

# Red operculum spots, body size, maturation and evidence for a satellite male phenotype in non-native European populations of pumpkinseed *Lepomis gibbosus*

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1 **Red operculum spots, body size, maturation and evidence for a satellite**  
2 **male phenotype in non-native European populations of pumpkinseed**  
3 ***Lepomis gibbosus***

4  
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35

36 **Running title:** Operculum spot of non-native pumpkinseed

37

38 **Abstract**

39 Carotenoid-based pigmentation is a striking feature of many taxa, yet the function, if any,  
40 of colour traits is often unclear. Pumpkinseed *Lepomis gibbosus*, a widely introduced  
41 freshwater sunfish that exhibits alternative male mating strategies, express a striking, red  
42 operculum spot. To investigate the potential function of this red spot as a signal in this  
43 species' mating system, we determined the presence and measured the size of red  
44 operculum spots in fish collected from 12 populations in five European countries in which  
45 pumpkinseed is an established non-native species. We subsequently related the  
46 presence and size of the red spot to body size and mating strategy, based on an analysis  
47 of relative gonad size (gonado-somatic index, GSI), using a mixed modelling approach.  
48 The study demonstrated that the presence of a red operculum spot in pumpkinseed is  
49 associated with sexual maturation, with GSI frequency distributions suggesting that  
50 cuckolders in some non-native populations comprised both sneaker and satellite males,  
51 the latter not having previously been reported for this species. Further, the size of red spot  
52 correlated strongly with body size in parental and cuckolder males, though there was no  
53 difference in the presence or size of the red operculum spot between male mating  
54 strategies. The function of a red operculum spot in females is not clear but may be partly  
55 mediated by pleiotropic genetic mechanisms. Red operculum spots appear to function as  
56 signals of male maturation and body size in pumpkinseed, irrespective of mating strategy.

57

58 **Keywords** alternative mating strategy · carotenoid · GLMM · sexual selection · visual  
59 signal · Centrarchidae

## 60 1 | INTRODUCTION

61 Visual signals play a key role in the environmental biology of teleost fishes (Marshall,  
62 2000; Wootton, 1998). Pigmentation is an important component of visual signals in fishes,  
63 both within and among species. The expression of colour, which often comprises multi-  
64 component signals, involves pigment-based and structural colours (Price, Weadick, Shim,  
65 & Rodd, 2008; Wootton & Smith, 2015). Carotenoid-based colouration, which typically  
66 appears as yellow, orange or red, has been of particular interest because these are costly  
67 to produce and thereby serve as a reliable indication of individual condition and foraging  
68 ability (Olson & Owens, 1998), with a potential role in mating system evolution (Wootton  
69 & Smith, 2015). Different carotenoid pigments are responsible for conveying different  
70 information to potential mates or rivals (McLennan, 2007). Colour traits can function in  
71 sexual selection; selection that acts on differences in fitness among individuals that arise  
72 due to the number and identity of their mates. Because sexual selection tends to act more  
73 strongly on males than females (Andersson, 1994), it is males that usually express the  
74 brightest and most elaborate visual traits, including nuptial colouration. Though a broad  
75 simplification, there is a tendency in many taxa for a preference to mate with more  
76 colourful and ornamented individuals (Andersson, 1994; Price et al., 2008).  
77 Understanding the adaptive value of mate choice (*sensu* Kokko, Brooks, Jennions &  
78 Morley, 2003) based on visual signals is a controversial field of research, but may arise  
79 through fitness benefits associated with mate choice, or an arbitrary mechanism  
80 (Andersson, 1994; Wootton & Smith, 2015).

