Nociception in chicken embryos, Part I: Analysis of cardiovascular responses to a mechanical noxious stimulus

Larissa Weiss[1], Anna M. Saller[1], Julia Werner[1], Stephanie C. Süß[1], Judith Reiser[1], Sandra Kollmansperger[2], Malte Anders[2], Thomas Fenzl[2], Benjamin Schusser[3], Christine Baumgartner[1*]

[1]Center for Preclinical Research, TUM School of Medicine, Technical University of Munich, Germany
[2]Clinic for Anesthesiology and Intensive Care, TUM School of Medicine, Technical University of Munich, Germany
[3]Reproductive Biotechnology, TUM School of Life Sciences, Technical University of Munich, Weihenstephan, Technical University of Munich, Germany

[*]Corresponding author: Prof. Dr. Christine Baumgartner, Center for Preclinical Research, TUM School of Medicine, Technical University of Munich, Munich, Germany, Christine.Baumgartner@tum.de

Keywords: blood pressure, heart rate, nociception, pain, chicken embryo, development, Gallus gallus domesticus

Abstract

While it is assumed that chicken embryos acquire the ability for nociception during the developmental period in the egg, an exact time point has not yet been specified. This study aimed to determine the onset of nociception during embryonic development in chicken. Changes in blood pressure and heart rate (HR) in response to a mechanical noxious stimulus at the base of the beak versus a light touch on the beak in chicken embryos between embryonic days (EDs) 7 and 18 were examined. Mean arterial pressure (MAP) was the most sensitive parameter for assessing cardiovascular responses. Significant changes in MAP in response to a noxious stimulus were detected in embryos at ED16 to ED18, while significant changes in HR were observed on ED17 and ED18. Infiltration anesthesia with the local anesthetic lidocaine significantly reduced reactions in MAP on ED18, so the cardiovascular changes can be assumed to be nociceptive responses.

Introduction

In present times animal welfare has increasingly become the focus of public attention with regard to farm and laboratory animals. Consequently, the culling of male day-old chicken for economic reasons is more and more ethically questioned. A large part of the male offspring in the layer industry is killed after hatching, as the fattening of male layer type chicken is not economically profitable[1]. In the EU,

330 million male chicks are killed annually through maceration or gasification[2], which is currently evoking a major discussion. Germany and France have already adapted their laws and banned the killing of male day-old chicks for economic reasons although there is not yet EU-wide regulation[3]. As an alternative, *in ovo* sex determination with subsequent killing of male embryos is already being practiced[4]. However, it is important for animal welfare reasons and for the public acceptance of *in ovo* sex determination that related culling be conducted at an early stage of development when nociception and the perception of pain are not yet possible[4,5].

Furthermore, chicken embryos are of great importance for biomedical research because of advantages provided in terms of fast growth and good accessibility in various research areas, such as developmental biology, toxicology, cancer research and drug development[6,7]. Under European regulations, interventions and treatments on chicken embryos are not considered animal experiments and even count as a replacement method in the context of the 3R principles[8]. At this time, there are no regulations regarding anesthesia and analgesia of chicken embryos during painful interventions[6,8]. Greater clarity regarding the period during which chicken embryos are capable of nociception and pain sensation would lead to improved animal welfare in research.

In pain research, a fundamental distinction is made between nociception and the perception of pain[9]. While nociception is the detection of a potentially tissue-damaging stimulus and its transmission by the nociceptive nervous system[10,11], pain is characterized by a subjective, conscious sensory perception, usually triggered by nociception[12,13]. Nociception and pain are progressive adaptive processes that gradually develop throughout the fetal period[14]. It is considered confirmed, that the chicken embryo acquires the ability for nociception at some point during the 21-day developmental period in the egg[8,15]. However, the question at which exact time point nociception or even pain sensation can be presumed is controversial. In several publications, researchers agree that nociception and pain perception are not possible in the first trimester of embryonic development of the chicken[4,15]. A requirement for the ability to perceive pain is the existence of functional pathways that enable the transmission of stimuli to the brain[12,14]. Although the first sensory afferent nerve fibers develop on

embryonic day (ED) 4, the closure of multisynaptic reflex arcs does not occur until ED7[16-18]. It is described in the literature that the chicken embryo develops a functional brain on ED13[15,19]. However, it is only confirmed that the brain does not show any electrical activity until 6.5 days of incubation[20]. Pain sensation is therefore considered to be impossible up to ED7, but beyond that, no specific time point can be defined from which the chicken embryo is capable of nociception and pain sensation[4,15].

Since self-reporting, which is the gold standard in humans to detect pain[21], is not possible as a direct method of pain evaluation in animals, indirect methods such as alteration of physiological and behavioral parameters must be resorted to[22]. Changes in heart rate and blood pressure are therefore used as clinical indicators of nociception and pain[23].

This study is part of a comprehensive study in which the nociceptive ability of chicken embryos was investigated using cardiovascular parameters, behavioral observations and EEG. Here, we present the results of the cardiovascular study and, in particular, the implemented cardiovascular measurement methods in regard to chicken embryos that were designed for investigation of the time point at which chicken embryos are able to respond to a noxious stimulus with a nociceptive cardiovascular response. The corresponding results of the EEG measurements and behavioral observations and the implemented techniques will be presented in further publications.

Results

Increasing arterial pressure and evolution of heart rate during embryonic development of the chicken

Systolic (SAP), diastolic (DAP) and mean arterial pressure (MAP) in the chorioallantoic artery and heart rate (HR) were recorded over one minute at ED7, ED9 and EDs 12 to 18. SAP, DAP and MAP increased with age of the embryos (Table 1). ED7 showed the lowest MAP with a value of 2.08 mmHg ± 0.40, and ED18 showed the highest MAP with a value of 17.28 mmHg ± 3.04.

