The role of associative learning process on the response of fledgling great tits (Parus major) to mobbing calls

Mylène Dutour, Jean-Paul Léna, Adeline Dumet, Vanessa Gardette, Nathalie Mondy, Thierry Lengagne

1 **The role of associative learning process on the response of**

2 **fledgling great tits (*Parus major*) to mobbing calls**

3

4 **Mylène Dutour[1] · Jean-Paul Léna[1] · Adeline Dumet[1] · Vanessa Gardette[1] · Nathalie**

5 **Mondy[1] · Thierry Lengagne[1]**

6 [1] Université de Lyon, UMR5023 Ecologie des Hydrosystèmes Naturels et Anthropisés,

7 Université Lyon 1, ENTPE, CNRS, 6 rue Raphaël Dubois, 69622 Villeurbanne, France

8 **Author for correspondence:**

9 Mylène Dutour

10 mylene.dutour@hotmail.com

11

12   **Abstract**

13   When they detect a predator, many species emit antipredator vocalizations. In some cases,

14   they emit mobbing calls, which are associated with the caller approaching and harassing the

15   predator while attracting others to join it. Surprisingly, although mobbing has been widely

16   reported in adults of numerous species, there has been no test of the role of learning in

17   mobbing call recognition, especially during ontogeny. Here, we exposed wild great tit (*Parus*

18   *major*) nestlings to playbacks of an unthreatening, novel sound either associated with

19   conspecific mobbing calls (experimental treatment) or with another unthreatening novel

20   sound (control treatment). We then tested them as nestlings and fledglings to see how

21   responses to the novel sound compared to conspecific mobbing calls. Results revealed that

22   fledglings in the experimental treatment behaved similarly to conspecific mobbing calls and

23   the novel sound associated with conspecific mobbing calls. Because mobbing efficiency is

24   often linked to interspecific communication, associative learning should be used by

25   heterospecifics as a mobbing calls recognition mechanism. Regardless of treatment during the

26   nestling phase, fledglings always were sensitive to the playback of conspecific mobbing calls.

27   However, fledglings from the control group were more likely to approach the loudspeaker

28   than those from the experimental group when mobbing calls were played, suggesting that

29   overexposure during the nestling phase altered mobbing learning. Overall, these results

30   suggest that learning could play a role in the recognition of calls, like heterospecific mobbing

31   calls, when paired with conspecific mobbing, and that mobbing is perceived as a threatening

32   stimulus from a very young age.

33

34   **Key words** Alarm call · Associative learning · Birds · Communication · Mobbing

35

Introduction

In many species of small birds and mammals, two contrasted anti-predator strategies, each associated to a specific alarm call, can be observed according to predator dangerousness: prey may flee or mob the predator (Marler 1957). Mobbing behaviour is usually exhibited when predators do not represent an immediate threat and is characterized by harassment or even attacks on the predator as well as vocalizations (i.e., mobbing calls) that quickly gather neighboring conspecifics and other prey species in the mob (Hartley 1950; Curio 1978). Both the evaluation of the threat associated with the predator and the recognition of mobbing calls, including heterospecific calls, are required to make mobbing behaviour an efficient anti-predator strategy.

Associative learning is a behavioural modification following reinforcement, based on associations between two stimuli, responses and events (Shettleworth 1998; Griffin et al. 2015). This process is essential to the development of vocalizations and several behaviours such as those involved in predator avoidance (Ferrari and Chivers 2011). Animals can learn to flee after associating an unfamiliar sound (i.e., novel sound) with (i) the appearance of a model predator (birds: Magrath et al. 2015a; mammals: Shriner 1999; Wheeler et al. 2019) or (ii) the hearing of alarm calls, without having to see the callers or a predator (Potvin et al. 2018). In mobbing behaviour, there is ample evidence of a process of associative social learning between conspecific mobbing calls and predators (Curio et al. 1978a; Maloney and McLean 1995; McLean et al. 1995; Griffin and Galef 2005; McIvor et al. 2018) but in all cases the training phase was based on visual cues; individuals were trained about predators through pairing model predator presentations with a training stimulus (i.e., live conspecific demonstrator engaging in mobbing, mobbing calls paired with mounts of conspecifics, or mobbing calls alone). However, visual associative learning needs the inexperienced learner to

61 be in the vicinity of the mobbing scene, so that the opportunities for learning while reducing

62 the risk of threat may be limited, especially for species living in dense vegetation. This

63 challenge is overcome in the case of acoustic-acoustic association, which may be efficient

64 even when the tutor and the threat are difficult to observe, possibly resulting in the rapid

65 spread of mobbing call recognition in natural communities.

66   Mobbing calls are usually characterized by loud and repetitive vocalizations, both

67 features that should facilitate associative learning, as there is ample opportunity to hear

68 mobbing calls while observing the threat, with less likelihood of immediate danger (Magrath

69 et al. 2015b). In birds, such association between a predator and the expression of a mobbing

70 behaviour could be achieved after young fledge leave their nest. In this case, young fledglings

71 can learn from their parents and several observations are in agreement with this statement

72 (Curio et al. 1978b; Griesser and Suzuki 2016). If nestlings however are able to associate

73 several acoustic signals from their nest cavities (i.e., they are able to associate the mobbing

74 calls emitted by parents with other vocalizations prevailing on a mobbing scene) they should

75 also be able to respond to mobbing calls appropriately as soon as they leave the nest. To our

76 knowledge, this capacity has never been investigated before. Indeed, until now, associative

77 learning has been examined using the association of several sensory channels, especially

78 visual and acoustic (e.g. Maloney and McLean 1995; McIvor et al. 2018). However, as

79 outlined above, young could also associate unknown vocalizations with conspecific mobbing

80 calls if both are simultaneously emitted in a mobbing scene. Such a mechanism could be

81 particularly relevant in learning to recognize heterospecific mobbing calls.

