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1 **Sniffing response to an odor through development in rats: modulatory role of repetition and**
2 **association with an aversive event**

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5

6 **Abstract (100-250 words)**

7 Sniffing has proven to be a useful behavioral readout for assessing olfactory performance in adult rats.
8 However, little is known about how sniffing response changes through ontogeny. This study thus aimed
9 at characterizing respiratory response to an odor through development in rats using paradigms
10 applicable to both young pups and adults. We first analyzed the sniffing response to the arrival of a
11 novel neutral odor. Then the value of the odor was changed through either its repeated presentation
12 (odor habituation), or its association with a foot-shock resulting in odor fear. In the habituation task,
13 we found that at the three ages, the first presentation of the novel odor induced a clear sniffing
14 response but the peak respiratory frequency was higher in adults than in juveniles and infants. When
15 the odor was presented repeatedly, the sniffing response gradually faded. This habituation of the
16 response took more trials as the animal's age increases. In the fear conditioning task, the odor induced
17 an increase in respiratory rate that persisted until the end of the session in adults and infants, while it
18 faded rapidly in juveniles. When the odor was explicitly unpaired with the foot-shock, at the three ages
19 the respiratory response to the odor lasted less over the session than in the paired condition. Finally,
20 we observed that shock delivery induced a similar respiratory response at the three ages in paired and
21 unpaired condition. Collectively these data show that sniffing response constitutes a faithful index to
22 assess rat's olfactory abilities through ontogeny.

23

24 **Introduction**

25 When a rat encounters a novel odor in its environment, it initiates an automatic orienting response
26 consisting in an active sampling of that odor achieved via directing its snout toward the odor source
27 and increasing its respiratory frequency resulting in so-called sniffing behavior. As nicely described by
28 Welker (1964) in his seminal study, sniffing in adult rats occurs with a precisely coordinated rhythmic
29 motor sequence involving nose, head, and whisker movements. When they occur together, these
30 movements take place at the same rate and exhibit a fixed temporal relationship to one another.
31 Kurnikova et al (2017) further showed that the onset of each breath initiates a "snapshot" of the
32 orofacial sensory environment. The authors suggest that respiration acts as a master oscillator to
33 phase-lock rhythmic orofacial motor actions. A consequence of the temporal regularity of these signals
34 would be to improve the fidelity of coding of the stimuli.

35 This stereotypical sniffing behavior undergoes considerable postnatal development. While rapid
36 sniffing is relatively rare and poorly maintained in pups less than 1 week old (Alberts and May, 1980a),
37 by the eighth day after birth, the four sniffing movements are present although not at their maximal
38 amplitude. Between the eighth and tenth postnatal days, exploratory behavior increases and appears
39 to direct the sniffing actions toward target sensory stimuli. Alberts and May (1980) reported that from
40 the second week of life onwards, sniffing becomes a finely-orchestrated pattern of sustained polypnea
41 combined with coordinated movement sequences.

42 Sniffing has a central role in olfaction since it enhances transport of volatile odorous molecules from
43 the entrance of the nares to the olfactory epithelium. Consequently, sniffing enhances detection and
44 localization of odorants and plays a critical role in odor information processing both in olfactory areas
45 and at higher levels (Buonviso et al. 2006; Mainland and Sobel, 2006; Verhagen et al, 2007; Wesson et
46 al, 2008b). Importantly, odor-induced sniffing proved to be a useful behavioral readout for evaluating
47 olfactory performance in adult unrestrained rats and mice (Macrides et al. 1982; Youngentob et al.
48 1987; Uchida and Mainen 2003; Kepecs et al. 2007; Wesson et al, 2008a; Courtiol et al, 2014; Lefèvre
49 et al, 2016; Boulanger-Bertolus et al, 2014; Shionoya et al, 2013; Dupin et al, 2020). Odor-induced
50 sniffing has also been used to investigate the ontogeny of olfactory perception in rodents (Alberts and
51 May, 1980b; Boulanger-Bertolus et al, 2014). Indeed, in contrast to classical olfactory tasks that require
52 extensive training and motoric skills that cannot be achieved by pups, odor-induced sniffing provides
53 a reliable index of odor sensitivity in neonates since it involves a spontaneous response to a perceived
54 change in olfactory environment. Using this measure, Alberts and May (1980b) showed that there was
55 a monotonic increase in chemosensitivity as the pups mature from 1 to 17 days of age, with a tendency
56 to a ceiling effect around PN11-13.

57 Although previous studies have used sniffing behavior to investigate the ontogeny of olfaction during
58 the first weeks of life on one side, and olfactory performances in adult animals on the other side, no
59 study has performed a longitudinal investigation from infancy to childhood with the same olfactory
60 paradigms. The present study was aimed at fulfilling this caveat by comparing the rat's respiratory
61 response to an odor at different ages of development, using experimental conditions readily applicable
62 to rat pups because they did not require complex movement skills. We first analyzed the sniffing
63 response to the arrival of a novel odor with no behavioral significance. Then the value of the odor was
64 changed through either its repeated presentation leading to odor habituation, or its association with
65 a foot-shock resulting in odor fear. In these two paradigms, respiratory rate was previously shown to
66 be a reliable indicator of the animal's performance both in pups and in adults (Alberts and May, 1980b;
67 Boulanger-Bertolus et al, 2014; Shionoya et al, 2013). We investigated three ages of development:
68 infant rats (postnatal day PN12-15), juvenile rats (PN 22-24) and adult animals (older than PN75).

69

70 **Methods**

71 **Animals**

72 The subjects were male and female Long Evans rats born and bred in the Lyon Neuroscience Research
73 Center (originally from Janvier Labs, France). A different dataset from a subset of these animals has
74 been published in an earlier study (Boulanger-Bertolus et al, 2014). A total of 20 litters were used. Only
75 one female and one male pup per litter per treatment/test condition were used for all experiments
76 and animals from the same litters were used in the different test conditions and ages. Three groups of
77 ages were used: post-natal day 12 to 15 (PN12-15, infants), PN22-24 (juveniles) and older than PN75
78 (adults). Day of birth was considered PNO. Pups were maintained with their litters up to the end of the
79 experiments, including juvenile pups. Adults were housed by pairs of same sex, at 23°C and maintained
80 under a 12h light-dark cycle (lights on from 6:00 am to 6:00 pm). Food and water were available ad
81 libitum and abundance of wood shavings was supplied for nest building. All experiments were
82 conducted in strict accordance with the European Community Council Directive of November 24, 1984
83 (84/609/EEC) and the French National Committee (87/848) for care and use of laboratory animals.
84 Care was taken at all stages to minimize stress and discomfort to the animals.