Table 1. SAP, DAP, MAP and HR at ED7 (n=3), ED9 (n=6), and ED12 to ED18 (n=10). Values are shown as the mean ± standard deviation.

	ED7	ED9	ED12	ED13	ED14	ED15	ED16	ED17	ED18
SAP (mmHg)	3.50 ± 0.65	6.04 ± 1.46	9.19 ± 1.32	9.88 ± 1.52	13.02 ± 1.60	16.54 ± 3.04	21.44 ± 2.78	24.46 ± 5.50	24.65 ± 4.36
DAP (mmHg)	1.07 ± 0.36	1.98 ± 1.10	2.20 ± 1.12	2.96 ± 0.61	3.95 ± 1.14	5.69 ± 1.82	7.80 ± 2.16	10.77 ± 3.53	11.43 ± 2.43
MAP (mmHg)	2.08 ± 0.40	3.44 ± 1.24	4.83 ± 1.05	5.52 ± 0.79	7.32 ± 1.26	10.11 ± 2.45	13.73 ± 2.38	16.79 ± 4.21	17.28 ± 3.04
HR (bpm)	128.97 ± 15.40	147.57 ± 9.03	159.08 ± 26.64	146.61 ± 19.99	179.10 ± 35.06	154.33 ± 33.12	151.35 ± 36.44	179.08 ± 29.18	176.07 ± 35.75

Increase in MAP in response to a noxious stimulus

The response in MAP to a noxious mechanical stimulus at the base of the beak (*Pinch*) was compared to a light touch at the base of the beak as a negative control (*Touch*) in embryos between EDs 7 and 18. As shown in Fig. 1, a significant increase in MAP was observed as a reaction to *Pinch* in embryos at ED16 (p=0.0008), ED17 (p=0.0020) and ED18 (p=0.0048). ED18 embryos showed the strongest response in MAP, with an increase of 15.52 % ± 12.36 from baseline. In comparison, a deviation from baseline of only 1.30 % ± 0.94 was detected in response to *Touch* on ED18. In embryos at ED7, ED9 and EDs 12 to 15, no significant changes in MAP in response to *Pinch* compared to *Touch* were detected, which can be seen in Supplementary Fig. 1 and Supplementary Fig. 2.