82   The great tit (*Parus major*) is a well-suited species to study the ontogeny of mobbing

83 behaviour in the wild. It is well known for its learning abilities (Exnerová et al. 2006; Cole et

84 al. 2011; Morand-Ferron et al. 2015) and its tendency to exhibit mobbing behaviour and emit

85 mobbing calls when confronted with a predator (Lind et al. 2005; Dutour et al. 2016; Carlson

86 et al. 2017a; Kalb et al. 2019). Furthermore, this altricial species provides a good system for

87 studying whether naïve nestlings are able to associate mobbing calls with unfamiliar stimuli in

88 the absence of any visual cues from adults or predators in the nest cavity.

89   The goal of this study was to determine whether associative learning might serve as a

90 mechanism for the development of mobbing calls recognition for free-living animals.

91 Specifically, we investigated whether experiencing a novel stimulus associated with

92 conspecific mobbing calls during the nestling period led great tit nestlings to associate this

93 novel sound with mobbing during the fledgling period. We designed playback experiments in

94 two groups: (i) an experimental group in which we trained individuals by broadcasting a

95 novel sound (i.e., associative learning stimulus) with mobbing calls (i.e., functional stimulus)

96 and (ii) a control group in which the associative learning stimulus was broadcast with another

97 novel sound (hereafter control stimulus).

98   In line with previous work showing that nestlings cease begging when they hear

99 playbacks of their parents' alarm calls (Davies et al. 2004; Hollén and Radford 2009; Barati

100 and McDonald 2017), we predict that at the end of the learning period, only nestlings

101 belonging to the experimental group should cease begging when they hear the associative

102 learning stimulus. We also measured responses at the fledgling stage after the playback of the

103 associative learning stimulus. We predict that the playback of the associative learning

104 stimulus should elicit a stronger mobbing response from the experimental group compared to

105 the control group (i.e., increases in scanning, calling and moving) because the experimental

106 group should associate the unfamiliar sound with mobbing and respond to the unfamiliar

107 sound as threatening, while the control group should not. Because it was important to ensure

108 that fledglings were sensitive to mobbing, we also tested whether they responded to the

109 playback of conspecific mobbing calls.

110

Material and methods

Study sites and species

We chose great tits, a common territorial passerine which breeds in secondary holes and wooden artificial nest boxes throughout Europe and parts of Asia and North Africa (Perrins 1965). The study was conducted in the Pierre Vérots Foundation, a private natural reserve located in the south-east France (4°91'E, 45°95'N). The study area covers approximately 150 ha and contains 120 nest boxes separated at minimum by 50 m and mainly occupied by great tits. This distance corresponds to the minimum distance separating tit territories during the breeding season in our study area and is far enough to ensure that the playbacks are not heard by neighbours (at 50 meters, the amplitude levels fluctuate from 33 to 39 dB, obtained from the loudspeaker, Solo 01dB Metravib, Z weighting, re: 20 µ m Pa; these levels are lower than the natural background noise levels in forest areas, fluctuate from 35 to 45 dB in quiet situations, 52 to 70 dB in noisy situations). The study took place during the breeding season (April-July 2017). We visited nest boxes at least every two days from the beginning of the breeding season onwards to determine the laying and hatching dates. When nestlings were 8 days old, they were ringed with a unique combination of color bands for individual identification. Nestlings spent 19.3 ± 1.4 days in the nest. Nestlings from the same nest all hatched on the same day and subsequently also fledged on the same day.

Experimental procedures: presentation of stimuli

The aim of our experiment was to assess whether chicks were able to associate a novel stimulus with conspecific mobbing calls. In 120 nest boxes, 31 were unoccupied, 14 occupied

136   by the blue tit (*Cyanistes caeruleus*) and one was occupied by the willow tit (*Poecile*

137   *montanus*). Each of the remaining 74 nest boxes occupied by great tits was randomly assigned

138   to one of the two treatments (balanced for laying date to ensure equal representation of both

139   treatment groups over the breeding season). In the first treatment (38 nests), the associative

140   learning stimulus was associated with conspecific mobbing calls (hereafter experimental

141   group) while in the second treatment (36 nests) the associative learning stimulus was

142   associated with unfamiliar stimulus (control sequence, hereafter control group). Behavioural

143   tests were conducted both during the development of nestlings and the fledging day.

144   Playbacks were always broadcasted once adults were silent and away from the nest (at least

145   15 m) or from the focal fledgling (at least 5 m). If parents responded to playback during tests,

146   we stopped the playback and restarted at least 30min after, but such cases were rare (~5% of

147   total tests).

148

149   **Choice of experimental stimuli and playback materials**

150

151   Three kinds of stimuli were used during playback experiments. First, we used mobbing calls

152   produced by four great tits in response to the Eurasian pygmy owl (*Glaucidium passerinum*)

153   and to conspecific mobbing calls. Calls were recorded with a Fostex FR2LE digital recorder

154   connected to a Sennheiser ME67-K6P microphone. We also used mobbing calls produced by

155   ten great tits obtained from the Xeno Canto online database (http://www.xeno-canto.org) (see

156   online supplement, Appendix 1). For the associative learning and the unfamiliar stimulus, we

157   used two musical instrument sounds (clarinet and trumpet sequences) obtained from an online

158   sound bank (http://www.universal-soundbank.com) to ensure the stimuli used had no

159   biological value and were unfamiliar. In order to ensure that control sounds were unfamiliar,

160   we first conduced a pilot study to assess the response of adult great tits (*n* = 9). None of them

161   approached and mobbed the loudspeaker. We matched the frequency characteristics and

162   temporal structure of the associative learning and the unfamiliar stimulus to obtain stimuli of