81 Pigmentation in North American sunfishes (Centrarchidae), in common with most other  
82 teleosts, is more extensive in males than in females (Scott & Crossman, 1973). In the  
83 pumpkinseed *Lepomis gibbosus* males have a striking, red operculum spot that is  
84 associated with male aggression (Stacey & Chiszar, 1978). In aquarium studies, breeding  
85 males were shown to attack fish models that intruded into their nesting area, and models  
86 with red colour on the operculum spot or iris received more attacks and aggressive  
87 displays than those without colour (Stacey & Chiszar, 1978). It is recognised that  
88 dominance relationships among sunfishes are related to colouration, with dominant,  
89 brightly-coloured parental (territorial) pumpkinseed males building and defending nests,  
90 and performing courtship behaviour to attract females to their nest for spawning. Male  
91 sunfishes also adopt alternative mating strategies (*sensu* Wootton, 1984), whereby sub-  
92 dominant 'cuckolder' males attempt to intrude into the nest of a parental male that is in  
93 the act of spawning (Gross, 1979, 1982). Cuckolder males are early-maturing, do not

94 perform courtship and avoid fitness costs associated with parental care (Smith & Wootton,  
95 1995). Small cuckolders, typically possessing disproportionately large testes, are referred  
96 to as ‘sneakers’ due to their rapid entry into the nest of a spawning territorial male to  
97 release sperm at the moment of spawning. Large cuckolders, which have testes of  
98 intermediate size relative to parental and sneaker males, are termed ‘satellites’ and  
99 participate in spawning by mimicking female characteristics and entering the nest of a  
100 territorial male as a potential mate (Gross, 1982; Garner & Neff, 2013). Despite aggressive  
101 defence of their nest by parental males, cuckolder males frequently intrude successfully  
102 during spawning events (Rios-Cardenas, 2003). In the close congener, bluegill *Lepomis*  
103 *macrochirus*, a model taxon for research on alternative reproductive strategies (Taborsky,  
104 2008), Gross (1982) characterised two cuckolder phenotypes, based on a population in  
105 Lake Opinicon, Canada. Sneaker bluegill are small and have relatively large testes relative  
106 to parental males, with a mean gonado-somatic index (GSI) of 4.6 (contrasting with  
107 parental males with a mean GSI of 1.1). In contrast, satellite male bluegill are larger than  
108 sneakers, with GSI values (mean 3.3) that are intermediate between those of parental  
109 males and sneakers. In applying these criteria to pumpkinseed from the same lake, Gross  
110 (1982) identified the sneaker phenotype, but not that of the satellite. In a subsequent  
111 paternity study, Neff and Clare (2008) noted that the satellite strategy had yet to be  
112 reported for pumpkinseed. In a subsequent genetic study of bluegill–pumpkinseed  
113 hybridisation, Garner and Neff (2013) did not distinguish between satellites and sneakers,  
114 referring to them collectively as cuckolders. Thus, there are no reports that the satellite  
115 phenotype (*sensu* Gross, 1982) is expressed in pumpkinseed and hereafter we use the  
116 term ‘cuckolder’ to refer to the alternative male mating strategy in pumpkinseed to the  
117 parental strategy.

118 The approach, onset and end of the reproductive period in fishes can often be identified  
119 by variation in GSI, the relationship between gonad weight and somatic weight (Wootton,  
120 1998). Male mating polymorphisms, particularly the cuckolder strategy, are associated  
121 with sperm competition, which occurs when the spermatozoa of different males compete  
122 for the same eggs (Parker, 1970). Males expressing alternative mating strategies tend to  
123 experience a higher “risk” of sperm competition; i.e. an elevated probability that their  
124 sperm will compete with the sperm of other males, because they usually mate in the  
125 presence of at least one competitor, typically the territorial male and often other cuckolder  
126 males (Wedell, Gage, & Parker, 2002; Wootton & Smith, 2015). An outcome is that  
127 cuckolders are predicted to show adaptations for sperm competition, including relatively

128 (or even absolutely) larger testis size, larger ejaculates, and faster swimming  
129 spermatozoa (Montgomerie & Fitzpatrick, 2009). Consequently, individual GSI can be  
130 used to identify male mating strategy.

131 Pumpkinseed have been widely established outside their native North American range.  
132 Most introductions have been in Europe, where the species was introduced in the late 19<sup>th</sup>  
133 and early 20<sup>th</sup> centuries, establishing populations in at least 28 countries (Copp & Fox,  
134 2007). Most of the research undertaken on pumpkinseed in Europe has focused on female  
135 growth and life-history traits (reviewed in Fox & Copp, 2014), with relatively few studies  
136 on males (e.g. Almeida, Tobes, Miranda, & Copp, 2012; Valente et al., 2016). In its native  
137 range, Neff & Clare (2008) observed temporal variability in the patterns of pumpkinseed  
138 paternity and suggested that these reflect temporal variation in mating opportunities,  
139 parental male defence ability, or cuckolder densities. Given this temporal variability,  
140 spatial variations are also feasible, possibly extending to visual signals, such as the  
141 operculum spot, which is assumed to be important for males for attracting mates (Stacey  
142 & Chiszar, 1978).