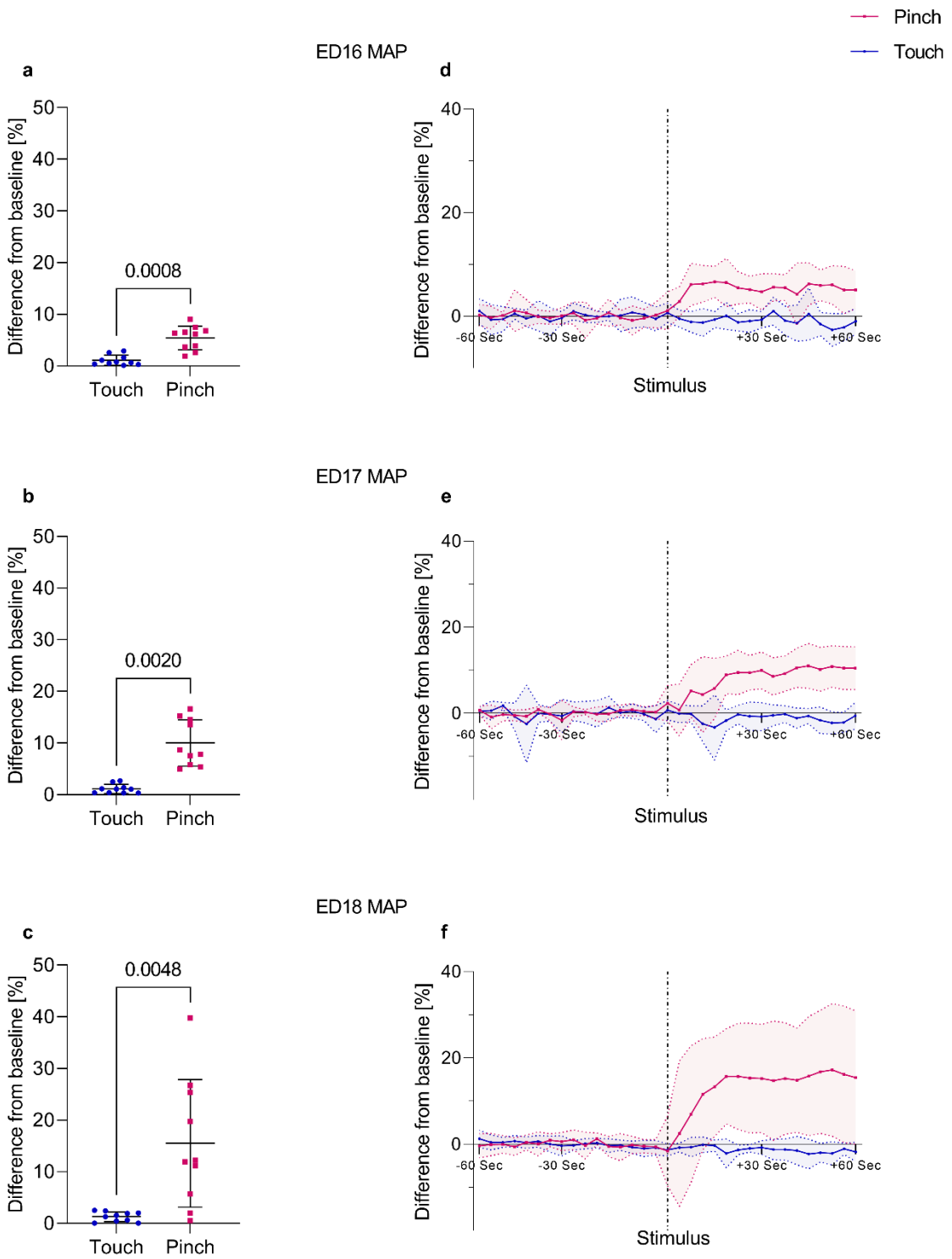


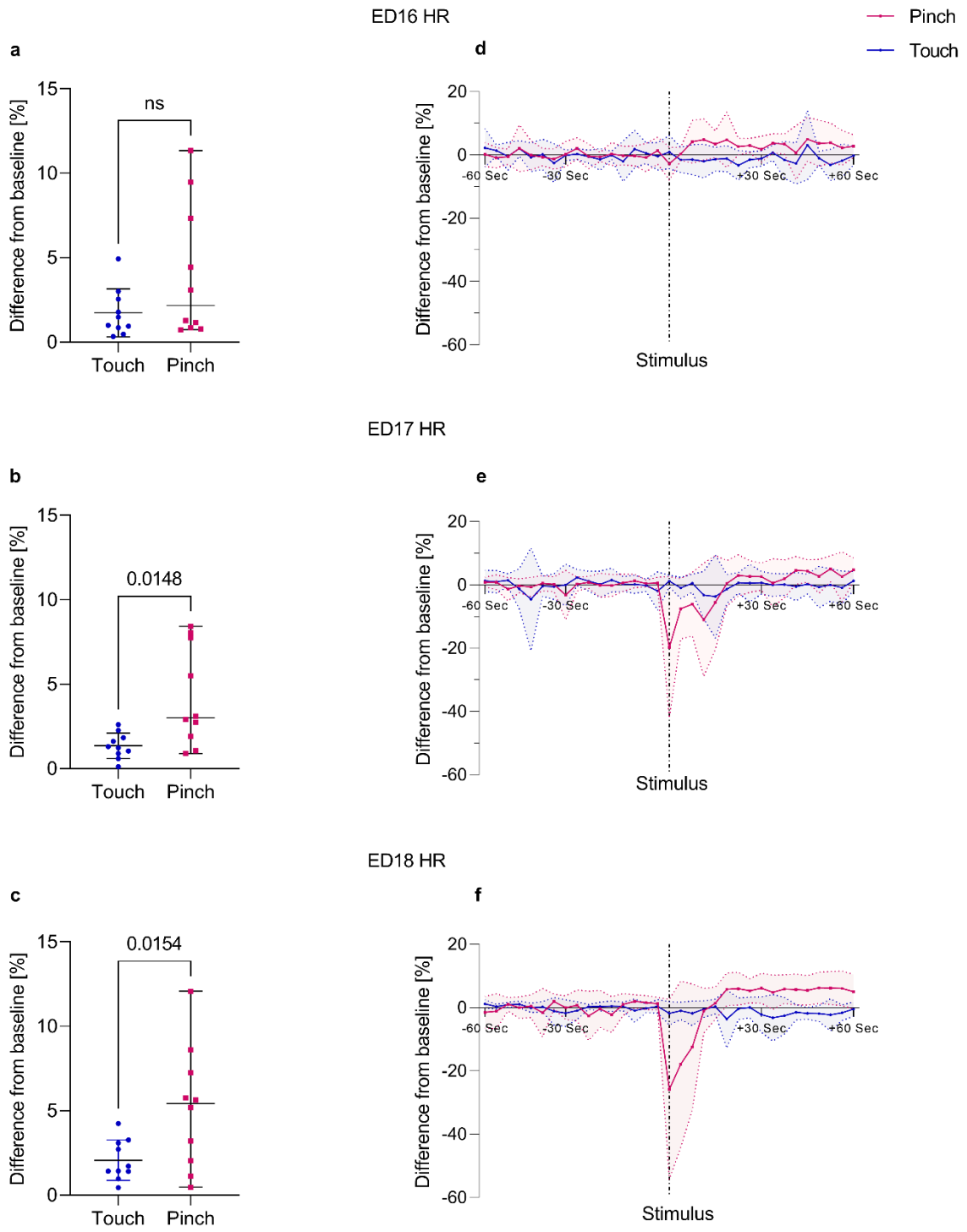
Fig. 1. Percent change in MAP post *Touch* and *Pinch.* Embryos at EDs 16 to 18 (n=10) received a mechanical noxious stimulus (*Pinch*) and a light touch as a negative control (*Touch*) at the base of the beak in randomized order. **a-c** Percent change to baseline in MAP post *Pinch* compared to *Touch*. Displayed as the mean ± standard deviation. Paired t test (normally distributed: a and c) or Wilcoxon signed-rank test (not normally distributed: b). P values shown; a: p=0.0008, b: p=0.0020, c: p=0.0048. **d-f** Percent change from the baseline mean value in MAP over time; values recorded every four seconds for one minute before and one minute after stimulation (*Pinch* and *Touch*); values shown as

the mean ± standard deviation (shaded).

Changes in HR up to a noxious stimulus

Regarding HR, two reaction patterns have been observed, particularly in ED17 and ED18 embryos. In some embryos, HR immediately increased post *Pinch*. In other embryos a hyperacute decrease in HR followed by an increase was observed in response to *Pinch*, as shown in Fig. 2. A decrease in HR of at least -15 % with a subsequent increase of at least 5 % from the baseline mean value post *Pinch* was observed in 80 % of ED18 embryos and in 30 % of ED17 embryos and was not detected post *Touch*. In embryos at ED18 HR decreased by up to -48.54 % ± 19.71 over 9.50 s ± 6.02 on average post *Pinch*. At ED17 these embryos showed a decrease in HR by up to -41.87 % ± 8.32 over 16.00 s ± 6.93 on average post *Pinch*. Simultaneous to the hyperacute decrease in HR, a slight decrease in MAP was also observed, particularly when the decrease in HR was large. In embryos at ED15 and ED16, the observations were inconsistent and could not be clearly distinguished from physiological variations in HR. In younger embryos, no hyperacute decrease in HR was observed in response to *Pinch*.

