-CPP stepping less than birds given saline (Fig. 3). There was
426   also an effect of time ($F_{(1,59)} = 5.55$, $p = 0.02$) with fewer steps made in the first minute of the test
427   (Table 1) but no interaction between time and dosing treatment group ($p = 0.29$) and this was
428   removed from the final model. There was an interaction between dosing treatment and time in
429   the total number of vocalizations made ($F_{(27,504)} = 1.64$, $p = 0.02$) and an overall effect of time
430   ($F_{(9,504)} = 7.93$, $p < 0.001$) with more vocalizations made in the first 4 minutes of the test (Table
431   1). There was no overall effect of dosing treatment group ($F_{(3,56)} = 1.0$, $p = 0.40$; mean ± SEM
432   saline: 3.69 ± 0.56, 0.5mg/kg: 8.4 ± 1.62, 1mg/kg: 3.73 ± 0.64, 2mg/kg: 6.96 ± 2.0). When
433   comparisons were made specifically on the number of short vocalizations made there was no

434   effect of treatment ($F_{(3,56)}$ = 1.18, p = 0.33; mean ± SEM saline: 3.18 ± 0.52, 0.5mg/kg: 8.19 ±
435   1.62, 1mg/kg: 3.57 ± 0.63, 2mg/kg: 6.15 ± 1.87), but more were made during the first 4 minutes
436   of the test ($F_{(9,531)}$ = 7.55, p < 0.001, Table 1) and there was no interaction between treatment and
437   time (p = 0.10) and this was removed from the final model. There was a significant interaction
438   between treatment and time for the long vocalizations ($F_{(27,504)}$ = 2.16, p < 0.001) with saline
439   birds making the most long vocalizations during the first 2 mins of the test (Table 1). Overall, the
440   2mg/kg birds made fewer long vocalizations than the saline and 0.5mg/kg birds ($F_{(3,56)}$ = 3.03, p
441   = 0.03) but the numbers of these calls were low (mean ± SEM saline: 0.36 ± 0.09, 0.5mg/kg:
442   0.23 ± 0.06, 1mg/kg: 0.14 ± 0.05, 2mg/kg: 0.03 ± 0.03) and reduced significantly across time
443   ($F_{(9,504)}$ = 4.95, p < 0.001, Table 1).
444
445

446   **Materials & Methods Experiment 3: Pharmacological validation of an**
447   **attention bias test**
448

449   **Habituation phase**
450   All research was approved by the University of New England Animal Ethics Committee (AEC
451   15-129) in compliance with the Australian Code for the Care and Use of Animals for Scientific
452   Purposes (National Health and Medical Research Council, 2004).
453   Fifty birds were used to pharmacologically validate an attention bias test. This sample size
454   included the remaining birds that were not previously used for pharmacological dosing trials in
455   Experiment 2 and also14 birds that had previously been given only a saline dose in the open field
456   test (Experiment 2). These previously-tested birds were subsequently divided evenly between
457   treatment groups for the attention bias test. However, to reduce the chance of birds responding to
458   the novelty of the test arena with freezing behavior (as observed in Experiment 2) rather than
459   responding to the alarm call playback, all birds were habituated to the open field arena at 21 to
460   22 weeks of age. This was to closer approximate the tests done by Brilot and colleagues (2012)
461   where an alarm call was played to birds in their home cages. For habituation, all birds were
462   caught from their home pen and individually placed into the front-center of the open field arena
463   with the lights off. Lights in the room were turned on and each bird was given 10 min to become
464   familiarized with the environment. Lights were turned off after 10 min and the bird was carried
465   back to the home pen (an additional leg band identified habituated birds to avoid recapture). This
466   was repeated three times for each bird across a period of six days. In these habituation trials, a
467   small pile of mixed poultry grain was placed center-back of the arena. Birds had been exposed to
468   the grain scattered in their home pens across three days prior to habituation and were observed to
469   consume the grain. The 14 saline birds that had been included from the drug dose trials only had
470   two habituation sessions with the grain, their first time in the open field arena during the
471   previous trials was considered their first habituation. As per Experiment 2, a video camera
472   recorded all bird behavior, and an out-of-sight experimenter watched live via a second camera
473   connected to a screen to ensure birds were becoming habituated to the test arena. Birds were also

474   feed-restricted the evening prior to habituation session 3. A reduced amount of food was
475   available within the home pen compared to previous *ad libitum* supply so that birds were limited
476   in the amount of food they could consume but they were not food-deprived. All birds were
477   observed to rapidly consume the grain by the third habituation session and thus were deemed
478   ready to be tested in the attention bias test.
479

Attention bias test
481   At 23 weeks of age, birds (*n* = 50) were randomly allocated to the 2mg/kg *m*-CPP treatment or
482   saline (control) group and individually weighed to calculate their dosage volume. The drug was
483   mixed into a saline solution at a dosing volume of 1:0.5 to reduce the volume of liquid given to
484   the birds. Birds were feed-restricted in the home pen the evening prior to testing to increase
485   motivation to eat, but were not food-deprived. Birds were then tested in the attention bias arena
486   at 23 weeks. Four birds were excluded due to unexpected external disturbance outside the facility
487   during testing (remaining test birds: saline *n* = 24; 2mg/kg *n* = 22).
488   The *m*-CPP or saline was administered sub-cutaneously as outlined in Experiment 2 and
489   birds were placed into the temporary pen. At 20 min following administration, birds were placed
490   front-center of the open field arena in the dark. The lights were then switched on and an attention
491   bias test began. An alarm call was played back for 8 sec immediately following lights-on at 75-
492   80 dB (as measured inside the box by a Digitech QM-1589 digital sound level meter, DigiTech,
493   UT, USA). The alarm calls were played from Latitude E7270 laptop speakers located on one side
494   of the open field arena. Two different alarm calls were randomly-allocated between birds but the
495   same alarm call was played up to twice for each bird. Alarm calls were selected from the same
496   set of recordings made from a group of hens prior to Experiment 1 but different calls were used
497   compared to the one used in Experiment 1. Following the first alarm call, an out-of-sight
498   observer watched on the connected screen until the bird reached the feed and consumed grain for
499   10 sec, then the alarm call was played a second time for 8 sec. This second alarm call was played
500   to gain a measure of whether the alarm call was sufficiently threatening to cause the bird to stop
501   feeding, and also to measure the bird's willingness to resume feeding again. If a bird did not
502   approach and consume the feed, no second alarm call was played as the second measure was
503   aimed at measuring their response once they had started eating. All tests were video-recorded for
504   later decoding of behavior. All tests concluded after 5 min and the bird was carried back to the
505   temporary pen. All birds were tested once across 2 days, and placed back in their home pen at the
506   conclusion of testing.
507