143 The aim of the present study was to determine whether the presence and size of the  
144 red operculum spot serves as a signal of sex and/or mating strategy in pumpkinseed. To  
145 do this we used estimates of GSI to categorise males as nesters or cuckolders and fitted  
146 models to test whether spot presence and size varied between the sexes and between  
147 males adopting different reproductive strategies. We predicted that parental males, which  
148 experience intrasexual contests and intersexual mate choice, would be more likely than  
149 cuckolder males and females to express a red operculum spot and that these males would  
150 also express larger red spots.

151

## 152 **2 | Methods**

153 Previously unpublished data for 1118 pumpkinseed were obtained from established non-  
154 native populations in Belgium, England, France, the Netherlands and Poland (Table 1),  
155 with all data elements being available for 924 specimens of which 465 were males, 387  
156 were females, and 266 were juveniles (Table 2). The fish were collected by a variety of  
157 sampling gear (Table 1), depending upon location characteristics and gear availability.  
158 Note that sampling of the two Netherlands populations was incorrectly reported as by  
159 funnel trap in Cucherousset et al. (2009). Where possible, the sampling aimed to obtain  $\approx$   
160 100 specimens for growth and life-history trait analysis (e.g. Villeneuve, Copp, Fox, &  
161 Stakénas, 2005; Cucherousset et al., 2009) and so was not quantitative. In the three

162 English sites, sampling was by catch-per-unit-effort (CPUE) with minnow traps (see Fox,  
163 Villeneuve & Copp, 2011). The data on female growth and life-history traits, but not  
164 operculum spot occurrence or size, have been previously published for all these  
165 populations (Villeneuve et al., 2005; Cucherousset et al., 2009; Fox et al., 2011; Valente  
166 et al., 2016), except the two populations from northwest Poland (Table 1): i) a former clay  
167 pit (Brodowski Pond) of  $\approx 0.9$  ha (maximum depth of 7 m) situated in Szczecin with a water  
168 temperature that ranges from 4 °C under thick ice cover in winter to 25 °C in summer; and  
169 ii) an artificial channel (4 km long, mean depth of 4.5 m), situated on the lower River Oder  
170 near the city of Gryfino that receives heated water effluent from a power plant, resulting  
171 in daily water temperature fluctuations of 5–15 °C, with maximum temperatures of 30 °C  
172 in summer and 15 °C in winter. There is currently no published study on the North  
173 American source populations of pumpkinseed in Europe.

174 For all populations, pumpkinseed were collected at the beginning of the spawning  
175 season (between May and June), immediately killed with a lethal dose of anaesthetic  
176 following licenced procedures in the country of collection and cooled to freezing for  
177 subsequent processing in the laboratory. After thawing, specimens were photographed  
178 (left side only) using a Nikon CoolPix 5000 camera for morphological analysis. The  
179 presence of operculum spots (denoted as present or absent) was recorded, including the  
180 rare occurrence of deviations from red (i.e. yellow or orange, or reddish shades thereof).  
181 Note that the reflectance spectra, hue or intensity of operculum spots were not measured,  
182 nor was the spectral sensitivity of pumpkinseed in the red region of the light spectrum  
183 known. The presence of an operculum spot was recorded for both sides of each specimen  
184 assessed, but only the left side of the fish (the standard for fish measurements) was used  
185 for quantifying the size of the red operculum spot, which was estimated as the surface  
186 area of the spot (ImageJ, 1.48v). Fish were measured for standard length (SL, nearest 1  
187 mm) and mass (nearest 1 g) and dissected to determine sex and gonad mass (in mg)  
188 when present. GSI was calculated as:  $100 \times \text{gonad mass} \div \text{total body mass}$  (Wootton &  
189 Smith, 2015).