Significant increases in HR in response to *Pinch* compared to *Touch* were detected in embryos at ED17 ($p=0.0148$) and ED18 ($p=0.0154$) (Fig. 2). Embryos at ED18 showed the largest increase in HR post *Pinch* with a deviation of 5.14 % ± 3.60 from baseline, compared to a deviation of only 2.07 % ± 1.20 from baseline post *Touch*. At ED7, ED9 and EDs 12 to 16, no significant changes in HR were observed, which can be seen in Supplementary Fig. 3 and Supplementary Fig. 4.



117

118 **Fig. 2. Percent change in HR post *Touch* and *Pinch***. Embryos at EDs 16 to 18 (n=10) received a
119 mechanical noxious stimulus (*Pinch*) and a light touch as a negative control (*Touch*) at the base of the
120 beak in randomized order. **a-c** Percent change to baseline in HR post *Pinch* compared to *Touch*.
121 Displayed as the mean ± standard deviation. Paired t test (normally distributed: b and c) or Wilcoxon
122 signed-rank test (not normally distributed: a). P values shown; b: p=0.0148, c: p=0.0154; ns=no
123 significant difference between the groups (a). **d-f** Percent change from the baseline mean value in HR
124 over time; values recorded every four seconds for one minute before and one minute after stimulation
125 (*Pinch* and *Touch*); values shown as the mean ± standard deviation (shaded).

126 **Reduction of cardiovascular response by local anesthesia**

127 The application of the local anesthetic lidocaine (*Lido*) at the base of the beak prior to stimulation
128 significantly reduced the MAP increase in response to *Pinch* in embryos at ED18. Compared to the
129 group without local anesthesia (*ED18 w/o Lido*), which showed an increase of 15.52 % ± 12.36 post
130 *Pinch*, the increase in MAP was reduced to 5.00 % ± 3.42 in the group that received lidocaine (*ED18*
131 *w/ Lido*). As represented in Fig. 3, the *ED18 w/o Lido Pinch* group showed the largest increase in MAP
132 in response to *Pinch* compared to *ED18 w/o Lido Touch* (p=0.0007), *ED18 w/ Lido Touch* (p=0.0031)
133 and *ED18 w/ Lido Pinch* (p=0.0397).

134 The changes in HR in response to *Pinch* were slightly reduced by the application of lidocaine. However,
135 a significant difference in HR was observed only between *ED18 w/o Lido Pinch* and *ED18 w/ Lido Touch*
136 (p=0.0097). In the group treated with lidocaine (*ED18 w/ Lido*), no embryo showed a hyperacute
137 decrease in HR of -15 % with a subsequent increase of 5 % from the baseline mean value post stimulus,
138 while this reaction pattern was observed in 80 % of the embryos in the group *ED18 w/o Lido Pinch*.
139 However, a slight decrease in HR post *Pinch* was also observed in the local anesthetic group (*ED18*
140 *w/ Lido Pinch*), but this could not be distinguished from physiological variations in HR (Fig. 3).