163   the same duration and the same frequency bandwidth using Avisoft-SASLab Pro. For the

164   experimental treatment, we built playbacks containing the associative learning stimulus (i.e.,

165   clarinet) with mobbing calls. For the control treatment, the associative learning stimulus was

166   associated with the unfamiliar stimulus (i.e., trumpet) (spectrograms of mobbing calls,

167   associative learning stimulus and unfamiliar stimulus are available in supplementary material

168   Appendix 2 Fig. A2). Each playback track consisted of 10 seconds of associative learning

169   stimulus followed by 10 seconds of the mobbing calls or the unfamiliar signal, and this

170   sequence was repeated three times in order to reach one-minute in total, time used in a recent

171   study conducted on nestling passerines in response to calls (Wheatcroft 2015). In order to

172   avoid habituation to playbacks, we constructed 21 one-minute playback samples for each

173   group and each sample was used only once (i.e., as nestlings spent 19.3 ± 1.4 days in the nest,

174   we used one stimulus by day) (Hurlbert 1984; Kroodsma et al. 2001). Because we used

175   mobbing calls produced by 14 great tits in total to construct 21 playback sequences, some

176   soundtracks are therefore only imperfectly independent from other ones, but the risk of

177   pseudoreplication was clearly minimized. We also built two additional 30-second playback

178   samples containing associative learning stimulus or mobbing calls to test the response of

179   nestlings and fledglings. We decided to keep the test duration shorter than was usual in

180   previous studies conducted on adults' great tit (including our own Dutour et al. 2017b; see

181   also Suzuki et al. 2012) in order to limit the risk of interference from parents during tests. The

182   average amplitude used for these playbacks was obtained with a sound level meter placed 1 m

183   from the loudspeaker (83.9 ± 3.51 dB, mean ± SD, Solo 01dB Metravib, Z weighting, re: 20 μ

184   m Pa). We broadcast sound tracks using a Shopinnov 20 W loudspeaker (frequency response

185   100 Hz -15 kHz).

186

Behavioural responses of nestlings

188

Begging calls suppression is described as a good measure of whether nestlings interpret

sounds as cues of danger (Magrath et al. 2010). To determine whether playback treatment

(i.e., associative learning stimulus with unfamiliar stimulus or associative learning stimulus

with mobbing calls) influenced nestling calling activity, every day, between 7am and 1pm,

and between hatching day and fledging day, we measured nestling production of begging calls

within the nest box during 30 seconds (begging call = 1, no begging call = 0) before and after

broadcasting the treatment, 1 minute being time by which passerine nestlings' behaviour

return to normal (Wheatcroft 2015). To measure this behaviour, one observer was placed ~

50 cm from each nest to listen to the calls of the nestlings, which were clearly heard at short

distance. Although there were multiple observers ($n = 8$) to measure begging call propensity,

we did not detect an observer effect (GLMM: $P > 0.05$). Additionally, at the end of the

nestling phase, between 19 days post hatch and fledging day, begging call propensity was

measured before and after playback of the associative learning stimulus. To create as natural a

situation as possible, the loudspeaker was placed on the top of the nest box for all trials. The

average amplitude used for the playback was obtained with a sound level meter placed in the

nest boxes (67.98 ± 0.94 dB, mean $\pm$ SD, Solo 01dB Metravib Z weighting, re: 20 μ m Pa).

205

Behavioural responses of fledglings

207

To test whether associative learning is involved in the development of mobbing calls

recognition, we studied fledglings' responses to the playback of either the associative learning

stimulus alone (control: $n = 30$ from 22 nests; experimental: $n = 31$ from 19 nests) or the

211   conspecific mobbing calls (control: n = 19 from 14 nests; experimental: n = 26 from 16 nests).

212   Tests were performed during the day of fledging (except 4 tests conducted the second day

213   after fledging corresponding to 3.8% of total tests). Each test was conducted by two field

214   assistants. One was assigned to the soundtrack preparation and playback operation, while the

215   other was kept unaware of the selected soundtrack (although they could hear it) and assigned

216   to the observation of the focal bird. In all experiments and before the playback, the identity of

217   the focal fledgling was determined using the unique leg color bands fitted to each fledgling

218   using binoculars. Neither of the two field assistants knew whether the fledging was in the

219   control or experimental group. Once a focal fledging was identified, the loudspeaker used to

220   broadcast the acoustic stimulus was placed ∼ 20 m from the fledgling at the base of a tree at

221   1.5 meter high. All observations were done using binoculars. Observations were made ∼10 m

222   from the loudspeaker and the focal fledging, a distance from which the tits' behaviour was not

223   disturbed. Trials were conducted mostly around 12am (range 8am - 5pm) to ensure good light,

224   in calm and dry weather. Playbacks were started when no bird was visible close to the speaker

225   and no bird was calling. To test fledglings' responses, we recorded the following behavioural

226   variables during the 30 s of playbacks: (i) number of horizontal scans (i.e., the number of

227   movements that birds made with their heads from left to right or right to left), (ii) calling (i.e.,

228   producing any mobbing vocalizations; call propensity: mobbing vocalization = 1; no mobbing

229   vocalization = 0), (iii) approaching the loudspeaker (i.e., we recorded whether birds

230   approached within 15 m of the loudspeaker during playback; see Dutour et al. 2017b for more

231   details), and (iv) fleeing (i.e., we recorded whether birds moved away more than 10 m from

232   their initial position). We measured these behaviours because they are good indicators of

233   perceived danger in birds (Curio et al. 1978a) and are common during mobbing events

234   (Suzuki et al. 2016; Carlson et al. 2017a; Carlson et al. 2017b; Dutour et al. 2017b; Suzuki et

235   al. 2017). There was 10-minute gap between playback of the associative learning stimulus and

236   playback of mobbing calls.