190

## 191 **2.1 | Statistical analysis**

192 To categorise males as parentals or cuckolders, a frequency distributions of male GSI  
193 was plotted by country. The resulting multimodal GSI frequency distributions were  
194 decomposed to normal distributions using the Bhattacharya method (Bhattacharya, 1967),  
195 with a meaningful separation of normal distributions identified with a separation index (SI)

196 > 2 (Gayaniilo, Soriano, & Pauly, 1989). GSI values below the threshold value were  
 197 categorised as parental and those equal to or above the threshold value were categorised  
 198 as cuckolders. Data were pooled by country, rather than population, to maximise the  
 199 precision with which threshold values could be identified.

200 To test whether parental males were more likely to express a red operculum spot than  
 201 other males and females, the presence of a red spot in pumpkinseed was modelled using  
 202 a Bernoulli Generalised Linear Mixed Model (GLMM). Fish SL and mass were collinear,  
 203 and mass was subsequently dropped from the analysis. Only a single fish that was  
 204 classified as a juvenile expressed a red spot, and juveniles were subsequently dropped  
 205 from the analysis. The distribution for the model response variable, link function and  
 206 predictor function were specified as:

$$\begin{aligned}
 207 \quad & Spot_{ij} \sim \text{Binomial}(\pi_{ij}, n_{ij}) \\
 208 \quad & E(Spot_{ij}) \sim n_{ij} \times \pi_{ij} \quad \text{and} \quad \text{var}(Spot_{ij}) = n_{ij} \times \pi_{ij} \times (1 - \pi_{ij}) \\
 209 \quad & \eta_{ij} = \beta_1 + \beta_2 \times SL_{ij} + \beta_3 \times \text{strategy}_{ij} + \text{population}_i \\
 210 \quad & \text{logit}(\pi_{ij}) = \eta_{ij} \\
 211 \quad & \text{population}_j \sim N(0, \sigma^2_{\text{population}})
 \end{aligned}$$

212 Where  $Spot_{ij}$  is the probability of fish  $i$  in population  $j$  having a red operculum spot in  $n_{ij}$   
 213 independent individuals, which was assumed to follow a binomial distribution with an  
 214 expected probability ( $E$ ) of expressing a spot of mean  $\pi_i N_i$  and variance  $\pi_i \times (1 - \pi_i)$ , with a  
 215 logit link function. The logit function ensures the fitted probability of a red spot falls  
 216 between 0 and 1. The variable  $\text{strategy}_{ij}$  is a categorical covariate with three levels,  
 217 corresponding with fish mating strategy; female, territorial or cuckolder. The model also  
 218 contained a linear effect for fish SL ( $SL_{ij}$ ). Exploratory analyses demonstrated differences  
 219 among populations in the size and expression of red operculum spots. To accommodate  
 220 this effect in the model, the random intercept  $\text{population}_j$  was included to introduce a  
 221 correlation structure between observations for different fish from the same population, with  
 222 variance  $\sigma_{\text{population}}$  distributed normally and equal to 0.

223 To test whether parental males expressed larger red operculum spots than other males  
 224 and females, the size of red spots was modelled on a zero-truncated subset of the data  
 225 using a Gaussian GLMM, specified as:

$$\begin{aligned}
 226 \quad & \log_{10}Spotsize_{ij} \sim N(\mu_{ij}, \sigma^2) \\
 227 \quad & E(\log_{10}Spotsize_{ij}) = \mu_{ij} \quad \text{and} \quad \text{var}(\log_{10}Spotsize_{ij}) = \sigma^2 \\
 228 \quad & \mu_{ij} = \beta_1 + \beta_2 \times \log_{10}SL_{ij} \times \beta_3 \times \text{strategy}_{ij} + \text{population}_i \\
 229 \quad & \text{population}_j \sim N(0, \sigma^2_{\text{population}})
 \end{aligned}$$



230 Where  $\log_{10}Spotsize_{ij}$  is the  $\log_{10}$  size of red spot on fish  $i$  in population  $j$  and  $\log_{10}SL_{ij}$  is  
231 the  $\log_{10}$  standard length of fish  $i$  in population  $j$ . Expected  $\log_{10}Spotsize_{ij}$  is  $\mu_{ij}$  with  
232 variance  $\sigma^2$ . Analysis was conducted using the *lme4* library (Bates, Maechler, Bolker, &  
233 Walker, 2014) in R (R Development Core Team, 2017). Model residuals were examined  
234 to ensure assumptions were met and that effects were adequately accounted for by the  
235 model.

