

26 Fish use different modalities to access mates for reproduction, often referred to as Alternative
27 Reproductive Tactics (ARTs). ARTs are an example of coexisting phenotypes, which have to
28 hold some degree of reproductive success to persist in a population. In the Mediterranean
29 damselfish (*Chromis chromis*), territorial males colonise nests on rocky reefs, competing for
30 females, while sneaker males attempt to parasitically spawn in those nests. Here we combine
31 behavioural observations in the field with molecular analyses, using bi-parentally and
32 maternally inherited markers, to investigate reproductive success patterns of the two observed
33 male ARTs in terms of number of eggs sired and number of females contributing to each nest.
34 Cuckoldry was observed in every nest sampled, with at least two and up to seven sneakers per
35 nest; however, the nesting male always significantly fathered the large majority of the eggs (on
36 average 49%) in each clutch. Each sneaker fathered around 7% of the clutch. The average
37 number of females whose eggs were fertilised by nesting males was 6.76 (ranging 2-13), while
38 each sneaker on average fertilised the eggs of 1.74 (range 1-8) females. Using this sibship
39 reconstruction, we investigated some of the factors involved in the regulation of the dynamic
40 equilibrium of reproductive success between the two ARTs showed by *C. chromis* males. Our
41 results show that the sneakers' reproductive success was positively linked to egg clutch size;
42 the density of individuals in the nesting area negatively affected the size of egg clutches; the
43 rate of defence behaviours performed by nesting males negatively influenced the number of
44 females contributing to each nest.

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46 Key words: *Chromis chromis*; microsatellites; mtDNA; parentage assignment; sneakers.

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INTRODUCTION

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52 Different modalities to access mates for reproduction, also called Alternative
53 Reproductive Tactics (ARTs, Oliveira *et al.*, 2008), are an example of coexisting phenotypes
54 (Taborsky, 1994; Heinze & Keller, 2000), where conspecifics of the same sex in one population
55 invest different amounts of energy and exhibit remarkably different strategies to ensure
56 reproduction (Taborsky *et al.*, 2008). Fish offer a great opportunity to investigate ARTs since
57 they show a huge diversity of reproductive modes (e.g., DeWoody & Avise, 2001, Avise *et al.*,
58 2002). In particular, males, depending on their mating system, can maximise their fitness
59 through scramble competition, resource defence (e.g., territory, nest, and/or females),
60 reproductive parasitism or even cooperation (Taborsky, 2001). Of these four strategies,
61 resource defence and reproductive parasitism are often coupled as ARTs: territorial males (also
62 called nesting males) conquer and defend a suitable territory and actively attract females, while
63 other males behave as “sneakers”, by parasitically spawning in the nest of a territorial male,
64 without providing any territorial defence or paternal care (Taborsky, 1994; Coleman & Jones,
65 2011).

66 While comprehensive knowledge of these mating strategies is available from a
67 behavioural point of view, less is known about the actual relative reproductive success
68 occurring as a consequence of coexisting ARTs (Garant *et al.*, 2001; Reichard *et al.*, 2004;
69 Cogliati *et al.*, 2013). Knowledge of the relative fitness among different tactics can help to
70 clarify if coexisting ARTs represent a plastic/conditional strategy (dependent mainly from
71 environmental conditions, resulting in unequal male fitness) or a genetic polymorphism
72 (maintained by negative frequency dependent selection, resulting in overall similar male
73 fitness) or, as recently proposed, a mixture of these two modalities, called a conditional
74 alternative strategy (Neff & Svensson, 2013; Cogliati *et al.*, 2014). Fitness equilibrium between
75 ARTs can be maintained by a complex interaction of multiple variables, including individual

76 traits, population features and environmental characteristics. According to literature, most of
77 the species with male nest-defence tactics exhibit a certain level of cuckoldry (i.e., part of their
78 egg clutch is fertilised by other males; Coleman & Jones, 2011). Yet, the relative number of
79 eggs fertilised by males other than the nesting one is rather limited (Mackiewicz *et al.*, 2005;
80 Rios-Cardenas & Webster, 2008; Alonzo & Heckman, 2010). Furthermore, the rate of multiple
81 maternity for these species is very high, indicating that polygyny is fairly common in these
82 species, but the average number of dams contributing to a single nest is low (three females on
83 average; Coleman & Jones, 2011).