Video observations
509   The third habituation session videos were watched by a single experimenter to record latency to
510   first step, vocalize and eat. The Observer XT 12.0 (Noldus Information Technology) was used by
511   a single trained experimenter who was blind to the treatments of each hen to decode all attention
512   bias videos. The experimenter measured latency to first step, first vocalize, and first eat as well
513   as latency to eat, step, and vocalize following the second alarm call. The total number of steps

514 and individual vocalizations were counted across the test duration. Finally the total time spent
515 feeding and duration of individual feeding bouts (following the second alarm call only) were
516 recorded where a single feeding bout was defined as the bird eating the food without raising its
517 head or walking away from the feed. Intra-observer reliability correlations between a selection of
518 10 videos observed twice were 93 to 96% as assessed by correlation analysis in Microsoft Excel.
519 Clear vigilance behavior with stretching of the neck as measured in Experiment 1 was not
520 observed in this different set of birds and thus was not assessed further.
521
522 **Data and statistical analyses**
523 To determine if the birds were perceiving the alarm call playback as a threat, the latency to first
524 step, vocalize, and eat were compared between habituation session 3 and the attention bias test
525 using Kaplan-Meier estimates with a log-rank test for differences between tests separately for
526 saline and 2mg/kg hens. The same Kaplan-Meier estimates were also used to compare the
527 latency (seconds) to first step, vocalize, and eat between the two treatment groups in the attention
528 bias test. Hens which failed to perform each behavior within 600 s were censored results. The
529 hens that never received a second alarm call were included in latency data analyses but excluded
530 from all subsequent analyses (saline: $n = 2$; 2mg/kg: $n = 5$, i.e. a total of 22 saline and 17 2mg/kg
531 birds were included). The total number of steps and total number of vocalizations were converted
532 to a rate of steps or vocalizations made per second of the test, excluding all time spent eating for
533 the two treatment groups. These data were logit transformed to approach normality and analyzed
534 using a two-tailed t-test. The total time (seconds) spent eating and duration of eating bouts
535 (following the second alarm call) were square-root transformed and analyzed using a two-tailed
536 t-test. Where applicable, the untransformed values are presented in the results as there was no
537 difference between the raw and back-transformed means. All analyses were conducted in JMP
538 13.0.0 (SAS Institute, Cary, NC) with α set at 0.05.
539

540 # Results Experiment 3: Pharmacological validation of an attention bias
541 # test
542
543 The Kaplan-Meier estimates showed a significantly slower latency to eat ($\chi^2 = 6.99$, df = 1, $p =$
544 0.008) during the attention bias test compared to habituation session 3 for the saline birds, but
545 significantly faster latency to vocalize ($\chi^2 = 16.77$, df = 1, $p < 0.001$) and no difference in the
546 latency to step ($\chi^2 = 1.67$, df = 1, $p = 0.20$, Fig. 4a-c). The 2mg/kg hens also showed significantly
547 slower latencies to step ($\chi^2 = 6.88$, df = 1, $p = 0.008$) and eat ($\chi^2 = 20.75$, df = 1, $p < 0.001$)
548 during the attention bias test compared to habituation session 3, but faster latencies to vocalize
549 ($\chi^2 = 19.40$, df = 1, $p < 0.001$, Fig. 4d-f).
550 There was a significant effect of dosing group for the latency to first eat during the attention bias
551 test following the first alarm call ($\chi^2 = 5.65$, df = 1, $p = 0.018$) with the 2mg/kg hens taking
552 longer to eat (Fig. 5a). Similarly, the 2mg/kg birds also took longer to eat following the second
553 alarm call ($\chi^2 = 11.23$, df = 1, $p < 0.0008$, Fig. 5b). There was no effect of dosing treatment

554   group in the latency to first step (χ^2 = 1.61, df = 1, p = 0.20; mean ± SEM 2mg/kg: 51.89 ± 14.51
555   seconds; saline: 42.32 ± 16.89 seconds) and the latency to first vocalize following the first alarm
556   call (χ^2 = 0.05, df = 1, p = 0.82; mean ± SEM 2mg/kg: 78.42 ± 23.40 seconds, saline: 110.21 ±
557   28.18 seconds) or the latency to first step following the second alarm call (χ^2 = 0.06, df = 1, p =
558   0.81; mean ± SEM 2mg/kg: 109.0 ± 16.24 seconds, saline: 115.31 ± 15.49 seconds). However,
559   there was a significant effect of dosing treatment group on the latency to vocalize (χ^2 = 4.83, df =
560   1, p = 0.03) following the second alarm call with the 2mg/kg hens vocalizing sooner (mean ±
561   SEM 2mg/kg: 118.84 ± 15.84 seconds, saline: 175.31 ± 16.77 seconds).
562   The 2mg/kg birds spent less time eating than the saline hens (t = 3.69, df = 43.93, p = 0.0003;
563   mean ± SEM 2mg/kg: 12.65 ± 4.12 seconds, saline: 31.0 ± 3.62 seconds) but there was no
564   difference between dosing treatment groups in the average eating bout length (t = 0.46, df =
565   93.80, p = 0.65; mean ± SEM 2mg/kg: 14.06 ± 2.0 seconds, saline: 14.64 ± 1.22 seconds).
566   There were significant effects of dosing treatment on the rate (steps or vocals per test time
567   excluding eating) of stepping (t = -4.63, df = 38.08, p < 0.001) and vocalizing during the test (t =
568   -3.65, df = 22.75, p = 0.001) with the 2mg/kg birds stepping and vocalizing more (Fig. 6).
569
570   **Discussion**
571
572   An attention bias test for chickens could be used as an indicator of anxiety. In laying hens, an
573   attention bias towards the surrounding environment following playback of an alarm call
574   simulating a threat, may be indicated by increased vigilance, reduced locomotion, and a reduced
575   willingness to feed, but behaviors of test birds may be impacted by their age and/or familiarity
576   with the testing environment. The attention bias test behaviourally distinguished indoor-
577   preferring and outdoor-preferring free-range hens at 51 weeks of age where indoor-preferring
578   hens showed a perceived heightened awareness of the simulated threat as indicated by increased
579   vigilance and a reduced willingness to eat. The drug meta-chlorophenylpiperazine (*m*-CPP)
580   affected behavior of 19 week old adult layers at a dose of 2mg/kg which manifested as reduced
581   locomotion in an open field arena. Hens habituated to the open field arena and given *m*-CPP at
582   23 weeks of age showed increased locomotion and vocalisations and reduced willingness to eat
583   in the attention bias test but no evidence of the same vigilance behavior as observed in the first
584   experiment with free-range hens.
585   Based on previous studies of behavioral assessment in free-range hens under controlled
586   settings, indoor hens display higher levels of fear than outdoor ranging hens (Campbell et al.,
587   2016; Hartcher et al., 2016; Hernandez et al., 2014a). The free-range environment is
588   unpredictable with exposure to predators. Thus birds that remain indoors may choose to do so
589   because of fear. But often these indoor birds are never registered to visit outdoors and thus their
590   choice to stay inside is likely due to a perceived threat which can be classified as anxiety. As
591   predicted, the indoor birds assessed in an attention bias test were more vigilant following the
592   alarm call and slower to start feeding. Both of these behaviors combined suggest increased
593   anxiety but no formal assessment was made of any potential differences in hunger states between