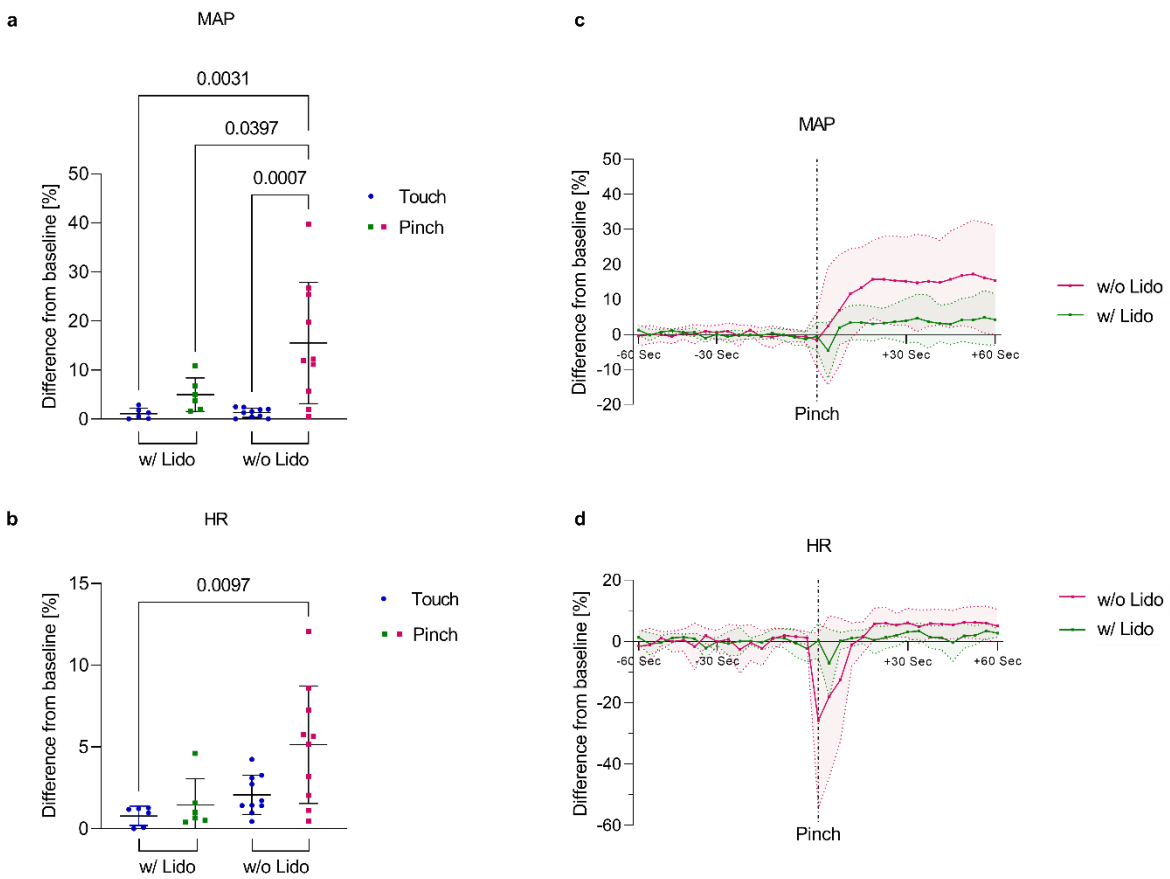


Fig. 3. Local anesthesia control group. Percent change in MAP and HR post *Touch* and *Pinch*. ED18 embryos either received a lidocaine injection (*ED18 w/ Lido*; n=6) or no lidocaine injection (*ED18 w/o Lido*; n=10) at the base of the beak prior to stimulation (*Touch* and *Pinch*). **a** Percent change to baseline in MAP post *Pinch* in the group without lidocaine (*ED18 w/o Lido Pinch)* compared to *ED18 w/o Lido Touch*, *ED18 w/ Lido Touch* and *ED18 w/ Lido Pinch*. Displayed as the mean ± standard deviation. One-way ANOVA (normally distributed); p values shown. **b** Percent change to baseline in HR post *Pinch* in the group without lidocaine (*ED18 w/o Lido Pinch)* compared to *ED18 w/o Lido Touch*, *ED18 w/ Lido Touch* and *ED18 w/ Lido Pinch*. Displayed as the mean ± standard deviation. Kruskal–Wallis test (not normally distributed); p values shown. **c-d** Percent change from the baseline mean value in MAP and HR post *Pinch* over time; values recorded every four seconds for one minute before and one minute after stimulation (*ED18 w/o* or *w/ Lido Pinch*); values shown as the mean ± standard deviation (shaded).

Discussion

This study successfully developed methods to record blood pressure and HR in chicken embryos between EDs 7 and 18. Cardiovascular changes in response to a mechanical noxious stimulus at the base of the beak were investigated with the aim of identifying the onset of nociception during embryonic development in chicken.

160 While there are many well-established noninvasive methods for determining HR in the chicken
161 embryo[24-26], the direct intraarterial measurement is the gold standard for recording blood pressure[27].
162 In the past, blood pressure in chicken embryos was measured using glass capillaries or needle catheters
163 inserted into an embryonic artery[28-30]. Corresponding to prior descriptions in the literature[28-30], an
164 increase in arterial blood pressure with increasing age of the embryos was observed in the present
165 study, while there were no major differences in HR between the EDs. Thus, the optical measurement
166 of arterial blood pressure and HR with a microtip catheter represents a reliable method for invasive
167 measurement of blood pressure and HR in chicken embryos. However, insertion of the catheter was
168 particularly challenging at ED7 and ED18 due to the small size of the chorioallantoic vessels at ED7 and
169 the beginning regression of the chorioallantoic vessels at ED18.

170 Since self-reporting is not possible, it is difficult to evaluate pain perception in animals[22]. Nociceptive
171 reactions to a noxious stimulus, on the other hand, can be measured[13]. The recording of cardiovascular
172 parameters is well suited for the clinical evaluation of nociception in animals, including birds[31,32]. In the
173 present study, the acquisition of cardiovascular parameters could be established for chicken embryos
174 between EDs 7 and 18. Blood pressure and HR are mainly influenced by the autonomic nervous
175 system[33]. Transmission of a noxious stimulus to the central nervous system results in activation of the
176 sympathetic nervous system, which usually leads to an increase in blood pressure and HR[33]. Therefore,
177 recording cardiovascular variables is considered the gold standard for the detection of nociception
178 under anesthesia[34].

179 As a means of assessing a cardiovascular response of the chicken embryo to a mechanical noxious
180 stimulus at the base of the beak, the MAP was found to be the most sensitive parameter in the present
181 study. Significant differences in MAP between *Pinch* and *Touch* were earliest detected on ED16, while
182 significant changes in HR were observed only in ED17 and ED18 embryos. While there was a distinct
183 increase in MAP in response to *Pinch* that reached over 10 % deviation from baseline in ED17 and ED18
184 embryos, the changes in HR were variable, and not necessarily were there any associations between
185 changes in MAP and HR. Similar observations have been reported in adult chickens[35]. MAP has also

186 been described in other studies concerning nociceptive responses in mammals as the most sensitive
187 indicator of nociception[33,36].

188 A prerequisite for a cardiovascular response to external stimuli is functional regulation of the
189 cardiovascular system. Blood pressure in the chicken embryo is mainly regulated by the sympathetic
190 nervous system[37]. An adrenergic tone on the cardiovascular system is considered to be present from a
191 point in time halfway through the incubation period[38,39]. Therefore, the sympathetic influence on blood
192 pressure is expected to be functional from approximately ED10[38]. In the heart, adrenergic and
193 cholinergic receptors are already functional on ED4[40]. Changes in HR due to alterations in
194 environmental conditions such as oxygen levels and temperature have already been observed on
195 ED3[41]. In the present study, significant changes in HR after a noxious stimulus were not observed until
196 ED17.

197 Another prerequisite for the assessment of a nociceptive response is functioning stimulus
198 transmission. Despite some differences in the nervous system, the processing of noxious stimuli in
199 birds is comparable to that in mammals[13]. C-fibers and A-delta fibers have been found in chickens,
200 innervating the beak, nasal and buccal mucosa as well as the legs[11,42]. High-threshold mechanothermal
201 nociceptors are polymodal and respond to mechanical lesions, elevated temperatures and chemical
202 insult[13]. It is considered that injuries to the beak can be highly painful for the bird[42] since the beak tips
203 are intensely innervated areas[43] and both the upper and the lower beak contain nociceptors[44].
204 Reflective reactions such as movements of the head to mechanical and thermal stimuli and to needle
205 punctures appear for the first time in the skin area of the beak on ED7[45]. Therefore, in the present
206 study, the application of a noxious stimulus to the base of the beak was chosen to evoke the highest
207 possibility for a nociceptive response.