84 Members of the family Pomacentridae are known to exhibit ARTs (Gronell, 1989;
85 Karino & Nakazono, 1993; Tyler, 1995; Barnett & Pankhurst, 1996). The Mediterranean
86 damselfish, *Chromis chromis* (Linnaeus, 1758), is characterised by male nest-defence. This is
87 a small (average standard length 6 cm; Bracciali *et al.*, 2014), sexually monomorphic species
88 commonly distributed in the Mediterranean Sea, extending to the Eastern part of the Atlantic
89 Ocean. It lives in shoals near rocky reefs or above seagrass meadows at depths between 3 and
90 30 m (Lythgoe & Lythgoe, 1971; Quignard & Pras, 1986). Throughout the reproductive season
91 (June-September), colonies go through several reproductive bouts. Some males (nesting males)
92 colonise nests and guard them until eggs hatch. They attract females to receive egg deposition
93 by vocalising and performing specific courtship-displays (Picciulin *et al.*, 2002, 2010).
94 Parasitic spawning by sneaker males is commonly observed in the Mediterranean damselfish;
95 sneakers do not establish nests, infiltrating a guarded nest, while the nesting male receives egg
96 deposition by a female. On average, nesting males can receive three intrusions over a 10 min
97 observation period (Picciulin *et al.*, 2004). However, the success rate of these “attacks” remains
98 unclear and molecular investigation is needed to unveil the real reproductive success of
99 sneakers. Additionally, parasitic spawning is known to be occasionally performed even by

100 nesting males (Picciulin *et al.*, 2004). *Chromis chromis* thus offers a model to study the relative
101 reproductive success attained by nesting males and sneakers.

102 In this study, a combination of bi-parentally and maternally inherited markers was used
103 to investigate reproductive patterns in *C. chromis*. In particular, the number of breeders
104 contributing to each nest was examined, with a special focus on the relative reproductive
105 success of nest-guarding males and sneakers in terms of percentage of egg clutch sired, and the
106 number of females contributing to each clutch. Additional factors, such as egg clutch size (often
107 linked to male success), density of the colony (which influences encounter rates between sexes
108 and male-male competition) and male aggressive behaviours (towards intruders) can also affect
109 reproductive outcomes. Thus, using sibship reconstruction, the following factors were also
110 investigated: a) the relationship between egg clutch size and the relative success of nesting
111 males and sneakers; b) the link between the density of individuals in the breeding colony and
112 egg clutch size; and c) the relationship between the frequency of nest-defence behaviours
113 performed by males and the number of females spawning in their nest.

114 MATERIALS AND METHODS

115 STUDY AREA AND SAMPLE COLLECTION

116 Two damselfish colonies were selected 5-10 m from the coasts of Palermo and Zingaro,
117 Sicily, Italy (Fig. 1) in May 2011. Twenty-five spherical flowerpots (18 cm diameter; Knapp
118 & Kovach, 1991) were placed at 7-12 m depth to provide males with artificial nests
119 (minimising differences in nests' quality and size; Fig. 1). Overall, twelve artificial nests (five
120 from Palermo and seven from Zingaro) were successfully colonised by *C. chromis*. Colonized
121 nests were monitored with video cameras during June and August 2011. At the end of the
122 behavioural observations, artificial nests (which contained all the eggs attached to the pot
123 surface on a single layer) were collected. Given their fast swimming behaviour, it was not
124 possible to catch the nesting males. Once on the boat, the relative quantity of eggs present in

125 each nest was estimated (given their small size, it was not possible to count all the eggs). All
126 egg clutches exhibited a diamond shape; minor and major axes were measured to calculate the
127 surface area of each egg clutch (cm²). All eggs were detached from the flowerpot using a
128 scalpel and immediately stored in 100% ethanol and subsequently at -20°C.