85

86 **Training apparatus**

87 The apparatus consisted of a whole body customized plethysmograph (diameter 20 cm, Emka
88 technologies, France) placed in a homemade sound-attenuating cage (L 60 cm, W 60 cm, H 70 cm). The
89 plethysmograph was used to measure respiratory parameters in behaving animals (see Hegoburu et
90 al., 2011 for further description of the plethysmograph). The height of the plethysmograph was
91 adapted to the age of the animal in order to optimize the signal-noise ratio, leading to a height of 30
92 cm for the adults and 16.5 cm for the infants.

93

94 **Odor fear conditioning procedure**

95 Conditioning took place in a sound attenuation chamber with deodorized air constantly flowing
96 through the cage (2 L/min). The odor CS was a 30-s peppermint odor (McCormick Pure Peppermint; 2
97 L/min; 1:10 peppermint vapor to air) and was controlled with a solenoid valve that diverted the airflow
98 to the peppermint air stream, thus minimizing pressure change. The 1-s mild electric shock was
99 delivered through a grid floor. Adult rats were handled for about 4 days and placed into the
100 conditioning chamber for context habituation. Juveniles received only one day of handling and
101 habituation while infants, for which conditioning to context is not yet developed (Rainekei et al., 2010),
102 were not handled to minimize distress from separation from the mother.

103 Three training conditions were used throughout the experiments (Figure 1): Odor-alone presentations
104 (Odor groups), Odor-shock pairings (Paired groups), Odor-shock unpaired presentations (Unpaired

105 groups). For all groups, animals were allowed a 4min- period of free exploration. Then, in the paired-
106 groups, the CS odor was introduced into the cage for 30s, the last second of which overlapped with
107 the shock. The animals received ten odor-shock trials, with an inter-trial interval of 4min. In the
108 Unpaired groups, the same procedure was carried out except that the shock and the odor were
109 explicitly unpaired using a fixed 180s-interval between the odor onset and the shock arrival. In the
110 Odor groups, the animals received ten 30s-odor alone presentations 4-min apart. In the following, for
111 the three conditions, the term trial will refer to the period starting 30s before odor onset until 210s
112 after odor offset.

113

114 **Data analysis**

115 In each experimental group, respiration was monitored throughout the acquisition session. Offline, the
116 respiratory signal was analyzed and momentary respiratory frequency was determined. Instant
117 respiratory frequency was averaged on a second by second basis, leading to 1-s time bins curves. The
118 resulting individual curves were then averaged among animals of the same experimental group. In
119 each experimental group, for each trial, three analysis periods were defined (Figure 1): In the Odor
120 group, 20s-Pre-Odor, 30s-Odor and 20s-Post-Odor; in the Paired and Unpaired groups, 20s-Pre-Odor,
121 30s-Odor and 20s-Post-Shock. The average respiratory frequency was calculated for each duration
122 period. In each experimental group, the obtained values were compared using three factors ANOVA
123 (Age, Period, Trial) followed by post-hoc pairwise comparisons when allowed by the ANOVA results.
124 For all the statistical comparisons performed, the significance level was set at 0.05.

125

126 **Results**

127 **1- Respiratory response to a neutral odor (Odor Group)**

128 In this experiment, we assessed the effect of a neutral odor presentation on the respiratory frequency.
129 We compared between the three ages the response to the first odor presentation, then to the
130 repeated presentation of this odor, progressively leading to odor habituation.

131 **1.1- First presentation**

132 Figure 2A shows individual examples of raw respiratory signal at the three ages. Figure 2B illustrates
133 the respiratory frequency curve (mean \pm SEM) with a 1-sec time bin. At the three ages, there was a
134 clearcut increase in respiratory frequency in response to odor arrival. However, the slopes of the three
135 curves were different, with a peak frequency reaching respectively 10Hz at 9s in adults, 8Hz at 10s in
136 juveniles, and 7Hz at 17s in infants.

137 Figure 2C represents the mean respiratory frequency at the three ages during the Pre-Odor, Odor and
138 Post-Odor periods (per 20s time bins) until the end of the trial. A two factors ANOVA revealed a
139 significant effect of Age ($F_{2,27}=12.26$, $p<0.001$), Period ($F_{10,270}=35.07$, $p<0.001$) and a significant Period

140 x Age interaction ($F_{20,270}=6.47$, $p<0.001$). In the three age groups, there is a significant increase in mean
141 respiratory frequency during the Odor period compared to pre-Odor. This increase is greater in adults
142 than in juveniles and infants but is not significantly different between the two younger age groups.
143 During the post-Odor period, in adults and juveniles the respiratory frequency decreases progressively
144 to reach pre-Odor levels from 70s after odor onset onwards in both age groups. In contrast, in infants
145 the respiratory frequency remains significantly higher than pre-odor levels until the end of the trial.

146 **1.2- Repeated presentation**

147 We then looked at the evolution of the mean respiratory frequency before (pre-Odor), during (Odor)
148 and after odor (post-Odor) across the 10 individual trials of the session. Figure 3A illustrates the
149 respiratory frequency curve with a 1-sec time bin at the three ages for the four first trials of the session,
150 the remaining trials being similar to the fourth trial at all ages. Figure 3B represents the mean
151 respiratory frequency for these trials during the pre-Odor, Odor and post-Odor periods. The three
152 factors ANOVA (Age, Period, Trial) carried out on the 10 trials of the session revealed a significant effect
153 of Age, Period, Trial and all the possible interactions (detailed statistics in Table 1). Within group post-
154 hoc comparisons showed that in adults, the mean respiratory frequency during odor was significantly
155 higher than in the pre-Odor period until the third trial. In juvenile animals, the increase during odor
156 lasted until the second trial while in infants it was only observed for the first trial. Thus, the older the
157 animal, the longer it takes for its respiratory response to a neutral odor to habituate.

158

159 **2. Response to an odor paired or unpaired with an aversive stimulus**

160 In this experiment, we assessed the respiratory response to an odor in two experimental conditions:
161 either the odor signaled the upcoming arrival of a foot shock (Paired condition) or the odor was not
162 predictive of the footshock delivery (Unpaired condition). In both conditions, we also analyzed the
163 respiratory response to the nociceptive footshock stimulus (i.e. Post-Shock period).

164 **2.1- Paired condition**

165 Figure 4A represents the mean respiratory frequency during the three defined periods across the 10
166 individual trials of the session, at the different ages in the Paired groups. The three factors ANOVA
167 (Age, Period, Trial) revealed a significant effect of Age, Period and Trial, and all the possible interactions
168 (See statistics Table 2 upper part, for the details).