208 Regarding HR, irregularities appeared spontaneously over the whole measurement period, even at
209 baseline. Mainly short decelerations in HR were observed, whereas MAP was not affected. It has
210 already been reported in several publications that HR irregularities physiologically occur at the end of

211 the second week of incubation[46-49]. Nevertheless, the HR irregularities did not have a great influence
212 on the calculation of the mean. Minor changes in DAP reflected the HR irregularities, but the analysis
213 of the MAP was not affected. In contrast to physiological variations in HR, a hyperacute decrease in HR
214 with a subsequent increase could be clearly identified as a response to *Pinch* in 30 % of ED17 and 80 %
215 of ED18 embryos. This reaction pattern could be distinguished from physiological variations in HR by
216 the finding that post *Pinch*, HR decreased by at least -15 % and was followed by a sustained increase
217 in HR of at least 5 % from the baseline mean value. The decrease in HR post *Pinch* was also
218 accompanied by a short decrease in the MAP followed by an increase. A decrease in HR as a reaction
219 to a noxious stimulus has been reported in adult chickens[35] and in mammals[50,51] and may be due to a
220 vasovagal reflex to a noxious stimulus[52]. However, only single embryos showed a hyperacute decrease
221 in HR post *Pinch*, which shows that the response in HR to a noxious stimulus is individual. Variable
222 responses in HR after a noxious stimulus have also been described in adult chickens[35].

223 In addition to a nociceptive response, it must also be considered that the measured cardiovascular
224 changes may be induced by other factors that influence the autonomic nervous system[53], or by
225 embryonic movements. Especially in birds, physiological variables can be influenced by many external
226 factors such as temperature, light or handling[53]. A correlation between fetal movements and HR
227 irregularities has been described in human fetuses[54]. In the present study, movements of the embryo
228 induced minor variations in HR and DAP, but the MAP was not affected. No sustained increase in MAP
229 and HR as observed in response to *Pinch* could be attributed to movements.

230 Infiltration anesthesia at the base of the beak could be used to verify that the measured changes in
231 MAP and HR may be classified as a nociceptive response and were not caused by embryonic
232 movements or factors that influence the autonomic nervous system. The application of local
233 anesthetics is one of the best methods to prevent the generation and transmission of nociceptive
234 impulses[55]. The anesthetics act by blocking sodium channels in the nerve axon[53]. The application of
235 lidocaine or bupivacaine has been described as an effective method of analgesia in birds[56]. However,
236 the time of onset of action and the duration of action are not defined for birds[53]. In the present study,

237 lidocaine was used because it has a rapid onset of action in mammals[55], and in chicken, a short onset
238 of action has also been described in spinal anesthesia[57]. Given that higher sensitivity to local
239 anesthetics is expected in birds than in mammals[58], embryos were intensively monitored for the
240 occurrence of toxic effects. No signs of side effects such as bradycardia, arrythmia or hypotension were
241 observed in the tested embryos. Since the increase in MAP was significantly reduced by the injection
242 of lidocaine, the cardiovascular reactions to *Pinch* in the embryos that did not receive local anesthesia
243 might be interpreted as a nociceptive response to the noxious stimulus. A limitation and possible
244 explanation for not entirely suppressing the reaction in all embryos was that injection into the beak of
245 the moving embryo was challenging and infiltration of the entire beak area could not always be
246 assured. Since the present study is explorative, further investigations would need to be performed to
247 ultimately exclude other factors as the cause of the measured cardiovascular changes. However,
248 assuming that it is a nociceptive response, further studies regarding anesthesia and analgesia protocols
249 are necessary to provide improved animal welfare for chicken embryos in research. Cardiovascular
250 variations are commonly used to determine the need for analgesia or sedatives[23] and so far there are
251 no EU-wide regulations regarding anesthesia and analgesia for chicken embryos in research.

252 Although no significant difference between *Pinch* and *Touch* was reached at ED15 in MAP and HR,
253 individual responses could be observed. Occasionally, embryos at ED15 showed reactions in MAP and
254 HR post *Pinch*. The measurements of these embryos were performed late in the day. The development
255 of the embryos could therefore have been more advanced compared to embryos examined in the
256 morning. In addition, embryonic development can be influenced by various factors, and some embryos
257 might progress faster in development than others[38]. Therefore, it has to be assumed that a nociceptive
258 cardiovascular response is possible in individual embryos at ED15.

259 A limitation of the study was that intra-arterial measurement of blood pressure and HR is an invasive
260 method. The measurements had to be performed on the opened egg, and it was inevitable to open
261 the egg membranes. Since chicken embryos are highly sensitive to external factors[28,41], special care
262 was taken to maintain standardized environmental conditions and to avoid blood loss during

263 preparation. In some embryos, severe bradycardia and hypotension were observed, or HR frequently
264 decreased to zero. These embryos had to be excluded from the analysis because reliable
265 measurements could not be completed. At ED7, reaching the beak was challenging, and a
266 measurement could only be performed in 3 embryos; severe arrythmias affecting the MAP were
267 observed. The microtip catheter is designed to measure low pressures, but the measurement accuracy
268 of 2 mmHg, according to the manufacturer, reached its limits at the extremely low blood pressure on
269 ED7. The results from ED7 should therefore be interpreted with caution.