129

130 DNA ISOLATION, PCR AMPLIFICATION, GENOTYPING AND SEQUENCING

131 Single eggs were separated under a stereomicroscope. DNA was isolated from a random
132 subsample of 48 eggs from each nest (total N = 576) using a modified salt extraction protocol
133 (Miller *et al.*, 1988) and 376 eggs (30-48 per nest) were successfully amplified at seven
134 microsatellite loci. Primers from four species of the Pomacentridae family were employed:
135 2AL2 (*Abudefduf luridus*; Carvalho *et al.*, 2000), Cm_D006 (*Chromis margaritifer*;
136 Underwood, 2009), Da360, Da542, Da589, Da590 (*Dascyllus aruanus*; Fauvelot *et al.*, 2009)
137 and SpTG53 (*Stegastes partitus*; Thiessen & Heath, 2007). Loci were amplified in two
138 multiplex polymerase chain reactions (PCR). Each reaction was carried out in a total volume
139 of 12 µl, using 6 µl of QIAGEN® Multiplex PCR kit, 4.4 µl of DNA template and 1.6 µl of
140 primer mix. Fluorescently-labelled primers (FAM, VIC, NED and PET) were added with the
141 following concentrations: Cm_D006 0.17 µM, 2AL2 0.17 µM, SpTG53 0.33 µM (multiplex
142 1); Da542 0.17 µM, Da589 0.17 µM, Da590 0.17 µM, Da360 0.08 µM (multiplex 2).
143 Amplification conditions were as follows: 95°C for 15 min; 37 cycles of 94°C for 45 s, 52°C
144 for 1 min, 72°C for 45 s and a final extension at 72°C for 45 min (multiplex 1); 95°C for 15
145 min; 37 cycles of 94°C for 45 s, 58°C for 1 min, 72°C for 45 s and a final extension at 72°C for
146 45min (multiplex 2). PCR reactions were performed using a Biometra T3000 thermocycler.
147 Allele sizes were determined on an ABI-3130xl Genetic Analyser (Applied Biosystems©) with
148 an internal size standard (600 LIZ, Applied Biosystems©). Genotypic data were acquired using

149 GeneMapper 4.0 (Applied Biosystems©). Following standard practice, we re-amplified and re-
150 scored genotypes for 10% (30 eggs) to check for consistency of genotype calling.

151 Furthermore, amplification of 355 bp of the mitochondrial control region (CR) was
152 carried out on a subsample of eggs from each nest (total N = 222) using the primers CR-A and
153 CR-E (Lee *et al.*, 1995, Domingues *et al.*, 2005) in a 25 µl reaction volume, according to the
154 following protocol: NH₄ Buffer X1, dNTPs 800 µM (200 µM each), CR-A 0.3 µM, CR-E 0.3
155 µM, MgCl₂ 2.5 mM, Taq 2.5 U (BIOLINE), 1 µl template. Amplification conditions were as
156 follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 1 min. Products
157 were sequenced commercially (Beckman-Coulter Genomics). D-loop sequences were
158 submitted to GenBank under the accession numbers KX442797-443014.

159

160 POPULATION GENETIC ANALYSES

161 Expected unbiased (H_e) and observed (H_o) heterozygosities and average number of
162 alleles (N_A) per nest were calculated using the Microsatellite Toolkit add-in available for
163 Microsoft Excel (Park, 2001). Allelic richness (A_R), departure from Hardy-Weinberg
164 equilibrium (by calculating F_{IS} and testing significance through 1320 permutations) and linkage
165 disequilibrium were estimated using FSTAT 2.9.3.2 (Goudet, 1995).

166 The effective number of breeders (N_b) for each nest was initially investigated by
167 computing the effective population size (N_e) for each nest, using the gametic disequilibrium
168 method implemented in the software LDNe 1.31 (Waples & Do, 2008). Additionally,
169 probability of individual identity, $P_{(ID)}$, was calculated for all seven loci with GIMLET
170 (Valière, 2002); $P_{(ID)}$ is defined as the chance that two individuals drawn at random from the
171 same population will share the same genotypic profile at multiple loci (Waits *et al.*, 2001).
172 GIMLET allows to calculate both $P_{(ID)sib}$ (the $P_{(ID)}$ of a population where siblings are found
173 and included, Evett & Weir, 1998) and $P_{(ID)unbiased}$ (the $P_{(ID)}$ after sample size corrections;

174 Paetkau *et al.*, 1998); the observed $P_{(ID)}$ lays between $P_{(ID)sib}$ and $P_{(ID)unbiased}$ and is estimated by
175 computing the proportion of all possible pairs of individuals that have identical genotypes.