237

238   **Data analysis**

239

240   Analyses were done using SAS 9.4 (SAS Institute Inc., Cary, NC, U.S.A.) and R v.2.15.1

241   softwares (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-

242   project.org). We first examined whether begging call propensity varied between treatment

243   groups during the nestling period. For this purpose, we analyzed call propensity recorded each

244   day before broadcasting the treatment using a logistic linear mixed model (LLMM) for which

245   the call propensity was treated as a repeated dependent binary variable. To this aim, nest was

246   considered as a random effect and the correlation between successive observations recorded at

247   the same nest was specified using an autoregressive covariance structure. The treatment

248   group, the time elapsed since hatch (i.e., nestlings age) as well as their interactive effect, were

249   introduced as fixed effects. The significance of each fixed effect was tested using a non-

250   sequential F test and the Kenward-Roger method was used to approximate the denominator

251   degrees of freedom (SAS 9.4). Non-significant terms were successively dropped to obtain the

252   final model. We used a similar model framework to compare the propensity of nestlings to

253   stop begging after the onset of the broadcasting treatment according to the treatment group

254   and the time elapsed since hatching. For this purpose, all observations for which nestlings

255   were not calling before the onset of the playback treatment were discarded from the analysis.

256   We used a Fisher exact test to test whether the begging calls propensity just after the playback

257   of the associative learning stimulus only when nestlings were between 19 days post hatch and

258   fledging day varied according to the group (experimental or control).

259   To investigate the behavioural responses of fledglings, we first examined whether the

260   number of horizontal scans varied between treatment groups and playback during the

261   fledgling period ($n = 106$). For this purpose, we ran generalized linear mixed models

262   (GLMM) which included the treatment group, the playback (associative learning stimulus or

263   mobbing calls) and their interactive effect as a fixed terms and nest as a random term. We

264   used a negative binomial error distribution and log-link function for this analysis (*glmer.nb* in

265   the package *lme4*; Bates et al. 2014). To investigate the call propensity, we used a binomial

266   error distribution and logit-link function (*glmer* in the package *lme4*; Bates et al., 2014)

267   (calling response = 1; no calling response = 0). As above, nests were included in the model as

268   a random effect. The treatment group, the playback and their interactive effect were

269   introduced as fixed effects. For both scanning and call propensity, we further conducted

270   pairwise comparisons (*glht* in the package *multcomp*). Finally, because approaching and

271   fleeing behaviours (two behaviours linked to movements) were opposed to immobility

272   behaviour (see online supplement Appendix 3, Fig. A3), all approaching and fleeing

273   individuals were regrouped within a unique category (hereafter moving individuals) and

274   compared to the individuals who stayed still. To compare the propensity of fledglings to move

275   *vs* individuals who stayed still according to treatment group and playback, we used a binomial

276   error distribution and logit-link function. We then focused our analysis on moving individuals

277   only (i.e., fleeing and approaching individuals; $n = 23$). Because only a small number of

278   fledglings moved, Fisher's exact test was used to investigate the variation in mobbing

279   propensity (i.e., approach) according to the treatment group in response to the mobbing call

280   playbacks ($n = 17$). Because only one of the control fledglings moved when exposed to the

281   playback of the associative learning stimulus, we discarded this group and we used a Fisher's

282   exact test to investigate the variation in mobbing propensity of individuals from the

283   experimental group between the two playbacks ($n = 16$).

284

285 **Data availability** The datasets generated during the current study are available from the

286 corresponding author on request.

287

288 # Results

289

290 **Behavioural responses of nestlings**

291

292 The calling behaviour before the broadcasting treatment did not significantly vary in function

293 to the time elapsed since hatching up to fledging but it was significantly higher for the

294 experimental group than the control group ($n = 1420$, treatment effect: $F_{1,341} = 4.17$; $P = 0.042$

295 ; time effect: $F_{1,426} = 0.66$; $P = 0.417$; interaction: $F_{1,425} = 0.40$; $P = 0.529$). The propensity to

296 stop calling after the onset of the broadcasting treatment significantly increased in function to

297 the time elapsed since hatching but did not vary between treatment groups although call

298 suppression tended to increase with time slightly more for the control group than for the

299 experimental group ($n = 353$, treatment effect: $F_{1,156} = 0.11$; $P = 0.745$; time effect: $F_{1,176.} =$

300 46.19; $p < 0.0001$; interaction: $F_{1,170} = 3.69$; $P = 0.057$). We did not detect a treatment group

301 effect on the nestlings responses to the playback of the associative learning stimulus ($n = 11$;

302 $P = 0.608$).

303

304 **Behavioural responses of fledglings**

305

306 Scanning was significantly affected by the interaction between the stimuli that were broadcast

307 (associative learning stimulus or mobbing calls) and the treatment group (experimental and

308 control) (Table 1; Fig. 1). In response to mobbing calls playbacks, the rate of horizontal scans

309 did not differ between the two groups ($P = 0.99$; Fig. 1). It was higher during playback of

310 mobbing calls than during playback of the associative learning stimulus for the control group

311 ($P = 0.004$; Fig. 1) while it did not differ for the experimental group ($P = 0.99$; Fig. 1).

312 Similar variations were found for call propensity as indicated by a significant interaction

313 effect between group and playback (Table 1; Fig. 2), although these variations were not

314 statistically different between both groups in response to the associative learning stimulus

315 playback ($P = 0.95$; proportion of calling: associative learning = 22.5%; control = 6.7%).

316 Furthermore, there was a significant effect of playback on the probability of moving (i.e.,

317 approaching and flight behaviours), whereas group had no significant effect (Table 1; Fig. 3).