169 Within age group's post-hoc comparisons showed that at the three ages, arrival of the odor induced
170 an increase in the mean respiratory frequency compared to the pre-Odor period. While this increase
171 was maintained for most of the trials of the session in adults and infants, it was only observed until the
172 third trial in juveniles. In regards to the respiratory response to shock arrival, it was similar at the three
173 ages: shock delivery induced a significantly increase in respiratory frequency compared to the pre-
174 Odor and Odor periods for all the trials until the end of the session.

175 Between ages comparisons carried out on the mean values obtained by averaging all the trials of the
176 session showed that in the three defined periods (pre-Odor, Odor, post-Shock), respiratory frequency
177 was higher in infants than in adults and juveniles (Figure 5A).

178 In summary, when an odor is explicitly paired with a foot-shock, arrival of the odor induces a transient
179 increase in respiratory frequency that was observed for each trial until the end of the session in adults
180 and infants, while it vanishes rapidly in juveniles. In contrast, shock delivery induced a similar response
181 at the three ages, consisting in a further increase in respiratory frequency for all the trials.

182 **2.2- Unpaired condition**

183 Figure 4B represents the mean respiratory frequency during the three defined periods, across the 10
184 trials of the session, at the different ages in the Unpaired groups. The three factors ANOVA revealed a
185 significant effect of Age, Period and Trial, and all the possible interactions (See statistics Table 2 lower
186 part, for the details). Within age group's post-hoc comparisons showed that in adults, the mean
187 respiratory frequency during odor was significantly higher than in the pre-Odor period until the fifth
188 trial. In contrast, in juveniles and infants the increase during odor was observed for the first trial only.
189 On the other hand, the respiratory response to shock arrival was similar at the three ages: the
190 respiratory frequency after shock delivery was significantly higher than in the pre-Odor and Odor
191 periods for most of the trials of the session.

192 Between ages' comparisons carried out on the mean values throughout the session showed that as for
193 Paired animals, in the pre-Odor and Odor periods, respiratory frequency was overall higher in infants
194 than in adults (Figure 5B).

195 In summary, the respiratory response to the odor lasted longer over the session in Paired than
196 Unpaired animals at all ages. In contrast, the respiratory response to the shock was similar in Paired
197 and Unpaired groups and consisted in a further increase in respiratory frequency for most of the trials
198 of the session.

199

200 **Discussion**

201 This study was aimed at characterizing the respiratory response to an odor through development in
202 rats using the same behavioral paradigms. The initial value of the odor was changed through either its
203 repeated presentation leading to odor habituation, or its association with a foot-shock resulting in
204 odor fear. These two paradigms were readily applicable to rat pups because they do not require
205 complex movement skills. In the habituation task, we found that at the three considered ages, the first
206 presentation of the novel odor induced a clear sniffing response. The peak respiratory frequency was
207 higher and occurred earlier after odor onset in adult animals than in juvenile and infant animals.
208 Despite being smaller in amplitude, the sniffing response of infants lasted longer after odor onset than
209 that of adults or juveniles. When the odor was presented repeatedly, the sniffing response gradually

210 faded and then disappeared. This habituation of the response took more trials as the animal's age
211 increases. In the odor fear conditioning task where the odor signaled the upcoming arrival of a foot-
212 shock, the odor induced an increase in respiratory rate that persisted over the trials until the end of
213 the session in adults and infants, while it faded rapidly in juveniles. When the odor was explicitly
214 unpaired with the footshock, the respiratory response to the odor lasted less long over the session
215 than in the paired condition at all three ages. Finally, we observed that shock delivery induced a similar
216 respiratory response at all three ages in paired and unpaired condition, consisting in a further increase
217 in respiratory frequency in most trials.

218 Using sniffing response as an index of learning allowed us to detect subtle differences within the
219 conditioning session that the classical measure of freezing would not have unveiled. Indeed, while
220 freezing is a robust and easily quantifiable response, it lacks temporal sensitivity and plasticity. Once
221 induced in response to a foot-shock, freezing often persists throughout the session thus precluding the
222 observation of subtle variations in animal's fear levels. Respiration in contrast is a more phasic signal
223 than freezing and could give access to transient changes due to odor arrival, otherwise overshadowed
224 by constant freezing (Hegoburu et al, 2011; Shionoya et al, 2013).

225

226 **Odor habituation task**

227 At all three ages, there was a sniffing response to the first presentation of the odor suggesting a good
228 perception of the stimulus in our experimental conditions. However, the slopes of the three curves
229 were different, with a peak frequency reaching respectively 10Hz at 9s in adults, 8Hz at 10s in juveniles,
230 and 7Hz at 17s in infants. Thus, the peak respiratory frequency was higher and occurred earlier after
231 odor onset in adults than in juveniles and infants. Importantly, the basal respiratory frequency before
232 odor delivery was not different between ages (Adults: 3Hz; Juveniles: 3.4Hz; infants: 3.6Hz), therefore
233 suggesting the sniffing response to the odor selectively changed through development. Alberts and
234 May (1980a) showed that from the second week of life onwards, sniffing behavior components are
235 well orchestrated. Our data suggest that the vigor of odor-induced sniffing is lower in infants and
236 juveniles, but this does not happen at the detriment of odor perception. Interestingly, while in adults
237 and juveniles the sniffing response induced by the odor returned to basal levels 70s after odor delivery,
238 which corresponds to the end of odor as assessed at the experimenter's nose, the infant sniffing
239 response persisted until the end of the 4min-trial. This is in accordance with data showing that
240 between 10 and 15 days of age, pups exhibit sustained exploratory behaviour to a novel environment
241 that may persist for several minutes, and they also show hyperreactivity to novel stimuli (Campbell
242 and Spear, 1972; Bolles and Woods, 1964). Thus, in the present study, the persisting sniffing response
243 observed in infants, is rather the result of the intense exploration triggered by the odor than an index
244 of odor sampling.

245 Upon repeated presentation of the odor, sniffing response progressively declined over the successive
246 trials. Habituation is classically defined as the progressive reduction of a behavioral response elicited
247 by repeated exposure to a novel stimulus not accompanied by any biologically relevant consequence
248 (Leussis and Bolivar, 2006; McNamara et al, 2008). Habituation is a simple form of non-associative
249 learning that underlies animals' capacity to tone down their response to familiar inconsequential
250 stimuli in favor of novel potentially more relevant events (Thompson and Spencer 1966). Classically in
251 rodents, habituation to an odor is assessed via measuring the investigation time, defined as the
252 amount of time the animal spent within 1-2 cm of the odorant source with its nose aimed toward it.
253 Over successive presentations of the odor, a progressive reduction in investigation time is observed,
254 indicating that the animal remembers its prior experience with the stimulus and no longer investigates
255 it as if it were novel (McNamara et al, 2008). Although easily applicable in adult animals, this measure
256 lacks precision and sensitivity when young animals are tested. In the present study, using exploratory
257 sniffing as a spontaneously expressed response to novel odorants, allowed us to compare habituation
258 through development.