236

### 237 **3 | RESULTS**

238 Male GSI distributions were multimodal within countries (Figure 1). With the exception of  
239 data from the Netherlands, the overlap of normally-distributed GSI groups was low; SI was  
240 always  $>2$  and thereby provided meaningful separation of modal groups, even for the  
241 Netherlands specimens (separation index = 2.68). Decomposition of GSI distributions  
242 showed the threshold GSI value that distinguished parental and cuckolder males varied  
243 among countries, from 2.3 (Poland) to 4.2 (England) (Table 2, Figure1). In Belgian, French  
244 and Polish populations, separation of male strategies was ambiguous, with Bhattacharya  
245 decomposition revealing two thresholds within these data (Figure 1). In each case, the  
246 higher threshold was used as the cut-off between male strategies.

247 The probability of expressing a red operculum spot was predicted by male SL, with a  
248 significantly greater likelihood of a red spot in larger males (Table 3; Figure 2). The  
249 probability of a red operculum spot was also predicted by sex, with both cuckolder and  
250 territorial males significantly more likely to express a red spot than females (Table 3;  
251 Figure 2). Among pumpkinseed that expressed a red spot, there was a significantly  
252 stronger interaction between SL and spot size in males than in females (Table 4; Figure  
253 3), though there was no difference between male strategies classified by GSI (Figure 3).  
254 Two specimens were observed to possess a yellow operculum spot, a mature female from  
255 Schoapedobbe Pond and a male from Brière Marsh (Table 1).

256

### 257 **4 | DISCUSSION**

258 Our analysis of introduced pumpkinseed populations across Europe demonstrated that  
259 the expression of a red operculum spot was more likely in mature males than females,  
260 (Table 3), whereas only a single sexually immature juvenile expressed a red spot. Further,  
261 a significantly stronger interaction was observed (Table 4) between red spot size and body  
262 size in parental and cuckolder males than in females. Taken together, these findings  
263 indicate that in pumpkinseed, the presence and size of a red operculum spot signals

264 sexual maturity and correlates strongly with body size in males. This signal, however,  
265 does not distinguish between the male mating strategies of parental and cuckold.

266 Colour signals can have multiple functions, potentially conveying information to rivals,  
267 mates or both. In pumpkinseed, the function of the red operculum spot is equivocal. The  
268 present findings are consistent with previous studies in demonstrating that a red spot has  
269 a potential role in male-male interactions by eliciting an aggressive response by males  
270 (Stacey & Chiszar, 1978). Notably, during aggressive intra-sexual contests, male  
271 sunfishes flare their opercula (Colgan & Gross, 1977), displaying the red spot to a rival  
272 facing them head-on. Our results support a role for the red operculum spot in male  
273 contests, with the expression of a red spot primarily associated with sexually mature males  
274 and correlating with body size. However, this finding contradicts our prediction that  
275 parental males, which experience intrasexual contests and intersexual mate choice,  
276 should be more likely than cuckold males to express a red operculum spot.

277 The expression of a red carotenoid-based colour signal may convey information about  
278 male condition, which could be functional in the context of male-male contests.  
279 Carotenoids are acquired solely in the diet by vertebrates and these compounds have a  
280 number of critical physiological functions, making carotenoid-based signals a potentially  
281 reliable indication of individual condition and fighting ability (Olson & Owens, 1998).  
282 Whether additional information is conveyed by the size, reflectance spectra and intensity  
283 of the red colour spot, or whether the red operculum spot comprises one component of a  
284 multimodal signal in pumpkinseed, remains to be investigated. Experimental studies  
285 would be particularly effective in differentiating these aspects of signal evolution in  
286 pumpkinseed.