318 In response to mobbing calls, fledglings were more likely to move than in response to the

319 associative learning stimulus playback. Results indicate that (i) in response to the mobbing

320 call playbacks, individuals from the control group were more prone to approach the

321 loudspeaker than experimental individuals ($n = 17$; Fisher test: $P = 0.049$; Fig. 3) (ii)

322 approaching behaviour of individuals from the experimental group did not differ between the

323 two playbacks ($n = 16$; Fisher test: $P = 0.999$; Fig. 3).

324

325 **Discussion**

326

327 We have investigated whether nestlings submitted to a novel stimulus associated with

328 mobbing calls enabled them to recognize this stimulus as an indicator of threat proximity

329 when becoming fledglings. This experiment, involving classical conditioning, showed that

330 fledglings learned to associate a novel stimuli with mobbing calls and exhibited vigilance

331 behaviours in response to the novel stimuli whereas they do not as nestlings.

332

333 **Effect of the associative learning during the nestling phase**

334

335 Several studies have shown that nestlings become silent when they hear playbacks of their

336 parents' alarm calls (Davies et al. 2004; Platzen and Magrath 2004; Madden et al. 2005; Haff

337 and Magrath 2012; Barati and McDonald 2017). However, most of these studies have

338 compared alarm calls to background noise or sympatric species stimuli (i.e., stimuli known).

339 In the present study, nestlings suppressed calling when submitted to associative learning

340 stimulus equally to mobbing call playbacks, suggesting that novelty (i.e., a stimulus never

341 heard before) alone may be used as a signal of danger (Schaller and Emlen 1961; Curio et al.

342 1978b). We found that, contrary to young nestlings, older nestlings reduced begging calls in

343 response to the associative learning stimulus and mobbing calls, suggesting that older

344 nestlings are less prone to recognition errors than younger ones (Davies and Brooke 1988;

345 Davies et al. 2004). This pattern of gradual acquisition is in accordance with previous studies

346 conducted on nestling great tits (Rydén 1978). We did not detect a group effect on nestling'

347 responses to playbacks of the associative learning stimulus at the end of the learning period,

348 but the low sample size may have reduced our ability to detect a significant pattern. A detailed

349 study of nestlings' behaviour would be insightful to actually address this question (e.g., if they

350 crouched down inside their nest cavity, suppressed their movements, increase their heart rate

351 and electromyographic activity after parental alarm calls; Rydén 1978; Ryden 1980; Suzuki

352 2011). Finally, another interesting aspect is related to the sound degradation. As we used hole

353 nesters, a sound degradation may occur that a sound will appear different within and outside

354 the nest box. However, quite low-frequency sounds have been used in this study, with

355 probably low alteration, and the sounds were broadcast using a loudspeaker placed on the top

356 of the nest box for all trials, with the only obstacle being the wall thickness of the nest box.

357 Future work could investigate which sound characteristics of the parent calls could be

358 perceived by offspring in their nest according to their nestling conditions (hole nesters vs

359 open nesters).

360

361 **Mobbing calls recognition and associative learning in fledglings**

362

363 Fledglings exhibited a higher rate of scanning when hearing the associative learning stimulus

364 but only if it was associated with mobbing calls during the nestling phase. A similar trend was

365 also observed for calling activity and the propensity to move. Together, these results indicate

366 that individuals associated the artificial sound with conspecific mobbing calls without having

367 to see the mobbing scene. Scanning rate is a good proxy of vigilance effort (Lendrem 1983;

368 Huang et al. 2012; Creel et al. 2014) suggesting that chicks actually perceived the associative

369 learning stimulus as an indicator of threat proximity only if it was associated with mobbing

370 calls during the learning phase. Whether fledglings associated the artificial stimulus as the

371 source of threat itself or as heterospecific mobbing calls (indicating the presence of a threat)

372 and whether the ontogeny of mobbing behaviour only relies on learning processes, remain

373 open questions. Positive correlation between the rate of response development and the

374 magnitude of exposure to heterospecific alarm calls suggest a learning process. For instance,

375 infant vervet monkeys (*Cercopithecus aethiops pygerythrus*) develop responses to superb

376 starling (*Lamprotornis superbus*) mobbing calls more quickly on territories where these birds

377 are common, suggesting that these young have more opportunities to learn about the calls than

378 young on territories where starlings are less common (Hauser 1988). Concerning adults, they

379 can associate novel sounds with a chorus of conspecific and heterospecific aerial alarm calls

380 (Potvin et al. 2018). One may thus expect that a learning process where individuals associate

381 the threat and/or heterospecific mobbing calls with the conspecific mobbing calls can operate

382 in the wild. Such mechanisms could make the set of recognized heterospecific mobbing calls

383   adjusted to the local composition of prey communities, which is also congruent with field

384   studies reporting local variations of the rate of responses towards heterospecific mobbing calls

385   (Wheatcroft and Price 2013).

386   In addition to such associative learning processes, our study also reveals that

387   fledglings responded to conspecific mobbing calls despite the treatment they experienced.

388   Indeed, in both groups, hearing conspecific mobbing calls resulted in increased vigilance, a

389   higher proneness to call as well as to move. Since all these tests were performed on fledging

390   day, we can safely assume that no fledgling could have had the opportunity to observe a

391   mobbing scene before the tests. Our results therefore indicate that fledglings do not need to

392   observe mobbing events to respond to mobbing calls, suggesting that conspecific mobbing

393   calls are inherently perceived as indicating a threat. However, two hypotheses, not mutually

394   exclusive, could explain the responses to mobbing calls: the response (1) is at least partly

395   innate, this result has been found in adult great tits in response to allopatric mobbing calls

396   (Randler 2012, Dutour et al. 2017b), or (2) is due to the impregnation of acoustic signals

397   heard during the nestling phase. Mobbing calls are composed of frequency modulated

398   elements, which are involved in vigilance, and D notes involved in foraging flocks or to

399   recruit partners during the breeding season, to which receivers respond by approaching the

400   caller (Dutour et al. 2019). In the present study, tests were performed during fledgling when

401   great tits form family groups and often use D notes to maintain group cohesion (Dutour, pers.