259 Upon repeated presentation of the odor, sniffing response in adults was no more detectable by the
260 fourth odor presentation. This is in agreement with data from the literature obtained in unrestrained
261 adult rodents using the sniffing response (Wesson et al, 2008; Coronas-Samano et al, 2016; Al Koborssy
262 et al, 2019). At younger ages, the animals' respiratory response to the odor habituates earlier during
263 the session: at the third trial in juveniles and second trial in infants. This observation suggests that the
264 intense exploratory period triggered in infants by the first odor presentation habituates rapidly.

265 It could be argued that this rapid habituation was due to the confounding effect of fatigue, particularly
266 in young animals. This might have been disambiguated using a habituation/cross-habituation task.
267 Indeed, in the habituation/cross-habituation test, animals are presented repeatedly with the same
268 odorant (habituation) after which a new odorant is introduced (cross habituation). An animal is
269 considered as perceiving the second odorant as novel when the exploration time is higher than for the
270 prior trial. In our case, the triggering of a sniffing response by the novel odor would demonstrate that
271 the rapid waning of response to the first odor in young animals was not due to fatigue, but to
272 habituation. In contrast, the absence of a sniffing response to the novel odor, would suggest an effect
273 of fatigue. However, another confounding effect in the habituation/cross-habituation test is the
274 possibility that odor discrimination abilities vary through development and an absence of response to
275 novel odor in infants might be explained by a lack of discrimination between the two odors at that age.
276 In the present study, we assume that the rapid habituation observed in infants cannot be accounted
277 for by fatigue because as discussed below, in the Paired condition, the infants' respiratory response to
278 the odor did not habituate throughout the session. Thus the present data suggest that young animals
279 tend to classify an odor as familiar more rapidly than adults.

280

281 **Odor fear conditioning task**

282 Odor fear conditioning has been well characterized during ontogeny and this literature has shown that
283 fear learning emerges in rat pups around PN10 and is caused by the recruitment of the amygdala during
284 the odor–shock conditioning (Sullivan et al. 2000a; Moriceau et al. 2006; Rainecki et al. 2009; Boulanger-
285 Bertolus et al, 2016).

286 The training paradigm used in the present study in the Paired condition has been shown to result in
287 good odor fear memory at the three developmental ages considered (Boulanger-Bertolus et al, 2014).
288 In contrast to what has been observed in Odor animals, adult and infant Paired animals displayed a
289 sniffing response to the odor until the last trial of the session, suggesting that as the trials accumulate,
290 the odor endowed the value of an arousing/alarming signal predicting shock arrival. In that sense, the
291 sniffing response transitioned from an unconditioned response to the new odor first presentation to a
292 conditioned response reflecting the learned emotional value of the odor. In juveniles, the sniffing
293 response to the odor did not persist after the third trial. Importantly, these animals have been shown
294 to have a good memory of the learned odor when tested 24h later (Boulanger-Bertolus et al, 2014).
295 Therefore, the difference in sniffing response to the odor cannot be ascribed to a difference in quality
296 of learning. Interestingly, based on the developmental literature, the peri-weanling period (PN17–23)
297 is the age range at which fear conditioning to the context emerges (Rainecki et al, 2010; Sullivan et al.,
298 2000; Brassler and Spear, 1998, 2004). A particularity of contextual learning at that developmental age
299 compared to adulthood, is that it is displayed by paired but not unpaired animals indicating a
300 potentiation of context learning by cue learning (Rainecki et al, 2010; Esmoris-Arranz et al., 2008). In
301 our study, contextual learning might have developed in juveniles as trials accumulate and compete
302 with the odor cue in terms of predictive value, leading to the loss of sniffing response to the odor after
303 the third trial. In contrast, in infants that do not yet learn the context and in adults for which context
304 learning occurs preferentially in unpaired condition, the odor cue keeps a strong predictive value and
305 triggers a sniffing response throughout the whole session.

306

307 **Difference between paired and unpaired condition**

308 Our data suggest that until the fifth trial, adult unpaired animals react to the odor in the same way as
309 paired animals. In contrast, from Trial 6 onwards, unpaired animals no longer react to the odor leading
310 us to assume that from there, the odor is no more considered as an alarming signal and could rather
311 constitute a safety signal because it is never causally associated with the shock (Rogan et al, 2005).
312 Although not assessed in the present study, we assume that as classically described in the literature,
313 adults unpaired rats have formed a fear to the contextual cues but not to the odor cue.

314 In juveniles and infants, no sniffing response to the odor was observed after the first trial suggesting
315 the animals clearly dissociated the odor from the shock. In addition, while no study systematically
316 explored context learning in animals younger than 16 days (our infant animals), previous studies have
317 shown that juvenile rats hardly learn context fear in an unpaired paradigm (Raineki et al, 2010). We
318 therefore suggest that unpaired infants and juveniles behave as control odor animals of the same age
319 with a lack of sniffing response to the odor after trial 1.

320

321 **Response to Shock delivery**

322 At all three ages in both Paired and Unpaired groups, shock delivery induced an increase in sniffing
323 response that did not habituate across trials. This increased sniffing response is associated with an
324 ensemble of unconditioned defense responses to the footshock. As nicely described by Fanselow
325 (1982), when a rat receives an aversive electric shock, it reacts with vigorous activity characterized by
326 reflexive paw withdrawal, jumping, and squealing. This activity persists for a brief period and then
327 gradually gives way to freezing behavior. The post-shock activity burst constitutes the unconditioned
328 response to aversive event while post-shock freezing is produced by conditioned fear elicited by cues
329 associated with shock (Fanselow, 1980). Here we show that the post-shock activity burst induced an
330 increase in sniffing behavior that was similar at the three ages, suggesting that perception of the
331 nociceptive stimulus is comparable at the different developmental ages tested in the present study
332 (Collier and Bolles 1980; Barr, 1995; Fitzgerald, 2005). In addition, the effect on sniffing is the same for
333 paired and unpaired animals suggesting that signaling the shock by an odor does not modulate the
334 reaction of the animal compared to an un signaled shock.