287 Surprisingly, we detected no significant difference between parental and cuckold  
288 males, either in the probability of expressing a red spot, or in the strength of the  
289 relationship between red spot size and male size. Alternative mating strategies in fish,  
290 which are exclusively shown by males in teleost fishes, are reflected most strikingly in  
291 reproductive behaviour, but also in external appearance and physiology. In contrast to  
292 Gross' (1982) interpretation of his sunfish model to a single native population of  
293 pumpkinseed, our analysis of non-native populations in Europe suggests that, while a  
294 clear distinction can be seen between the parental and cuckold male reproductive  
295 strategies in relative testes size, the GSI value threshold that distinguishes parental and  
296 cuckold males varies among populations, ranging from 2.3 (Poland) to 4.2 (England).

297 A possible alternative explanation for the observed range of GSI values may relate to  
298 variation in pumpkinseed population density, which has consequences for the  
299 reproductive success of cuckold males. In water bodies where the populations occurred  
300 at high density, traits associated with sperm competition, including large testes size, are  
301 favoured, thereby potentially selecting for relatively larger gonads (high GSI) in both  
302 parental and cuckold males, with a consequent upward shift in the threshold between  
303 them. In the three ponds in England for which quantitative density data were available  
304 (Fox et al., 2011), pumpkinseed was virtually the only species present in the two ponds  
305 where the species have been observed in high density and males in these ponds had  
306 higher mean and maximum male GSI values than observed in the low-density pond. In  
307 water bodies where populations occur at low densities, where the risk of sperm  
308 competition for parental males is low, relative testes size is predicted to be smaller and  
309 the GSI threshold between parental and cuckold males lower (Wootton & Smith 2015).

310 A caveat to these conclusions was the finding that many females also expressed red  
311 spots in their opercula, albeit with lower probability than males (Figure 2). The size of red  
312 operculum spots in females also conveyed less information about size than in males  
313 (Figure 3). If red operculum spots function primarily in male-male contests, then it is  
314 unclear why females would display this trait. For example, the red operculum spot may  
315 have multiple functions, potentially signalling male size and fighting ability, but also  
316 undergoing intersexual selection in the context of mate choice. If the red operculum spot  
317 is selected through intersexual selection, then it is possible that females express the trait  
318 for the same reason as males. Female ornamentation is surprisingly common in teleosts,  
319 and in the context of the pumpkinseed mating system, in which males are the sole  
320 providers of parental care, the evolution of male mate choice and female ornamentation  
321 is possible (Wootton & Smith 2015).

322 The expression of red operculum spots in males is adaptive solely through intra-sexual  
323 selection, whereas in females the expression of red spots may be through a genetic  
324 correlated response. In threespine stickleback (*Gasterosteus aculeatus*), red throat  
325 colouration is known to function in intra- and intersexual selection on males and is a  
326 reliable indicator of body condition and parasite resistance (Milinski & Bakker, 1990;  
327 Barber, Arnott, Braithwaite, Andrew, & Huntingford, 2001). The red ornament, which has  
328 a strong genetic component, is also found in females and its expression in females is  
329 consistent with pleiotropy (Yong, Peichel & McKinnon, 2016). Pleiotropy may also mediate  
330 the expression of a red operculum spot in female pumpkinseed, and possibly also in

331 cuckold males, though this conclusion relies on there being a shared genetic basis to  
332 red spots in males and females and no adaptive value in expressing a red spot in females  
333 and cuckolders, and these assumptions remain to be tested.

334 The presence of two male GSI thresholds in pumpkinseed populations in Belgium,  
335 France and Poland provides evidence that, contrary to Gross, (1982), both the satellite  
336 and sneaker strategies may be present in some non-native European pumpkinseed  
337 populations (Figure 1). Alternatively, these findings may simply reflect the greater  
338 heterogeneity in age at maturity within some European populations (Copp & Fox, 2007;  
339 Fox & Copp, 2014), which is a possible outcome of multiple sources of introduction or  
340 adaptation to conditions outside the natural range of the species. Behavioural studies,  
341 complemented with morphological analyses, are needed to clarify the existence of a  
342 satellite strategy in European pumpkinseed populations. Similarly, variation in predation  
343 among populations has the capacity to modify the selective landscape for red operculum  
344 spots, since sexual selection for visual signals can be limited through natural selection by  
345 predators in teleosts (Endler, 1980; Wootton & Smith, 2015). Although outside the scope  
346 of the present study, the effect of selective predation on pumpkinseed in response to the  
347 expression of the red operculum spot is not known and warrants further research.