270 **Conclusion**

271 In conclusion, significant differences in a cardiovascular response to a mechanical noxious stimulus at
272 the base of the beak compared with a light touch at the base of the beak were detected in chicken
273 embryos on EDs 16 to 18. For individual embryos, a cardiovascular response to a mechanical noxious
274 stimulus is considered to be already possible on ED15. Infiltration anesthesia with the local anesthetic
275 lidocaine 2 % could significantly reduce reactions in MAP to a mechanical noxious stimulus at the base
276 of the beak in ED18 embryos, indicating that the measured cardiovascular changes can be interpreted
277 as nociceptive responses.

278 **Material and Methods**

279 **Animals:**

280 Fertilized eggs from a breeding of Lohman Selected Leghorn chicken eggs were obtained from the TUM
281 Animal Research Center (Thalhausen) and stored at 15 °C. ED0 was considered the day when eggs were
282 transferred to the incubator (Favorit – Olymp 192 Spezial, HEKA – Brutgeräte, Rietberg, Germany). The
283 eggs were incubated for 7 to 18 days at 37.8 °C and 55 % humidity and turned six times a day until they
284 were fenestrated.

285 At ED3 of incubation, the egg shells were fenestrated[59]. For this purpose, the egg was placed
286 horizontally for at least two minutes, and then 5 to 7 ml albumen was withdrawn from the apical pole
287 of the egg using a 5 ml syringe and an 18 G needle. The top of the egg was then covered with tape. A
288 hole was cut in the shell, and the vitality of the embryo was verified. Then, 0.5 ml
289 penicillin-streptomycin (10,000 units penicillin, 10 mg streptomycin/ml, P4333-100 ml Sigma–Aldrich)
290 was added before the egg was resealed with cling film and was further incubated in horizontal position.
291 The vitality of the embryos was checked daily until the end of the experiment. Experiments were
292 conducted between 9:00 a.m. and 7:00 p.m. so that the variance in the age of the embryos within an
293 ED was limited to a maximum of 10 hours.

294 **Experimental design:**

295 The study was an explorative study. For ED12 to ED18, n=10 embryos of each embryonic day were
296 measured. Due to higher losses in younger embryos, a group size of n=6 (ED9) and n=3 (ED7) embryos
297 was chosen. Furthermore, to study the effect of local anesthesia, n=6 ED18 embryos were used.

298 Experiments were performed under standardized conditions in a specially designed heating chamber
299 equipped with a heating lamp (ARTAS GmbH, Arnstadt, Germany) and an air humidifier (HU4811/10
300 Series 2000, Philips, Amsterdam, the Netherlands). The eggs were placed on a heating mat
301 (ThermoLux, Witte + Sutor GmbH, Murrhardt, Germany) in a bowl filled with warmed Armor Beads

302 (Lab Armor Beads™, Sheldon Manufacturing, Cornelius, USA). The mean temperature and mean
303 humidity during all experiments were 37.7 °C ± 0.8 and 55.5 % ± 4.3.

304 First, the cling film was removed from the egg, and the shell was carefully opened to the level of the
305 chorioallantoic membrane. Using a microscope (Stemi SV6, Zeiss, Oberkochen, Germany), the allantoic
306 and amniotic membranes were opened over the head of the embryo, avoiding any large vessels so that
307 the beak could be reached in the further course of the experiment. A side branch of the chorioallantoic
308 artery was prepared, temporarily ligated to avoid blood loss, and incised with microsurgical scissors. A
309 microtip catheter (FISO-LS Fiber Optic Pressure Sensor, FOP-LS-PT9-10, FISO Technologies Inc.,
310 Quebec, Canada) was then inserted into the vessel and fixed in place with a ligature. SAP, DAP, MAP
311 and HR were recorded continuously every four seconds (PLUGSYS module, EIM-B, EIM-A, HAEMODYN
312 Software, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany, FFP-LS and
313 Evolution Software, FISO Technologies Inc., Quebec, QC, Canada). The beak of the embryo was
314 carefully placed on a Desmarres lid retractor. For younger embryos at ED7 and ED9, the beak was
315 carefully placed on a custom-made wire loop.

316 After implementation of the catheter, a two-minute waiting period followed. Then, two mechanical
317 stimuli were applied at the base of the beak. In randomized order, either a mechanical noxious stimulus
318 was applied with a surgical clamp (*Pinch*) or, as a negative control, a light touch (*Touch*) was applied
319 first. Between the two stimuli, a period of five minutes was maintained to normalize the parameters.
320 After the second stimulus, measurements were continued for five more minutes. The measurement
321 time between both stimuli and after the second stimulus was reduced from five to three minutes for
322 ED13 and younger embryos due to the increasing sensitivity of the organism.

323 For the *Pinch*, a surgical clamp was applied to the base of the beak and squeezed. For the *Touch,* the
324 beak was only lightly touched with the surgical clamp. For both stimuli, a mosquito clamp was used for
325 ED12 to ED18 embryos. For embryos at ED7 and ED9, the surgical clamp was too large, and
326 microsurgical forceps were used instead for both stimuli. To ensure comparability, the stimuli were

327 always applied by the same person. In the further course of the study, an analgesia meter (BIO-RP-M,
328 BioSeb, Vitrolles, France) with customized tips of the mosquito clamp was used to monitor the pressure
329 applied by the mechanical stimulus.

330 To verify whether the measured cardiovascular responses could be classified as nociceptive responses,
331 in n=6 ED18 embryos, a local anesthetic was applied before stimulation. For this purpose, after the
332 preparation and placement of the microtip catheter, 0.02 ml of lidocaine 2 % (Xylocitin® 2 %, Mibe
333 GmbH Arzneimittel, Brehna, Germany) was injected into the upper and lower beak using a 30 G needle
334 (*ED18 w/ Lido Touch* and *Pinch*). The measurements were carried out following the same experimental
335 protocol as for ED14 to ED18 embryos with the exception that a waiting period of three minutes was
336 added prior to the measurement. During this time, blood pressure and heart rate were monitored for
337 the occurrence of side effects of lidocaine, such as bradycardia, arrythmia or hypotension. As a
338 comparison group without lidocaine, the already measured ED18 embryos were used (*ED18 w/o Lido*
339 *Touch* and *Pinch*).