335

336 **Conclusion**

337 What does the analysis of sniffing behavior bring to the field of olfactory perception assessment,
338 particularly during ontogeny? Sniffing behavior is a spontaneous unlearned response, functional at
339 early ages of development. This measure allowed us to compare olfactory performances through
340 development using the same index, which would not have been possible using for example classical
341 freezing assessment in the odor fear conditioning paradigm since freezing response is immature in
342 infants. Indeed, in adult rats freezing is defined as an immobile crouched posture with the ventral
343 surface elevated above the floor (Blanchard and Blanchard, 1969). However, while freezing can be
344 observed in postweaning animals (Bolles and Woods, 1964), young preweaning rats are not capable of
345 assuming a crouched posture with their limbs extended to support their body trunk due to their
346 immature musculoskeletal system (Takahashi, 1992), thus precluding the use of freezing behavior as a
347 common index through ontogeny.

348 Despite its above-mentioned advantages, sniffing behavior as assessed through the lens of respiratory
349 frequency, does not replace overt behavior analysis. Indeed, the behavior of the animal cannot be
350 inferred from its respiratory frequency only since for example during freezing, respiratory rate is
351 around 3-4Hz in rats (Hegoburu et al, 2011; Dupin et al, 2019; Boulanger-Bertolus et al, 2014), which
352 is the same frequency range as that of quiet waking state (Girin et al, 2021). In that case, visual
353 observation of the animal's behavior is necessary to differentiate between the two states. However, it
354 is important to keep in mind that while the present study was centered on respiratory frequency, other
355 parameters of the respiratory cycle like amplitude, volume, peak flowrate or shape, might bring
356 additional information that could help identifying the ongoing behavior (Youngentob, 1987).
357 Sniffing response is not exclusively observed in response to an odor. Indeed, it is part of the orienting
358 response and it is triggered whenever the animal detects something new and unexpected in its
359 environment. Sniffing response thus constitutes a useful index to assess rat's perceptual abilities in
360 different sensory modalities in complement to classical behavior markers.

361

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369

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- 450

451 **Figure legends**

452

453 **Figure 1: Behavioral paradigms and data analysis periods in the three experimental conditions.** Odor
454 group: the animals received 10 presentations of an odor lasting 30s. Respiratory frequency was
455 analyzed 20s before odor onset (Pre-Odor period), 30s during odor (Odor period) and 20s after odor
456 offset (Post-Odor period). Paired group: the animals received 10 Odor-Shock pairings using a 30 s
457 Odor-Shock interval. Respiratory frequency was analyzed 20s before odor onset (Pre-Odor period), 30s
458 during odor (Odor period) and 20s after shock delivery (Post-Shock period). Unpaired group: the
459 animals received 10 presentations of the odor and the shock with a 180s-interval between the two
460 events. Respiratory frequency was analyzed 20s before odor onset (Pre-Odor period), 30s during odor
461 (Odor period) and 20s after shock delivery (Post-Shock period).

462

463 **Figure 2: Time course of the respiratory response to the first presentation of an odor across**
464 **development** A- Individual examples of raw respiratory signals at the three developmental ages in the
465 Odor groups. Following odor delivery, the respiratory frequency increases drastically at the three ages.
466 B- Respiratory frequency time course at the three developmental ages in the Odor group. The time
467 course of respiratory frequency is represented with a 1-s bin precision, from 20 s before odor onset
468 (black vertical line) to 20s after Odor offset (black dotted vertical line). C- Histograms representing the
469 average respiratory frequency during the Pre-Odor (white bars), Odor (plain color bars) and Post-Odor
470 periods (transparent color bars), per 20s time bins until the end of the trial. o: Significant difference
471 with Pre-Odor period; §: Significant difference with Adult at the same period; *: Significant difference
472 with Adult and Juvenile at the same period; $p < 0.05$.

473

474 **Figure 3: Sniffing response habituation to a neutral odor.** A- Respiratory frequency time course for
475 the first four trials (T1 to T4) of the session at the three developmental ages in the Odor groups (from
476 top to bottom: Adult, Juvenile and Infant). The time course of respiratory frequency is represented
477 with a 1-s bin precision, from 20 s before odor onset (black vertical line) to 20s after Odor offset (black
478 dotted vertical line). B- Histograms representing the average respiratory frequency during the Pre-
479 Odor, Odor and Post-Odor periods at the three developmental ages in the Odor groups. o: Significant
480 difference with Pre-Odor period; #: Significant difference with Odor period; $p < 0.05$.

481

482 **Figure 4: Evolution across trials of sniffing response to an odor in paired and unpaired conditions.** A-
483 Histograms representing the average respiratory frequency during the Pre-Odor, Odor and Post-Shock
484 periods for the 10 trials of the session, at the three developmental ages in the Paired group (from top
485 to bottom : Adult, Juvenile and Infant). B- Histograms representing the average respiratory frequency

486 during the Pre-Odor, Odor and Post-Shock periods for the 10 trials of the session, at the three
487 developmental ages in the Unpaired group. o: Significant difference with Pre-Odor period; #:
488 Significant difference with Odor period; $p < 0.05$.

489

490 **Figure 5: Mean respiratory frequency during paired and unpaired sessions.** A- Histograms
491 representing the average respiratory frequency during the Pre-Odor, Odor and Post-Shock periods
492 over the whole session, at the three developmental ages in the Paired groups. B- Histograms
493 representing the average respiratory frequency during the Pre-Odor, Odor and Post-Shock periods
494 over the whole session, at the three developmental ages in the Unpaired groups. *: Significant
495 difference with Adult at the same period; #: Significant difference with Juvenile at the same period; o
496 : Significant difference with Pre-Odor period at the same age; $p < 0.05$.

497 **Table legends:**

498

499 **Table 1:** Statistical results of the three factors ANOVA (Condition, Age, Period, Trial) carried out on the
500 data from the Odor groups presented on Figure 3B. Numbers in red signal significant values of p .

501

502 **Table 2:** Statistical results of the three factors ANOVA (Age, Period, Trial) carried out on the data from
503 the Paired (upper part) and Unpaired (lower part) groups presented on Figure 4A and 4B. Numbers in
504 red signal significant values of p .