402   observation). The responses of fledglings could be related to the context (i.e., presence of

403   family members in the vicinity), or could be due to sensitivity to the D notes. Furthermore,

404   contrary to fledglings belonging to the control group, those belonging to the experimental

405   group were more prone to flee than approach the loudspeaker. It could be from the playbacks

406   which were always presented to fledglings in the same order (i.e., associative learning sound,

407   then mobbing calls), creating biases that differed between treatments. Indeed, this could be a

408   carry-over from the experimental group hearing the unfamiliar sound, which is threatening to

409   them, before mobbing calls, while the control group heard a non-threatening sound and then

410   the mobbing calls. The situation seemed more dangerous for the experimental group if the two

411   cues had an additive effect. However, this result suggests that reinforcement with mobbing

412   calls during the nestling phase altered mobbing learning. Such impairment could be either due

413   to call overexposure, as previously shown in vocalization learning (Tchernichovski et al.

414   1999; Brainard and Doupe 2013), or because of the absence of a threat associated with the

415   mobbing call during exposure. An alternative explanation is that maybe fleeing is the adaptive

416   response to mobbing calls for young juveniles. This should be addressed in further studies. It

417   would be feasible in relatively resident birds like great tits where dispersal distances are small.

418   Flee would not be an impairment when expressed by a young, inexperienced, and physically

419   clumsy individual compared with more experienced, agile and older juveniles (~ 120 days

420   post fledging) (Kullberg & Lind, 2002) or adults. In that case, being exposed to additional

421   mobbing via playbacks may have sped up the development of this response.

422

423   **Conclusion**

424   This study shows that nestling great tits do not discriminate between two different acoustic

425   signals but demonstrates that they associate mobbing calls with a novel sound when exiting

426   the nest cavity as fledglings. Nevertheless, fledglings did not respond with typical mobbing

427   behaviour (i.e., approach and harass), which likely require experience of parental visual

428   response (i.e., cultural transmission) (Kullberg and Lind 2002). The present findings raise

429   questions about how nestlings extract information about the nature of predators and how

430   finely they can discriminate between different acoustic stimuli.

431

References

Barati A, McDonald PG (2017) Nestlings reduce their predation risk by attending to predator-information encoded within conspecific alarm calls. Sci Reps 7:11736. https://doi.org/10.1038/s41598-017-11528-y

Bates D, Maechler M, Bolker B, Walker S (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-7. http:// CRAN.R-project.org/package=lme4

Brainard MS, Doupe AJ (2013) Translating birdsong: songbirds as a model for basic and applied medical research. Annu Rev Neurosci 36:489–517. https://doi.org/10.1146/annurev-neuro-060909-152826

Carlson NV, Healy SD, Templeton CN (2017a) A comparative study of how British tits encode predator threat in their mobbing calls. Anim Behav 125:77–92. https://doi.org/10.1016/j.anbehav.2017.01.011

Carlson NV, Pargeter HM, Templeton CN (2017b) Sparrowhawk movement, calling, and presence of dead conspecifics differentially impact blue tit (*Cyanistes caeruleus*) vocal and behavioral mobbing responses. Behav Ecol Sociol 71:133. https://doi.org/10.1007/s00265-017-2361-x

Cole EF, Cram DL, Quinn JL (2011) Individual variation in spontaneous problem-solving performance among wild great tits. Anim Behav 81:491–498. https://doi.org/10.1016/j.anbehav.2010.11.025

Creel S, Schuette P, Christianson D (2014) Effects of predation risk on group size, vigilance, and foraging behavior in an African ungulate community. Behav Ecol 25:773–784. https://doi.org/10.1093/beheco/aru050

Curio E (1978) The adaptive significance of avian mobbing. I. Teleonomic hypotheses and predictions. Z Tierpsychol 48:175–183. https://doi.org/10.1111/j.1439-0310.1978.tb00254.x

457   Curio E, Ernst U, Vieth W (1978a) Cultural transmission of enemy recognition: one function

458   of mobbing. Science 202:899–901. https://doi.org/10.1111/j.1439-0310.1978.tb00255.x

459   Curio E, Ernst U, Vieth W (1978b) The adaptive significance of avian mobbing. II. Cultural

460   transmission of enemy recognition in blackbirds: effectiveness and some constraints. Z

461   Tierpsychol 48:184–202. https://doi.org/10.1126/science.202.4370.899

462   Davies NB, Brooke MD (1988) Cuckoos versus reed warblers: adaptations and

463   counteradaptations. Anim Behav 36:262–284. https://doi.org/10.1016/S0003-

464   3472(88)80269-0

465   Davies NB, Madden JR, Butchart SHM (2004) Learning fine-tunes a specific response of

466   nestlings to the parental alarm calls of their own species. Proc R Soc Lond B Biol Sci

467   271:2297–2304. https://doi.org/10.1098/rspb.2004.2835

468   Dutour M, Lena JP, Lengagne T (2016) Mobbing behaviour varies according to predator

469   dangerousness and occurrence. Anim Behav 119:119–124. http://

470   dx.doi.org/10.1016/j.anbehav.2016.06.024

471   Dutour M, Lena JP, Lengagne T (2017a) Mobbing behaviour in a passerine community

472   increases with prevalence in predator diet. Ibis 159:324–330.