340 Immediately after the end of the experiments, the embryos were euthanized by intravenous injection
341 of pentobarbital sodium (Narcoren®, 16 g/100 ml, ED7-ED12: 0.1 ml; ED13-ED19: 0.2 ml) followed by
342 decapitation.

343 **Analysis:**

344 SAP, DAP, MAP and HR were recorded every four seconds. For the evaluation of the reactions to the
345 stimuli, the means of MAP and HR were calculated over one minute before (= baseline) and one minute
346 after the respective stimulus. To avoid an influence by the approach of the clamp, the 15 seconds
347 immediately before the respective stimulus were not included as part of the baseline. In embryos
348 showing a hyperacute decrease in HR with a subsequent increase in HR post *Pinch,* the decrease was
349 not included in the calculation and was evaluated separately to avoid compensation of opposite
350 reactions. The percentage deviation of the response after the stimulus (*Pinch/Touch*) to the baseline
351 was then calculated. Differences in the percent changes to baseline in MAP and HR after *Pinch* and

Touch were tested for statistical significance. For normally distributed data, a paired t test (two-tailed) was used. For data that failed the normality test, a Wilcoxon signed-rank test (two-tailed) was performed. For the comparison of multiple groups, either a one-way ANOVA (normally distributed) or a Kruskal–Wallis test (not normally distributed) was used.

Data availability

Raw data are available up on reasonable request to the corresponding author.

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482 **Acknowledgments**

483 This work was financially funded by the German Federal Ministry of Food and Agriculture (BMEL) based
484 on a decision of the Parliament of the Federal Republic of Germany, granted by the Federal Office for
485 Agriculture and Food (BLE; grant number 2821HS005).

486 The authors thank the scientific advisory board with Prof. Dr. Dr. Michael Erhard, Prof. Dr. Wolf Erhardt,
487 Prof. Dr. Harald Luksch, Prof. Dr. Heidrun Potschka, Prof. Dr. Hans Straka and Dr. Britta Wirrer for their
488 excellent scientific contribution as well as Dr. Johannes Fischer, Stefanie Fitzner and Dr. Hicham Sid for
489 their technical support.

490 **Author contributions**

491 Conceptualization: CB, AMS, JW, JR, TF, BS; Data curation: LW, AMS; Formal analysis: LW, AMS, JW;
492 Funding acquisition: CB, TF, BS; Investigation: LW, SCS, AMS, JW, JR; Methodology: CB, AMS, JW, JR,
493 SK, MA, TF, BS; Project administration: CB; Resources: CB, BS; Supervision: CB, TF, BS; Writing - original
494 draft: LW; Writing - review & editing: LW, AMS, JW, SCS, JR, SK, MA, TF, BS, CB. All authors have read
495 and agreed to the published version of the manuscript.

496 **Competing interests**

497 The authors state no competing interests.

498 **Figure Legends**

499 Figure 1. Percent change in MAP post Touch and Pinch. Embryos at EDs 16 to 18 (n=10) received a
500 mechanical noxious stimulus (Pinch) and a light touch as a negative control (Touch) at the base
501 of the beak in randomized order. a-c Percent change to baseline in MAP post Pinch compared
502 to Touch. Displayed as the mean ± standard deviation. Paired t test (normally distributed: a
503 and c) or Wilcoxon signed-rank test (not normally distributed: b). P values shown; a: p=0.0008,
504 b: p=0.0020, c: p=0.0048. d-f Percent change from the baseline mean value in MAP over time;
505 values recorded every four seconds for one minute before and one minute after stimulation
506 (Pinch and Touch); values shown as the mean ± standard deviation (shaded).

507 Figure 2. Percent change in HR post Touch and Pinch. Embryos at EDs 16 to 18 (n=10) received a
508 mechanical noxious stimulus (Pinch) and a light touch as a negative control (Touch) at the base

of the beak in randomized order. a-c Percent change to baseline in HR post Pinch compared to Touch. Displayed as the mean ± standard deviation. Paired t test (normally distributed: b and c) or Wilcoxon signed-rank test (not normally distributed: a). P values shown; b: p=0.0148, c: p=0.0154; ns=no significant difference between the groups (a). d-f Percent change from the baseline mean value in HR over time; values recorded every four seconds for one minute before and one minute after stimulation (Pinch and Touch); values shown as the mean ± standard deviation (shaded).

Figure 3. Local anesthesia control group. Percent change in MAP and HR post Touch and Pinch. ED18 embryos either received a lidocaine injection (ED18 w/ Lido; n=6) or no lidocaine injection (ED18 w/o Lido; n=10) at the base of the beak prior to stimulation (Touch and Pinch). a Percent change to baseline in MAP post Pinch in the group without lidocaine (ED18 w/o Lido Pinch) compared to ED18 w/o Lido Touch, ED18 w/ Lido Touch and ED18 w/ Lido Pinch. Displayed as the mean ± standard deviation. One-way ANOVA (normally distributed); p values shown. b Percent change to baseline in HR post Pinch in the group without lidocaine (ED18 w/o Lido Pinch) compared to ED18 w/o Lido Touch, ED18 w/ Lido Touch and ED18 w/ Lido Pinch. Displayed as the mean ± standard deviation. Kruskal–Wallis test (not normally distributed); p values shown. c-d Percent change from the baseline mean value in MAP and HR post Pinch over time; values recorded every four seconds for one minute before and one minute after stimulation (ED18 w/o or w/ Lido Pinch); values shown as the mean ± standard deviation (shaded).

Tables

Table 1. SAP, DAP, MAP and HR at ED7 (n=3), ED9 (n=6), and ED12 to ED18 (n=10). Values are shown as the mean ± standard deviation.