348 In conclusion, the present study demonstrates that the presence of a red operculum  
349 spot in pumpkinseed is associated with sexual maturation, primarily in males. The size of  
350 red spot correlates with body size in parental and cuckold males, although there was no  
351 difference in the presence or relative size of the red operculum spot between male mating  
352 strategies. The observed GSI distributions suggest the possible existence of the satellite  
353 mating strategy in some European populations, but alternatively this may be due to  
354 variations in pumpkinseed population density or to selective predation. The function, if  
355 any, of a red operculum spot in females is not clear but is consistent with pleiotropy.

356

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365

366 **Author contribution**

367 The study was conceived by GHC and MGF. Specimens were collected and processed  
368 by GHC, GZ, MGF, SY, EZ, GM, JC, HV and HHvK. Data were analysed by GHC, SY,  
369 GZ, MP and CS. The initial draft of the manuscript was prepared by SY, EZ, GZ and GHC,  
370 then extensively revised by GZ, CS and MP, with co-author contributions.

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480 **TABLE 1.** Country, population, location (WGS84 latitude and longitude coordinates) and capture method for male pumpkinseed  
 481 *Lepomis gibbosus*, including number of specimens (*n*), mean and SD of standard length (mm) and the mean, minimum (min.) and  
 482 maximum (max.) of the gonado-somatic index (GSI) for each population by water body.  
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Country	Population	Latitude	Longitude	Method <sup>†</sup>	n	SL (SD)	GSI mean	min.	max.
Belgium	Webbekomsbroek Pond	50.975833N	5.076111E	ELF	76	61.2 (15.7)	2.4	0.2	5.3
France	Briere Marsh	47.366111N	2.312500W	ELF	63	90.9 (16.4)	1.9	0.5	4.6
	Grand-Lieu Lake	47.083333N	1.650000W	ELF	51	69.4 (21.2)	3.1	0.2	8.3
Netherlands	Schoapedobbe Pond	52.951944N	6.258333E	NET	30	124.2 (16.6)	2.0	0.7	5.6
	Meeuwven Pond	51.391111N	5.499722E	NET	58	67.3 (3.8)	3.5	1.3	10.7
Poland	Brodowski Pond	53.450342N	14.565856E	ELF	24	77.1 (11.1)	2.0	0.2	5.2
	Power Plant discharge channel	53.213983N	14.468975E	ELF	25	77.1 (18.4)	1.8	0.1	5.7
England	Cottesmore Pond (Lower)	51.089167N	0.219444W	TRC	31	64.5 (12.5)	2.3	0.4	9.5
	Cottesmore Pond (Upper)	51.088056N	0.219444W	TRC	82	64.9 (12.3)	2.6	0.8	8.3
	Cottesmore Pond (School)	51.087222N	0.219444W	TRC	56	64.4 (16.2)	1.8	0.2	2.9
	Batts Stream at Cackle Street	51.018611N	0.074444W	ELF	28	75.2 (12.4)	1.7	0.7	6.4
	Tanyard Specimen Lake 3	51.019843N	0.012377W	ANG	39	92.7 (16.4)	2.4	0.9	12.5

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 498 <sup>†</sup>ANG = angling; ELF = continuous electrofishing; NET = small seine netting; TRC = minnow traps, catch-per-unit-effort (see Fox et al. 2011)

499 **TABLE 2.** Mean (SD) pumpkinseed body mass, standard length (SL) and gonado-somatic  
500 index (GSI; n/a = not available) among countries of origin and reproductive status. Male  
501 reproductive strategy (either cuckolder or parental) was identified by separation of GSI  
502 frequency distributions by Bhattacharya's (1967) method.