176 All CR sequences were manipulated on ProSeq 3.0 (Filatov, 2002), and subsequently
177 aligned with ClustalX 2.1 (Larkin *et al.*, 2007) using the default parameters, whilst resulted
178 alignments were verified by eye. Summary statistics of haplotypes (K), number of polymorphic
179 sites (n), haplotype diversity (h), nucleotide diversity (π) and standard deviations (SD) (Nei,
180 1987), for the pooled dataset and per nest, were calculated with Arlequin 3.11 (Excoffier *et al.*,
181 2005). Finally, a median joining network was examined on NETWORK 4.6.1.0 (Bandelt *et al.*,
182 1999; <http://www.fluxus-engineering.com>).

183

184 RELATEDNESS ANALYSIS AND PARENTAGE ASSIGNMENT

185 Since genetic data from parents were not available, sibship and parentage of offspring
186 were assigned in Colony 2.0 (Jones & Wang, 2009) using the built-in maximum-likelihood
187 method for microsatellite data. Half- and full-sib pairs with a probability equal to 1.0 (100%)
188 were selected and manually grouped under two inferred parents of unknown sex. Mitochondrial
189 haplotype information was then used to determine the sex of each parent: offspring assigned to
190 one parent could share the same haplotype (thus the parent was classified as female) or exhibit
191 more than one haplotype (parent classified as male; Sefc *et al.*, 2008).

192 For those parent-offspring groups with no clear pattern (CR haplotypes were
193 determined only for 59% of the eggs used to infer sibship), a manual cross-check across the
194 whole data-set was performed: every offspring was shared by two parents, if one of them was
195 clearly classified as either sex, the other had to belong to the opposite sex. The male with the
196 highest number of offspring assigned within a nest was assumed to be the nesting male
197 (Coleman & Jones, 2011). The number of offspring fertilised by each male in a nest was
198 recorded and the relative proportion of eggs fathered was estimated. The average of the number

199 of females whose eggs were fertilised by each male was calculated, weighted by the number of
200 offspring per nest.

201

202 BEHAVIOURAL OBSERVATIONS

203 Clear parentage information was successfully obtained for nine out of the 12 nests (N
204 = 6 for Palermo, N = 3 for Zingaro). For these nests, behavioural data had been obtained from
205 video cameras installed by a scuba diver in front of each nest, at a distance of 80-120 cm. The
206 central 75 min of each 90 min video were scored to determine the number of defence-related
207 behaviours performed (thrusts: focal male moves toward the opponent with a rapid movement
208 and turns immediately back upon reaching the adversary; chases: focal male does not turn back
209 after the thrust but instead chases the adversary for several metres; Verginella *et al.*, 1999). The
210 nine focal males' behaviour was followed for a total of approximately 11 h of observation using
211 JWatcher 1.0 (Blumstein & Daniel, 2007). The number of defence-related behaviours per hour
212 were quantified considering only the time spent by focal males in sight of the camera view (i.e.
213 [defence behaviours * (total time min) - time spent out of sight min]⁻¹) * 60 min). Damselfish
214 density (i.e., number of individuals per colony) varied across locations. At the end of randomly
215 chosen sampling sessions, video files were recorded to estimate density expressed as number
216 of individuals per breeding colony.

217

218 DATA EXPLORATION AND STATISTICAL ANALYSES

219 The clutch size in each nest can be assumed to be a proxy of the success of the nesting
220 male (Carriço *et al.*, 2014), but this can be hampered by high presence of sneakers (Reichard
221 *et al.*, 2004) and/or by high density of individuals in the colony (Mück *et al.*, 2013). Thus,
222 nesting males should increase the number of defence behaviours against sneakers to maximize

223 their fertilization rate. This defence behaviour could become even more predominant as number
224 of females visiting the nest increases (eggs can be deposited in a nest by a single or multiple
225 females). Starting with these assumptions and using the available variables estimated for each
226 nest (number of parents, number of sneakers, number of females, egg clutch size, proportion
227 of eggs fathered by the nesting male and defence behaviours; Table I), a principal component
228 analysis (PCA) was run. Although the dataset did not strictly meet all PCA assumptions
229 (Budaev, 2010), the variables factor map generated was used to visualise and gather a better
230 understanding of the complex relationships among the variables of this system. Such
231 relationships were also tested by fitting generalised linear models (GLM) and linear models, to
232 confirm their significance.