594 the two groups of birds. There was a drop in peripheral temperature from baseline to indicate
595 stress-induced hyperthermia (Herborn et al., 2015), and the indoor birds also had lower comb
596 temperatures overall. Chickens have previously shown a decrease in peripheral temperature as a
597 result of air-puffs and handling stress (Edgar et al., 2011, 2013; Herborn et al., 2015) validating
598 this as a measure of distress, although Moe and colleagues (2012) did demonstrate that arousal in
599 anticipation of a reward also resulted in a decrease in peripheral temperature. Edgar and
600 colleagues (2013) documented a temperature drop 1 minute following handling but as the free-
601 range birds tested in the current study were accustomed to regular handling, the temperature
602 decrease is interpreted to be in response to the alarm call. This temperature drop suggests higher
603 arousal in the indoor birds, but the valence (positive/negative) of the response is equivocal.
604 Additional physiological measures could be made in future testing scenarios and that may
605 confirm valence. Catheter blood-sampling could further characterize the physiological stress
606 responses such as production of plasma catecholamines, or corticosterone (Korte et al., 1997;
607 Larsen et al., 2018). However, caution would need to be placed on the interpretation of
608 physiological measures that could be affected by both a state of anxiety and activity.
609   Dosing of *m*-CPP in adult laying hens affected their behavior in the open field arena.
610 Anecdotally, in the home pen, birds were observed to walk cautiously/slowly around the pen and
611 several birds exhibited an odd behavior of picking up feathers/litter off the ground and trying to
612 place it on their backs. It is difficult to prove or dis-prove any hallucinogenic effects of the drug
613 in hens, but it is a potential side-effect to be considered (Tancer and Johanson, 2001). These odd
614 behaviors were not seen in saline-dosed birds. However, we do not know if there was any social
615 transmission of negative affective states or stress between tested/untested or saline/*m*-CPP dosed
616 birds kept within the same housing pen prior to testing (de Haas et al., 2012). Purposeless oral
617 movements as a side effect of *m*-CPP have been reported in rodents (Kreiss and De
618 Deurwaerdère, 2017) and humans can suffer from migraines followed administration of *m*-CPP
619 (Panconesi, 2008). To the best of the author's knowledge, the pharmacokinetics of *m*-CPP in
620 laying hens has not previously been documented or the presence of side effects which may not be
621 overtly displayed. In humans, the bioavailability and half-life varies substantially between
622 subjects, which may only partly be explained by genetic variation in the metabolizing enzymes
623 (Feuchtl et al., 2004). The cytochrome P450 enzymes are responsible for the metabolism of *m*-
624 CPP (Rotzinger et al., 1998) and are present in chickens (Watanabe et al., 2013) but research to
625 document the pharmacokinetic pathways is still needed. Further pharmacological validation of
626 behavioral tests with layers using alternative anxiogenic drugs with different modes of action
627 such as caffeine or FG-7142, for example, would be valuable.
628 The open field arena was selected as an environment to clearly assess behavior at the individual-
629 bird level. At higher doses of *m*-CPP, the birds in this arena exhibited prolonged freezing and
630 reduced locomotion. Freezing in an open field arena is a typical response of laying hens
631 experiencing an actual threat (visible human threat: Suarez and Gallup, 1982), as well as birds in
632 the arena that have not specifically been presented with a threat (Gallup and Suarez, 1980). In
633 this study, *m*-CPP accentuated the freezing response for birds placed in the open field arena for