473   http://dx.doi.org/10.1111/ibi.12461

474   Dutour M, Léna JP, Lengagne T (2017b) Mobbing calls: a signal transcending species

475   boundaries. Anim Behav 131:3–11. https://doi.org/10.1016/j.anbehav.2017.07.004

476   Dutour M, Lengagne T, Léna JP (2019) Syntax manipulation changes perception of mobbing

477   call sequences across passerine species. Ethology. https://doi.org/10.1111/eth.12915

478   Exnerová A, Štys P, Fučíková E, Veselá S, Svádová K, Prokopová M, ... Landová E (2006)

479   Avoidance of aposematic prey in European tits (Paridae): learned or innate?. Behav

480   Ecol 18:148–156. https://doi.org/10.1093/beheco/arl061

481   Ferrari MCO, Chivers DP (2011) Learning about non-predators and safe places: the forgotten

482   elements of risk assessment. Anim Cogn 14:309–316. https://doi.org/10.1007/s10071-

483   010-0363-4 PMID: 21203793

484   Griesser M, Suzuki TN (2016) Kinship modulates the attention of naïve individuals to the

485   mobbing behaviour of role models. Anim Behav 112:83–91.

486   https://doi.org/10.1016/j.anbehav.2015.11.020

487   Griffin AS, Galef Jr BG (2005). Social learning about predators: does timing matter?. Anim

488   Behav 69:669–678.

489   Griffin AS, Guillette LM, Healy SD (2015) Cognition and personality: an analysis of an

490   emerging field. Trends Ecol Evol 30:207–214. https://doi.org/10.1016/j.tree.2015.01.012

491   Haff TM, Magrath RD (2012) Learning to listen? Nestling response to heterospecific alarm

492   calls. Anim Behav 84:1401–1410. https://doi.org/10.1016/j.anbehav.2012.09.005

493   Hartley PHT (1950) An experimental analysis of interspecific recognition. Symp Soc Exp

494   Biol 4:313–336

495   Hauser MD (1988) How infant vervet monkeys learn to recognize starling alarm calls: the

496   role of experience. Behaviour 105:187–201. https://doi.org/10.1163/156853988X00016

497   Hollen LI, Radford AN (2009) The development of alarm call behaviour in mammals and

498   birds. Anim Behav 78:791–800. https://doi.org/10.1016/j.anbehav.2009.07.021

499   Huang P, Sieving KE, St Mary CM (2012) Heterospecific information about predation risk

500   influences exploratory behavior. Behav Ecol 23:463–472.

501   https://doi.org/10.1093/beheco/arr212

502   Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol

503   Mono 54:187–211. https://doi.org/10.2307/1942661

504 Kalb N, Anger F, Randler C (2019) Subtle variations in mobbing calls are predator-specific in

505   great tits (*Parus major*). Sci Rep-Uk 9:6572. https://doi.org/10.1038/s41598-019-

506   43087-9

507 Kroodsma DE, Byers BE, Goodale E, Johnson S, Liu WC (2001) Pseudoreplication in

508   playback experiments, revisited a decade later. Anim Behav 61:1029–1033.

509   https://doi.org/10.1006/anbe.2000.1676

510 Kullberg C, Lind J (2002) An experimental study of predator recognition in great tit

511   fledglings. Ethology 108:429–441. https://doi.org/10.1046/j.1439-0310.2002.00786.x

512 Lendrem DW (1983) Predation risk and vigilance in the blue tit (*Parus caeruleus*). Behav

513   Ecol Sociol 14:9–13

514 Lind L, Jöngren F, Nilsson J, Schönberg Alm D, Strandmark A (2005) Information, predation

515   risk and foraging decisions during mobbing in great tits *Parus major*. Orn Fennica

516   82:89–96

517 Madden JR, Kilner RM, Davies NB (2005) Nestling responses to adult food and alarm calls:

518   1. Species specific responses in two cowbird hosts. Anim Behav 70:619–627.

519   https://doi.org/10.1016/j.anbehav.2004.11.019

520 Magrath RD, Haff TM, Horn AG, Leonard ML (2010) Calling in the face of danger: how

521   predation risk affects acoustic communication by parent birds and their offspring. Adv

522   Stud Behav 41:187–253. https://doi.org/10.1016/S0065-3454(10)41006-2

523 Magrath RD, Haff TM, McLachlan JR, Igic B (2015a) Wild birds learn to eavesdrop on

524   heterospecific alarm calls. Curr Biol 25:2047–2050.

525   https://doi.org/10.1016/j.cub.2015.06.028

526 Magrath RD, Haff TM, Fallow PM, Radford AN (2015b) Eavesdropping on heterospecific

527   alarm calls: from mechanisms to consequences. Biol Rev 90:560–586.

528   https://doi.org/10.1111/brv.12122

529 Maloney RF, McLean IG (1995) Historical and experimental learned predator recognition in

530   free-living New Zealand robins. Anim Behav 50:1193–1201.

531   https://doi.org/10.1016/0003-3472(95)80036-0

532 Marler P (1957) Specific distinctiveness in the communication signals of birds. Behaviour

533   11:13–38. https://doi.org/10.1163/156853956X00066

534 McIvor GE, Lee VE, Thornton A (2018) Testing social learning of anti-predator responses in

535   juvenile jackdaws: the importance of accounting for levels of agitation. R Soc Open Sci

536   5:171571. https://doi.org/10.1098/rsos.171571

537 McLean IG, Hoelzer C, Studholme BJS (1999) Teaching predator-recognition to a naive bird:

538   implications for management. Biol Conserv 87:123–130. https://doi.org/10.1016/S0006-

539   3207(98)00024-X

540 Morand-Ferron J, Hamblin S, Cole EF, Aplin LM, Quinn J L (2015) Taking the operant

541   paradigm into the field: associative learning in wild great tits. PLoS One 10:e0133821.