Country of origin	Reproductive status	n	Mass (g)	SL (mm)	GSI
Belgium	Female	61	8.33 (9.89)	61.3 (16.0)	n/a
	Juvenile	10	3.85 (1.63)	52.5 (6.5)	n/a
	Cuckolder	21	4.81 (2.59)	55.9 (7.3)	3.60 (0.86)
	Parental	24	14.86 (10.22)	75.2 (16.4)	1.26 (0.59)
France	Female	66	17.81 (11.68)	81.1 (18.5)	n/a
	Juvenile	16	1.28 (0.75)	38.9 (6.1)	n/a
	Cuckolder	15	5.03 (2.95)	55.6 (8.9)	4.90 (1.11)
	Parental	91	22.58 (14.21)	88.2 (17.1)	2.00 (0.83)
Netherlands	Female	62	23.77 (23.99)	86.7 (30.2)	n/a
	Juvenile	1	7.00	66.6	n/a
	Cuckolder	33	7.25 (1.16)	66.3 (4.5)	4.25 (1.32)
	Parental	51	36.35 (26.58)	101.3 (30.4)	2.10 (0.63)
Poland	Female	18	20.25 (11.76)	76.4 (14.5)	n/a
	Juvenile	0	n/a	n/a	n/a
	Cuckolder	10	11.01 (7.24)	64.2 (10.3)	4.19 (1.05)
	Parental	39	23.79 (12.42)	80.4 (14.4)	1.26 (0.62)
England	Female	180	14.46 (14.64)	76.5 (19.9)	n/a
	Juvenile	45	2.56 (3.14)	46.2 (13.4)	n/a
	Cuckolder	16	5.69 (7.08)	59.4 (14.4)	6.20 (2.23)
	Parental	165	12.29 (10.04)	76.1 (16.2)	n/a

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505 **TABLE 3.** Summary of Bernoulli GLMM to model the probability of pumpkinseed  
506 expressing a red operculum spot. Fish from different populations were fitted as random  
507 intercepts, with standard deviation of 1.47.  $N_{\text{obs}} = 897$ .

Model parameter	Estimate	SE	<i>P</i>
Intercept <sub>(female)</sub>	-8.02	0.80	<0.001
SL <sup>†</sup>	0.08	0.01	<0.001
Strategy <sub>(cuckolder)</sub>	2.30	0.34	<0.001
Strategy <sub>(territorial)</sub>	2.35	0.23	<0.001

508 † SL = standard length

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512 **TABLE 4.** Summary of a Gaussian GLMM to model the size of pumpkinseed red  
 513 operculum spots. Fish from different populations were fitted as random intercepts, with  
 514 standard deviation of 0.24.  $N_{\text{obs}} = 344$ .

Model parameter	Estimate	SE	<i>P</i>
Intercept <sub>(female)</sub>	-7.43	0.99	<0.001
Log <sub>10</sub> SL <sup>†</sup>	1.82	0.22	<0.001
Strategy <sub>(cuckolder)</sub>	-5.31	2.77	0.055
Strategy <sub>(territorial)</sub>	-4.38	1.06	<0.001
Log <sub>10</sub> SL x Strategy <sub>(cuckolder)</sub>	1.40	0.66	0.033
Log <sub>10</sub> SL x Strategy <sub>(territorial)</sub>	1.18	0.24	<0.001

515 † SL = standard length  
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518 **Figure legends**

519 **FIGURE 1.** Observed gonado-somatic index (GSI): frequency plots for pumpkinseed  
520 among country of origin with Gaussian distribution curves and separation indices (SI),  
521 generated using Bhattacharyya's (1967) method. Threshold GSI values for separating  
522 frequency distributions for male mating strategies are indicated with arrows.

523 **FIGURE 2.** Mean fitted probability (solid line) of pumpkinseed expressing a red operculum  
524 spot as a function of standard length (SL in mm) with 95% confidence intervals (shaded  
525 area) for females, cuckolder males and parental males. Data were modelled with a  
526 Bernoulli GLMM. Fish from different populations were fitted as random intercepts in the  
527 model.

528 **FIGURE 3.** Mean fitted  $\text{Log}_{10}$  area ( $\text{mm}^2$ ) of red operculum spot (solid line) of  
529 pumpkinseed as a function of  $\text{log}_{10}$  standard length (SL in mm) with 95% confidence  
530 intervals (shaded area) for females, cuckolder males and parental males fitted with a  
531 Gaussian GLMM. Fish from different populations were fitted as random intercepts in the  
532 model.