634 the first time with no specific known threat presented. The open field test instigates a trade-off in
635 chickens between the desire for social reinstatement and a reduction in locomotion/freezing to
636 avoid detection (Gallup and Suarez, 1980). The vocalizations emitted at the different doses of *m*-
637 CPP were indicated that the type of vocalization made by the bird could be most informative
638 versus total number of vocalizations, but further detailed spectrographic analysis would be
639 required which was beyond the scope of the current study. Additional dosing trials using *m*-CPP
640 could document the behavioral responses of birds both when the environment is novel, and then
641 when the environment is familiar.
642   In comparison to the final habituation session, the playback of the alarm call during the
643 attention bias test reduced the willingness to feed in both saline and *m*-CPP birds indicating the
644 birds were perceiving the alarm call as threatening. However, the birds in Experiment 3 did not
645 exhibit the same vigilance behavior (stretching of the neck and looking around) as did the birds
646 in the first experiment when comparing between indoor and outdoor free-range hens. This could
647 be the result of a multitude of differences between the testing groups such as age, breed, housing
648 environments, or prior testing experience. Early life experience with cognitive stimulation has
649 been demonstrated to affect displays of vigilance as adults (Zidar et al., 2017). Vigilance is also
650 defined differently across laying hen experiments (Newberry et al., 2001; Odén et al., 2005) and
651 thus one definition may not fit all testing scenarios. Further refinement and use of an attention
652 bias test is needed to develop a set of standard behavioral measures. Although the birds in
653 Experiment 3 did not exhibit vigilance similar to the free-range hens, the *m*-CPP birds were less
654 willing to eat which suggests an increased attention bias. Both sheep and cattle dosed with *m*-
655 CPP also showed reduced willingness to feed during an attention bias test (Lee et al., 2016,
656 2018; Monk et al., 2018a). The *m*-CPP drug is however, also an appetite suppressant. Humans
657 report a reduced appetite while dosed with *m*-CPP (Thomas et al., 2014), but in one study it only
658 affected consumption of a palatable snack when human subjects were already satiated (Thomas
659 et al., 2018). Feeding behavior alone in this study is likely not sufficient to distinguish between
660 the dosing groups, but in combination with the increased locomotion and vocalizations the *m*-
661 CPP birds could be interpreted as being more anxious following the playback of the alarm calls.
662 Similar to this study, in other tests with livestock, the *m*-CPP dosed individuals also exhibited
663 other changes supplement to feed-related behavior such as paying more attention to location
664 where the dog was presented, more vigilance, and increased head and tail-related behaviors in
665 cattle (Lee et al., 2018). In further refinements of an attention bias test for laying hens, a different
666 reward could be used that would avoid any confounds of pharmacological side-effects on
667 appetite. A practical test developed to be potentially used on-farm for sheep successfully used an
668 image of a conspecific rather than feed (Monk et al., 2018b). Willingness to forage in new
669 substrate for example, could be an alternative to a food reward for hens.
670   In contrast to the freezing behavior that was observed with dosed birds placed into the
671 open field arena for the first time, 2mg/kg *m*-CPP birds previously habituated to the open field
672 arena showed increased locomotion and vocalizing relative to control birds. In sheep, m-CPP did
673 not affect locomotion or vocalizations in comparison to control animals (Lee et al., 2016; Monk

674 et al., 2018a), but number of zones crossed increased for cattle dosed with *m*-CPP (Lee et al.,
675 2018) and in rats, higher doses of *m*-CPP reduced (Kennett and Curzon, 1988; Kennett et al.,
676 1989) or tended to reduce locomotion (Kreiss and De Deurwaerdère, 2017). If freezing is an
677 indication of high anxiety, we would have predicted the playback of an alarm call would have
678 increased anxiety and resulted in reduced locomotion as observed in the open field test. Given
679 the contrast in responses between non-habituated and habituated birds (of the same breed/flock
680 and similar age) that change in locomotion is unlikely to be an effect of the drug per se, but an
681 indication of how familiarity of the environment impacts the responses the birds exhibit. When
682 hens were more familiar with the test environment, the anxiety manifested as increased
683 movement, potentially the hens were looking for escape from the arena and reinstatement with
684 their peers. Pacing behavior has been observed in birds believed to be trying to escape during
685 behavioral testing (Bolhuis et al., 2009), and exhibited by hens frustrated by non-delivery of an
686 expected food reward (Zimmerman and Koene, 1998). Although the hens during the test were
687 not observed to be stereotypically pacing in the arena, there are possibly similar underlying
688 affective states that led to increased movement in the current study. These results suggest that
689 extreme behavioral responses (freezing/high locomotion) in an open field test may both be
690 indicative of negative affective states. Further comparisons with more groups of birds would be
691 highly valuable as individual groups of birds in the same housing environments can display
692 different behavioral patterns (Campbell et al., 2018).
693

Conclusions

695 Overall, an attention bias test could be used with laying hens to measure anxiety but it requires
696 further refinement. The familiarity of the birds with the testing environment will likely affect
697 their behavioral responses where habituation to the testing environment may result in improved
698 detection of anxiety specifically in response to the alarm call playback. Subsequent iterations of
699 the test may result in a set of standardized behavioral measures to be used. The drug *m*-CPP
700 appears to be most effective at a dose rate of 2mg/kg for pharmacological validation of this
701 affective state in laying hens. Measuring anxiety will provide understanding of how hens may be
702 responding to their housing environments and the impacts it may have on their welfare. Extreme
703 behavioral responses of freezing or high locomotion exhibited in individual-bird behavioral tests
704 are possibly both indicators of negative states.
705
706
707

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714

715

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Figure 1

The time to first eat following the second alarm call

This Kaplan-Meier curve shows the latency in seconds to first eat for daily ranging (outdoor) and non-ranging (indoor) hens following a second alarm call played in the attention bias test. The plot indicates the probability that a bird would not eat at a given time point for the duration of testing.