Behavioral paradigm

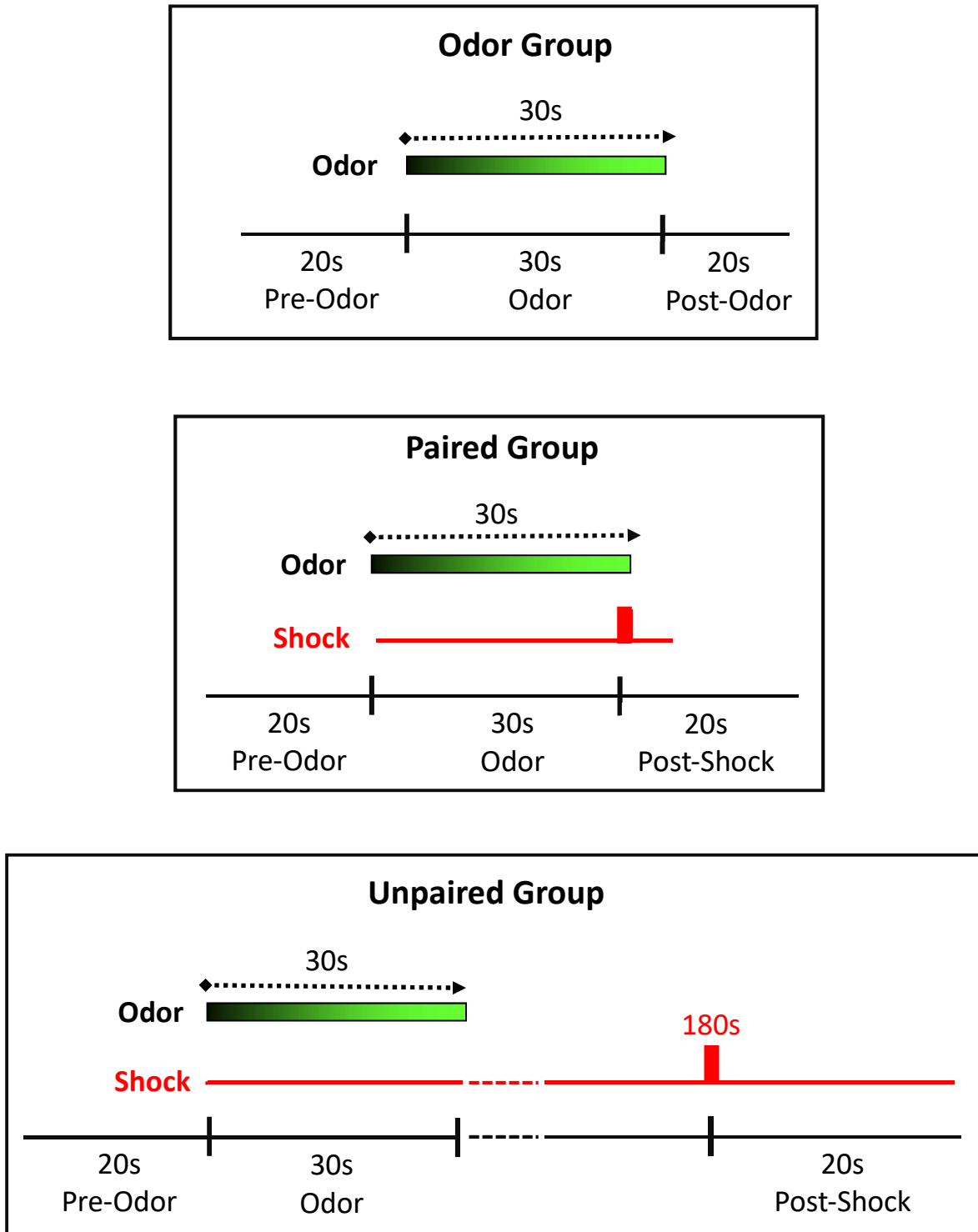
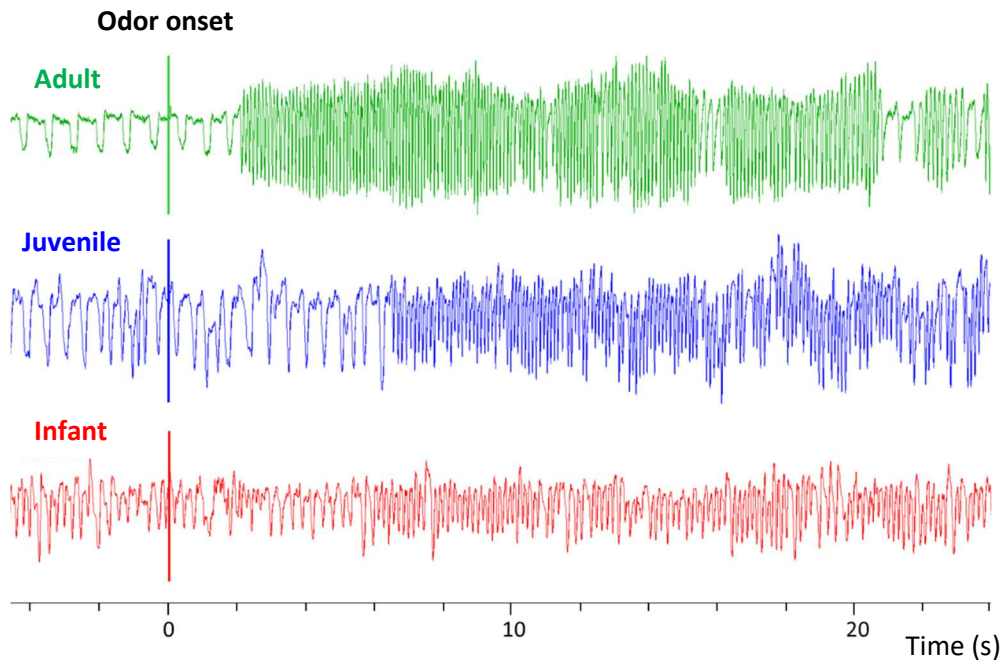
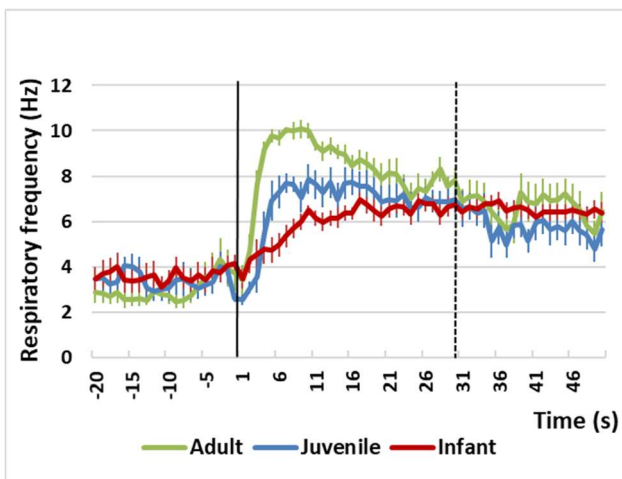