542   https://doi.org/10.1371/journal.pone.0133821

543 Perrins CM (1965) Population fluctuations and clutch-size in the Great Tit, *Parus major* L. J

544   Anim Ecol 601–647. https://doi.org/10.2307/2453

545 Platzen D, Magrath RD (2004) Parental alarm calls suppress nestling vocalization. Proc R

546   Soc Lond B Biol Sci 271:1271–1276. https://doi.org /10.1098/rspb.2004.2716

547 Potvin DA, Ratnayake CP, Radford AN, Magrath RD (2018) Birds learn socially to recognize

548   heterospecific alarm calls by acoustic association. Cur Biol 28:2632–2637.

549   https://doi.org/10.1016/j.cub.2018.06.013

550 Randler C (2012) A possible phylogenetically conserved urgency response of great tits (*Parus

551   major*) towards allopatric mobbing calls. Behav Ecol Sociobiol 66:675–681.

552   https://doi.org/10.1007/s00265-011-1315-y

553

554 Rydén O (1978) Differential responsiveness of great tit nestlings, *Parus major*, to natural

555 auditory stimuli. Ethology 47:236–253. https://doi.org/10.1111/j.1439-

556 0310.1978.tb01834.x

557 Rydén O (1980) Heart rate response in great tit nestlings (*Parus major*) to an alarm call.

558 Journal of comparative and physiological psychology, 94:426.

559 http://dx.doi.org/10.1037/h0077680

560 Schaller GB, Emlen JT (1961) The development of visual discrimination patterns in the

561 crouching reactions of nestling grackles. The Auk 73:125–137

562 Shettleworth SJ (2010) Cognition, evolution, and behavior. Oxford University Press.

563 Shriner WM (1999) Antipredator responses to a previously neutral sound by free-living adult

564 golden-mantled ground squirrels, *Spermophilus lateralis* (Sciuridae). Ethology

565 105:747–757. https://doi.org/10.1046/j.1439-0310.1999.00454.x

566 Suzuki TN (2011) Parental alarm calls warn nestlings about different predatory threats.

567 Current Biology 21: R15-R16. https://doi.org/10.1016/j.cub.2010.11.027

568 Suzuki TN (2012) Referential mobbing calls elicit different predator-searching behaviours in

569 Japanese great tits. Animal Behaviour, 84(1), 53–57.

570 https://doi.org/10.1016/j.anbehav.2012.03.030

571 Suzuki TN, Wheatcroft D, Griesser M (2016) Experimental evidence for compositional

572 syntax in bird calls. Nat Commun 7:10986. https://doi.org/10.1038/ncomms10986

573 Suzuki TN, Wheatcroft D, Griesser M (2017) Wild Birds Use an Ordering Rule to Decode

574 Novel Call Sequences. Curr Biol 27:2331–2336. https://doi.org/10.17632/r7v96zf5pp.1

575 Tchernichovski O, Lints T, Mitra PP, Nottebohm F (1999) Vocal imitation in zebra finches is

576 inversely related to model abundance. Proc Natl Acad Sci U S A 96:12901–12904.

577 https://doi.org/10.1073/pnas.96.22.12901

578   Wheatcroft D, Price TD (2013) Learning and signal copying facilitate communication among

579   bird species. Proc R Soc Lond B Biol Sci 280:20123070.

580   https://doi.org/10.1098/rspb.2012.3070

581   Wheatcroft, D (2015) Repetition rate of calls used in multiple contexts communicates

582   presence of predators to nestlings and adult birds. Anim Behav, 103, 35–44.

583   https://doi.org/10.1016/j.anbehav.2015.02.009

584   Wheeler BC, FahyM, Barbara Tiddi (2019) Experimental evidence for heterospecific alarm

585   signal recognition via associative learning in wild capuchin monkeys. Anim Cogn, 1–9.

586   https://doi.org/10.1007/s10071-019-01264-3

587

596

Compliance with ethical standards

598 **Conflict of interest** The authors declare that they have no conflict of interest.

599

600 **Ethics approval** Our work was carried out under permission from the Prefecture du Rhône

601 (Ref 2015-13), Prefecture de l'Ain (DDPP01-15-230) and with the approval of the ethics

602 committee at Lyon 1 University, France (permit number: 2017012410184917). All authors are

603 accredited for performing experiments with living animals (French diploma "Experimentation

604 animale" first level for researchers). After ringing, all nestlings were readily accepted back by

605 their parents.

606 **Human and animal rights** This article does not contain any studies with human participants

607 performed by any of the authors.

608

609 **Table 1**

610 Generalized linear mixed models (GLMM) results for playback (associative learning stimulus

611 or mobbing calls) and group (experimental or control) as predictors of variation in fledglings

612 response behaviours (scanning, calling, approaching + fleeing). The "*" character indicates

613 the interaction between the explanatory terms. Significant P values are indicated in bold.

Behaviours	Fixed effects	df	χ^2	P
scanning	playback	1	5.8243	**0.0158**
	group	1	6.1785	**0.0129**
	playback*group	1	5.4487	**0.0196**
calling	playback	1	0.9227	0.3368
	group	1	0.0247	0.8752
	playback*group	1	4.8979	**0.0269**
approaching + fleeing	playback	1	8.9545	**0.0028**
	group	1	1.6161	0.2036
	playback*group	1	0.8797	0.3483

614

615

FIGURE CAPTIONS

617

618 **Fig. 1** Number of horizontal scans made by experimental and control fledglings during the

619 playbacks of associative learning stimulus and mobbing calls (mean ± SE)

620

621 **Fig. 2** Experimental and control fledglings' probability of calling during playbacks of

622 associative learning stimulus and mobbing calls

623

624 **Fig. 3** Response of experimental and control fledglings to playbacks of associative learning

625 stimulus (white) and mobbing calls (grey)

626