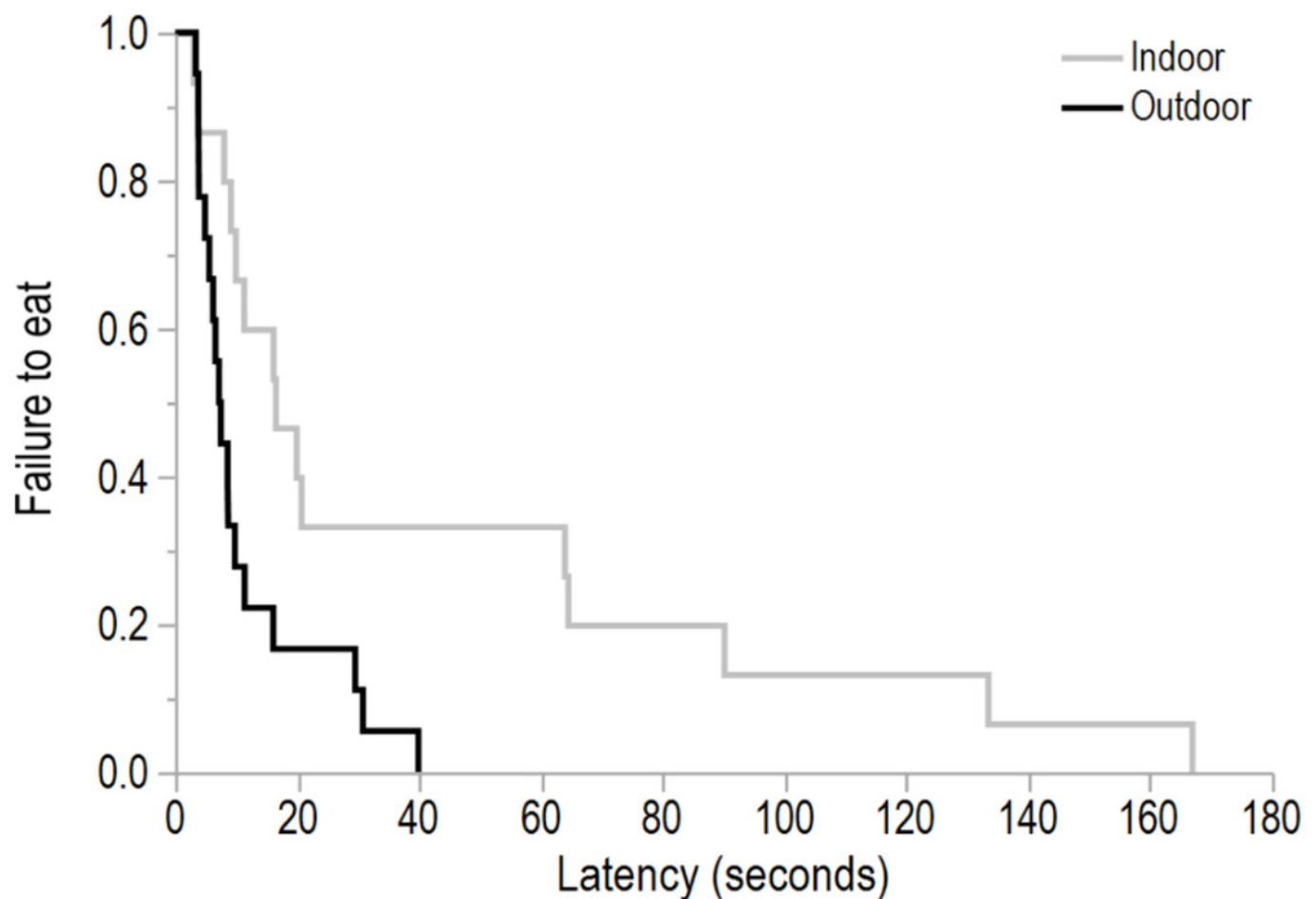



Figure 2

Time to first move for hens from four dosing groups

The Kaplan-Meier curve shows the latency for hens that were dosed with saline (control), 0.5, 1, or 2mg/kg of *m*-CPP) to first move (seconds) in an open field test. This plot indicates the probability that a hen would not move at a given time point for the duration of testing.