Figure 1

A



B



C

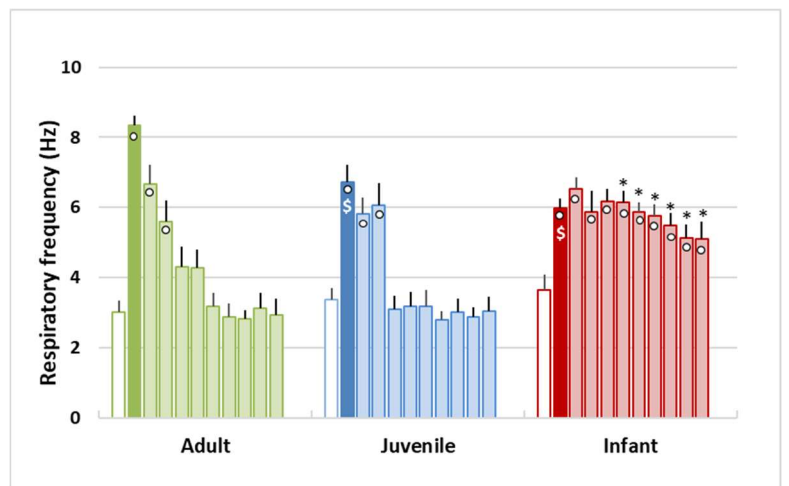


Figure 2

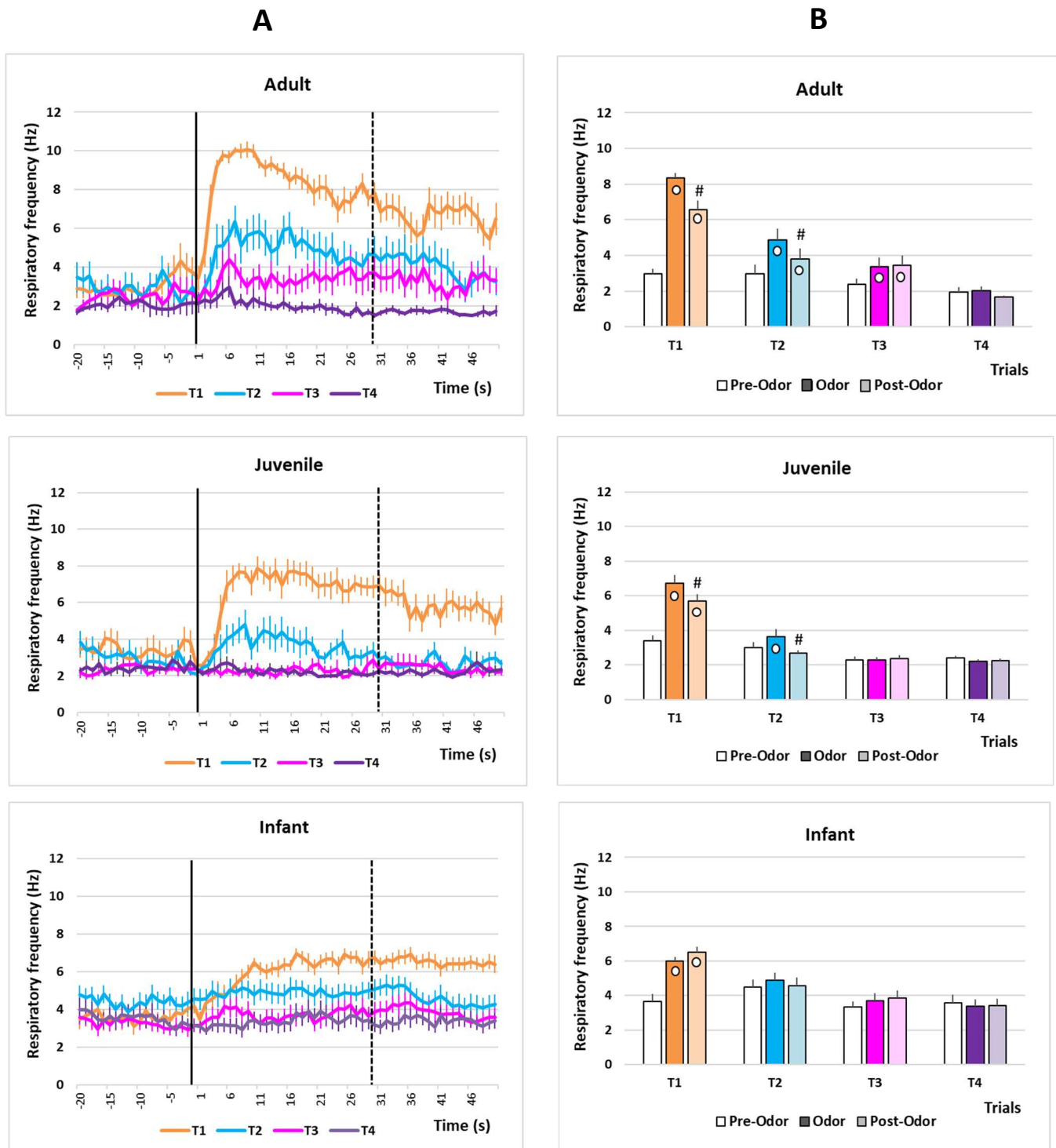


Figure 3

A- Paired group

B- Unpaired group

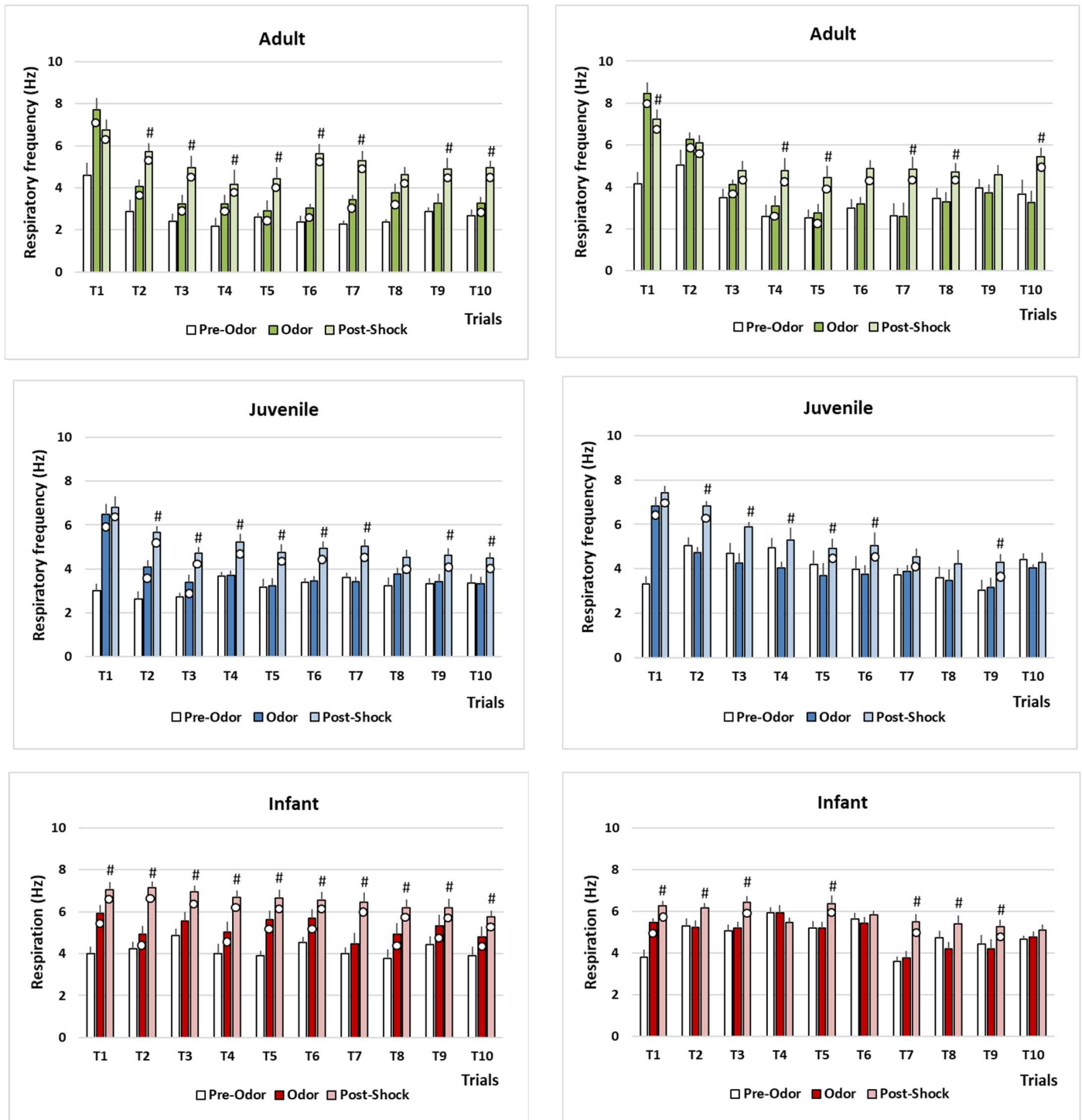
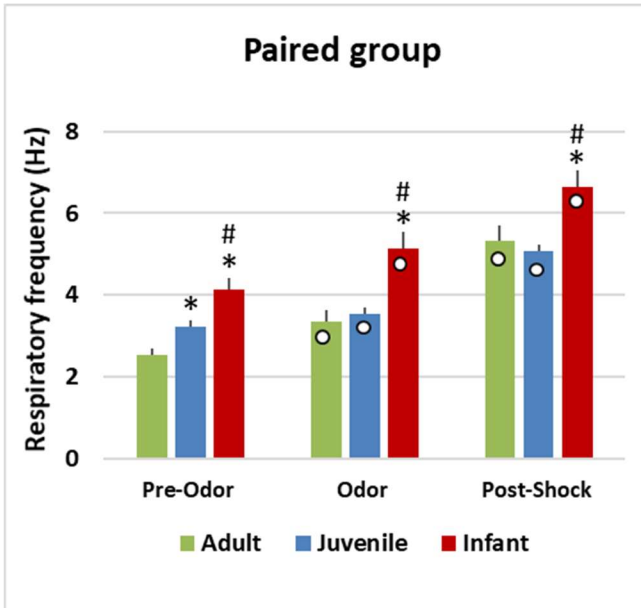


Figure 4

A



B

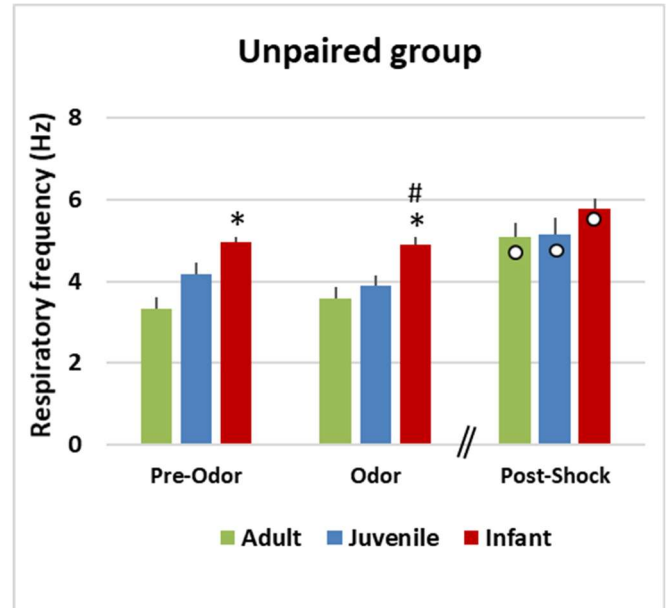


Figure 5

Odor group	Pre-Odor vs Odor vs Post-Odor	
Respiration	F	p
<i>Between subjects</i>		
Age	F(2,27)=4,396	0.022
<i>Within subjects</i>		
Period	F(2,54)=82.75	<0.001
Period*Age	F(4,54)=10.44	<0.001
<i>Within subjects</i>		
Trial	F(3,81)=68.41	<0.001
Trial*Age	F(6,81)=3.39	0.005
<i>Within subjects</i>		
Period*Trial	F(6,162)=45.28	<0.001
Period*Trial*Age	F(12,162)=2.43	0.006

Table 1

3 factors ANOVA		
Paired group	Pre-Odor vs Odor vs Post-Shock	
Respiration	F	p
<i>Between subjects</i>		
Age	F(2,33)=10.35	<0.001
<i>Within subjects</i>		
Period	F(2,66)=240.874	<0.001
Period*Age	F(4,66)=1.786	0.142
<i>Within subjects</i>		
Trial	F(9,297)=18.359	<0.001
Trial*Age	F(18,297)=4.673	<0.001
<i>Within subjects</i>		
Period*Trial	F(18,594)=7.93	<0.001
Period*Trial*Age	F(36,594)=2.541	<0.001
Unpaired group	Pre-Odor vs Odor vs Post-Shock	
Respiration	F	p
<i>Between subjects</i>		
Age	F(2,20)=3.41	0.053
<i>Within subjects</i>		
Period	(2,40)=148.056	0.001
Period*Age	F(4,40)=5.352	0.002
<i>Within subjects</i>		
Trial	F(9,180)=16.138	<0.001
Trial*Age	F(18,180)=4.938	<0.001
<i>Within subjects</i>		
Period*Trial	F(18,360)=10.126	<0.001
Period*Trial*Age	F(18,360)=2.844	<0.001

Table 2