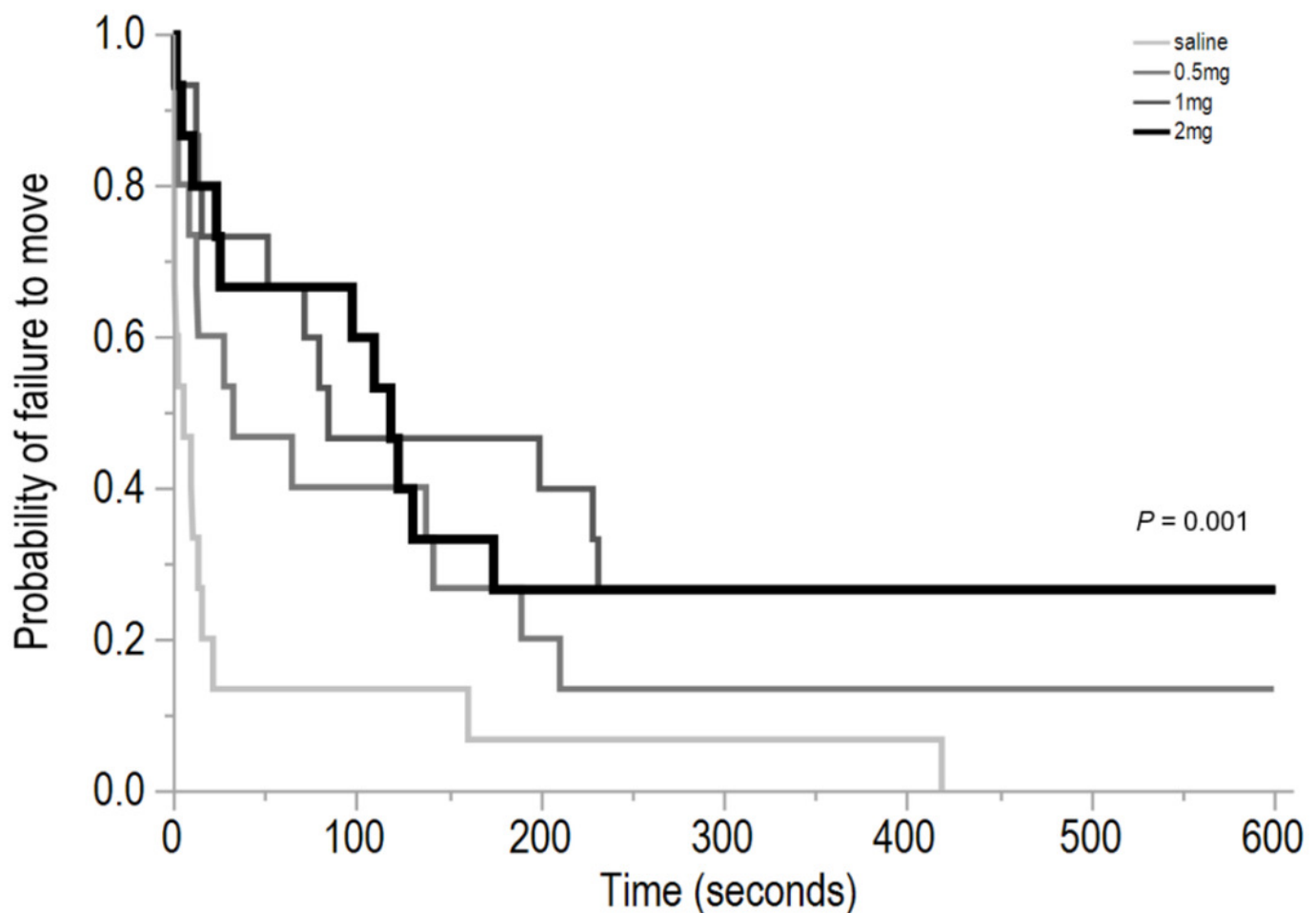
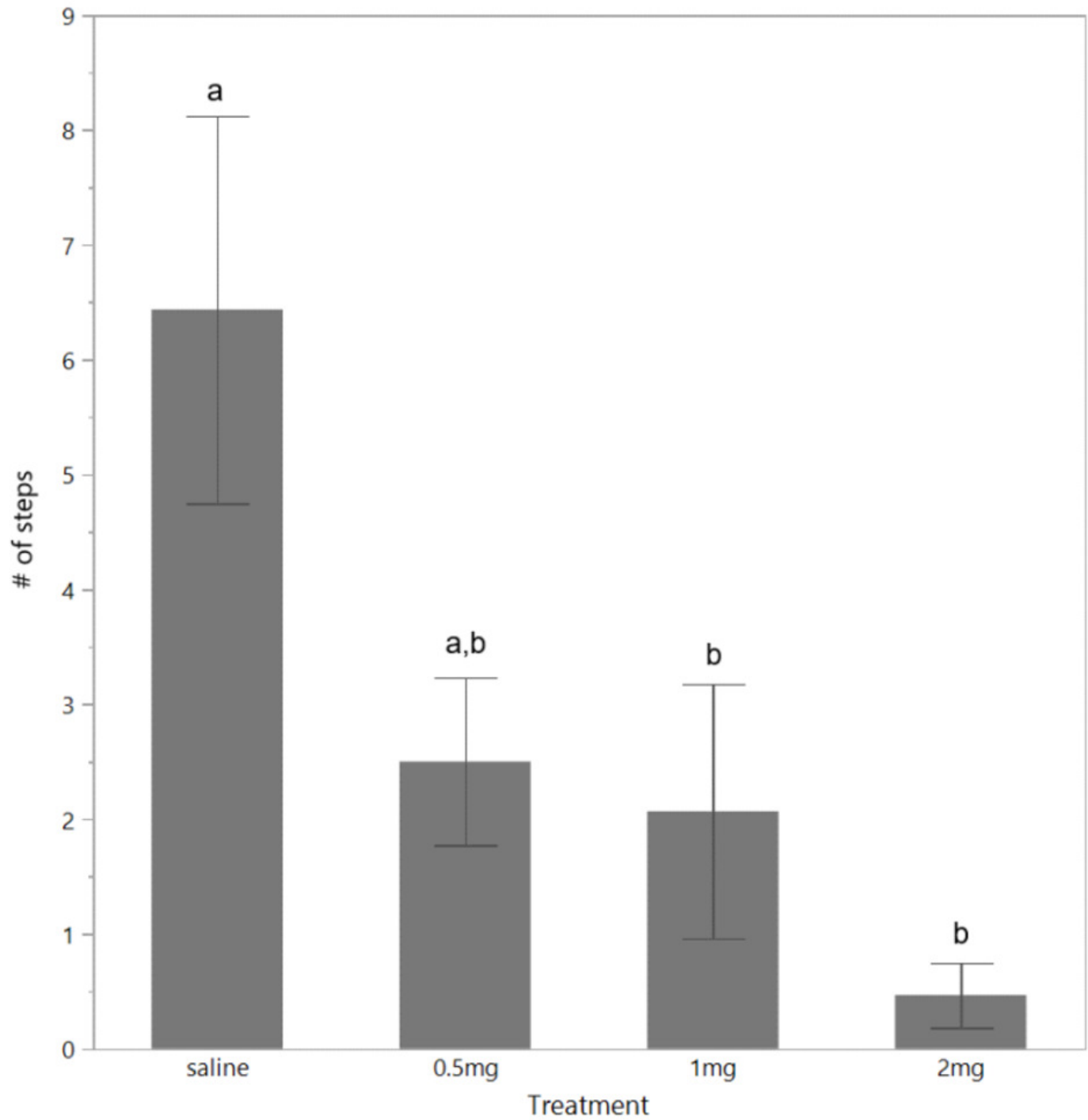



Figure 3

Steps made in the open field test

The mean (± SEM) number of steps made in the first 2 mins of the open field test by hens dosed with saline (control), 0.5, 1, or 2mg/kg of m-CPP. Different superscript letters indicate significant differences between treatment groups at $P < 0.05$.

Mean ± s.e.m of the number of steps or vocalizations (total, short,long) made by hens from four treatment groups across a 10 minute open fieldtest.

[1] Values are presented for all dosing groups combined (saline, 0.5mg/kg, 1mg/kg, 2mg/kg).

[a,b,c,d] Different superscripts within rows indicate significant differences between times as determined using post hoc analyses ($P < 0.005$)

		0-1 min	1-2 min	2-3 min	3-4 min	4-5 min	5-6 min	6-7 min	7-8 min	8-9 min	9-10 min
All dosing treatments [1]	# steps	2.01 ± 0.60[c]	3.72 ± 0.98[b,c]	5.47 ± 1.32[a,b]	6.32 ± 1.34[a]	7.1 ± 1.64[a]	5.85 ± 1.33[a,b]	5.83 ± 1.53[a,b]	6.1 ± 1.38[a]	5.15 ± 1.03[a,b]	5.15 ± 1.13[a,b]
	# total vocals	7.25 ± 1.57[a,b]	8.27 ± 1.90[a,b]	10.78 ± 3.35[a]	7.92 ± 1.94[a,b]	3.53 ± 0.93[b,c]	2.75 ± 1.0[c]	1.95 ± 0.96[c]	5.38 ± 2.60[c]	5.65 ± 2.48[c]	3.47 ± 3.05[c]
	# short vocals	6.82 ± 1.55[a,b]	7.72 ± 1.89[a,b,c]	10.48 ± 3.35[a]	7.52 ± 1.94[a,b]	3.3 ± 0.89[b,c,d]	2.67 ± 1.0[d]	1.88 ± 0.96[d]	5.37 ± 2.60[c,d]	3.6 ± 1.60[d]	3.38 ± 3.05[d]
	# long vocals	0.47 ± 0.14[a]	0.48 ± 0.19[a,b]	0.3 ± 0.11[a,b,c]	0.13 ± 0.06[b,c,d]	0.23 ± 0.10[a,b,c,d]	0.08 ± 0.04[b,c,d]	0.07 ± 0.05[c,d]	0.02 ± 0.02[d]	0.07 ± 0.05[c,d]	0.03 ± 0.03[d]

Table 1: Mean ± s.e.m of the number of steps or vocalizations (total, short, long) made by hens from four treatment groups across a 10 minute open field test.

[1] Values are presented for all dosing groups combined (saline, 0.5mg/kg, 1mg/kg, 2mg/kg).

[a,b,c,d] Different superscripts within rows indicate significant differences between times as determined using post hoc analyses ($P < 0.005$)

Figure 4

The time to first step, eat, and vocalise for hens dosed with saline or *m*-CPP

The Kaplan-Meier curves show the latency (seconds) for hens to first step (a), eat (b), and vocalise (c) during habituation session 3 and the attention bias test when dosed with saline solution during the attention bias test only, and the latencies to first step (d), eat (e), and vocalise (f) during habituation session 3 and the threat perception test for hens when dosed with 2mg/kg *m*-CPP during the threat perception test only.

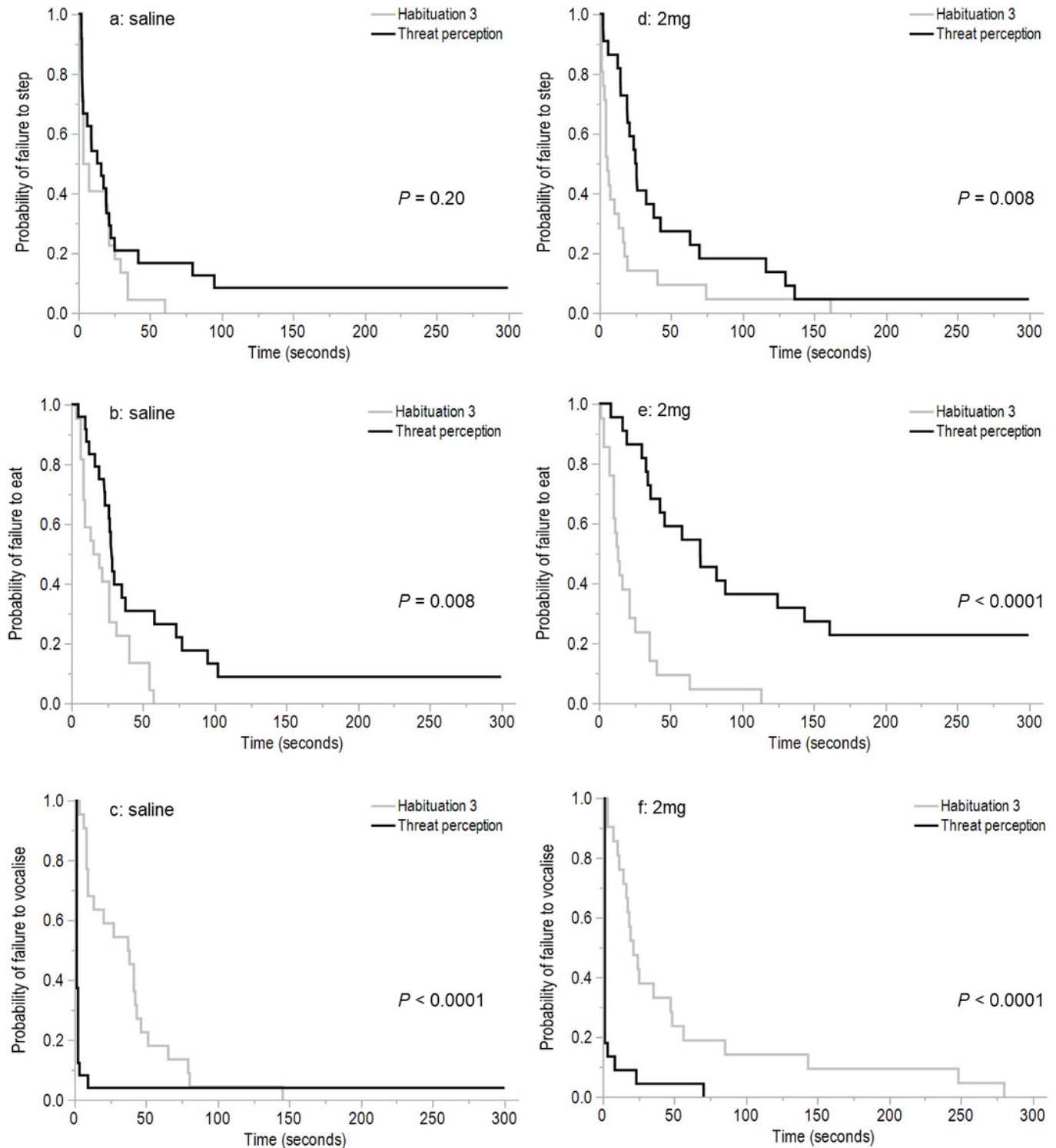



Figure 5

The time to eat following the first and second alarm calls

The Kaplan-Meier curves show the latency to eat (seconds) following the first alarm call (a) and latency to eat (seconds) following the second alarm call (b) for hens dosed with 2mg/kg m-CPP or a saline solution in the attention bias test. Two saline-dosed and five 2mg/kg-dosed birds did not eat and thus never received a second alarm call playback (n = 7 birds not included in graph 'b').

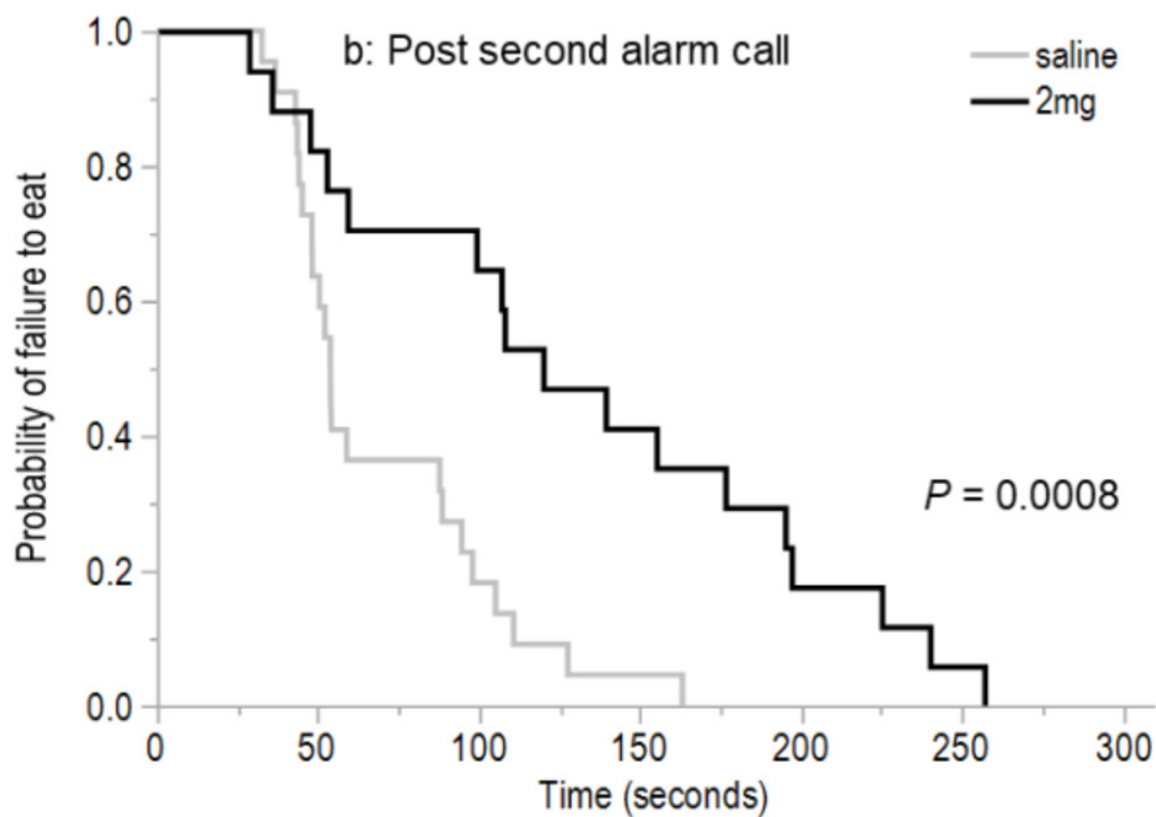
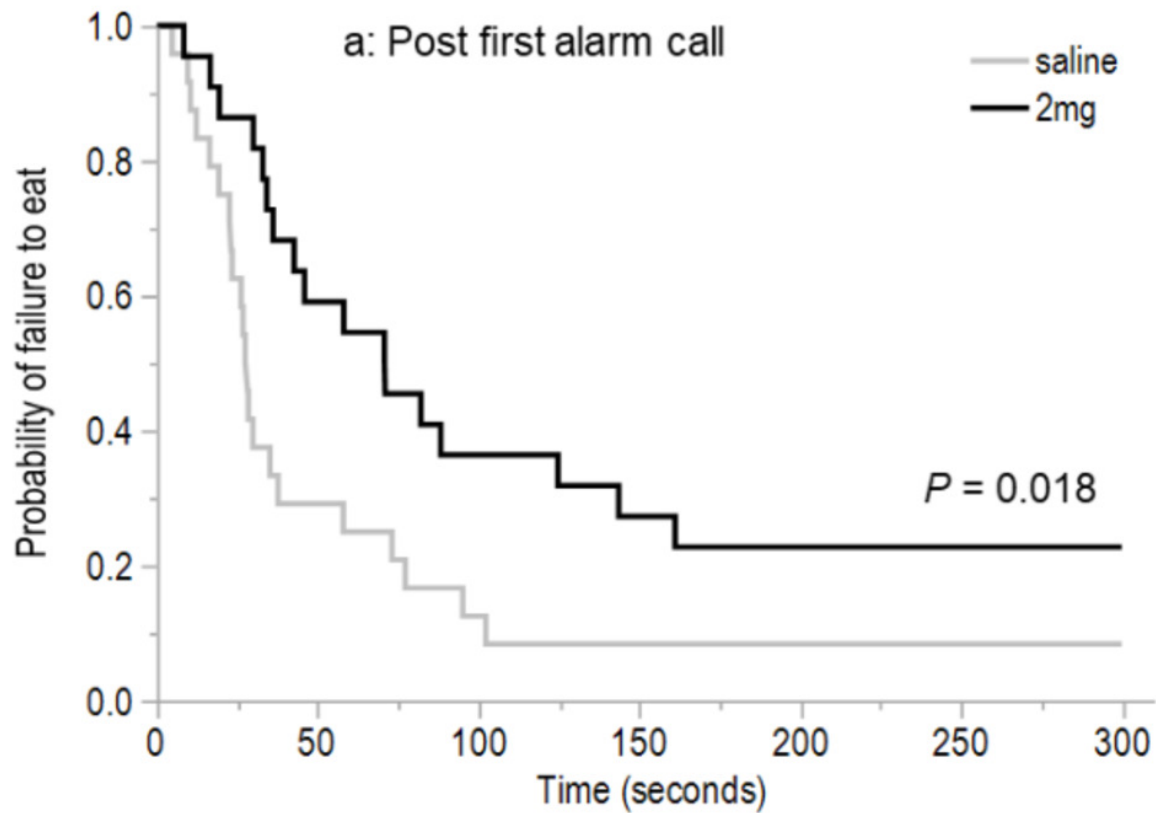



Figure 6

Proportional steps and vocalizations for each second of the attention bias test when not eating

The data are displayed for hens dosed with 2mg/kg of *m*-CPP or a saline solution. Asterisks indicate significant differences ($P < 0.001$) between dosing treatment groups for each measured behavior.