233 The analyses performed were: a) the relationship between the relative reproductive
234 success of nesting males and size of the egg clutch (GLM with a binomial distribution and a
235 logit link function: a column-bind matrix was created with the cbind function linking the
236 number of eggs fertilized by the nesting male with those fertilized by sneakers to consider the
237 relative frequency of nesting male success using the actual number of eggs and not proportions)
238 ; b) the relationship between the density of individuals and the size of the clutch (GLM with a
239 negative-binomial distribution and log link function, due to overdispersion of data; O'Hara &
240 Kotze, 2010); c) the relationship between the density of individuals and the reproductive
241 success of the sneakers (number of eggs not fathered by the nesting male) using a linear model.

242 Given that the main source of variability in the number of parents per nest was the
243 number of females (see results below and Table I), a Pearson correlation was run between the
244 number of females and the total number of parents per nest. A GLM (Poisson distribution and
245 log link function) was fitted using the number of females per nest as the response variable and
246 the number of defence-related behaviour per hour as the explanatory variable.

247 All GLM were tested with and without the study area as a covariate to take into account
248 general differences between the two areas; the two models for each test were compared and the
249 best one was chosen according the Akaike information criterion (AIC; Burnham, 2011). Data
250 exploration and statistical analyses were performed using R statistical software (R 2.13; R
251 Development Core Team, 2011).

252

253

RESULTS

254 Overall, 376 offspring were successfully amplified at all seven microsatellite loci (data
255 is available upon request). Microsatellite markers showed no evidence of linkage-
256 disequilibrium. H_o , on average, was not significantly different from H_e (0.77 and 0.75
257 respectively). Average number of alleles per nest was 8.08, with nest E showing the lowest and
258 nest K showing the highest number of alleles and allelic richness respectively (Table II). The
259 average number of breeders per nest, estimated by N_b , was 13.97, ranging from 2.3 (nest L) to
260 29.7 (nest K). Overall $P_{(ID)sibs}$ and $P_{(ID)unbiased}$ were respectively 4.130×10^{-4} and 2.588×10^{-13}
261 indicating that two individuals had less than 0.04% probability of sharing the same multilocus
262 genotypic profile (Table II).

263 In total, 218 mtDNA sequences were generated (Accession numbers: KX442797-
264 443014), including 49 variable sites and 38 haplotypes. The CR haplotype diversity ranged
265 from high values (0.931) to null (0.000), whilst values of nucleotide diversity ranged from
266 0.018 to 0.000 (Table II). The individual haplotype network reflected the high haplotype
267 diversity across areas (Fig. 2).

268 Of the original 376 eggs, 360 (173 from Palermo and 187 from Zingaro) showed a
269 probability of 100% to be either full- or half-sib with at least another offspring and thus, they
270 were used to infer sibship within each damselfish colony. Moreover, $P_{(ID)sibs}$ and

271 $P_{(ID)}$ unbiased indicated a negligible probability that two individuals could share the same
272 multilocus genotypic profile.

273 Overall, 201 offspring sequenced for mitochondrial CR (95 from Palermo and 106 from
274 Zingaro) were used to assign sex to the parents previously inferred by COLONY. It was possible
275 to extract clear information for nine of the twelve original nests. It was not possible to define a
276 clear pattern for the remaining three, because either the information provided by mtDNA was
277 not sufficient to discriminate between males and females (all offspring shared the same
278 haplotype, nests A and E), or provided unrealistic scenarios (both parents for each given
279 offspring showed more than one haplotype, e.g. nest B, in contrast with the assumption that
280 females transmit the same mitotype to all of their offspring). However, even in these instances,
281 we were able to gauge an idea of the number of parents contributing to each nest.

282 Cuckoldry was observed in every nest sampled. One male fathered most of the eggs in
283 each nest, on average 49% (range 27-75%) of the egg clutch; we assumed that male to be the
284 nesting one (Table I). The weighted average number of sneakers per nest was 6.48 (range 2-7);
285 on average, each sneaker fathered 7% of the egg clutch (Supplementary Table SI). The
286 weighted average number of females whose eggs were fertilised by nesting males was 6.76
287 (range 2-13), while each sneaker on average fertilised the eggs of 1.74 (range 1-8) females
288 (Supplementary Table SI). Female polygamy was also observed: 2 females (out of the 72 that
289 contributed to our sample) were found to have laid their eggs in two different nests, D and K,
290 during the same reproductive bout.

291 The first two principal components of the PCA explained more than 85% of the
292 variance of the dataset (PC1: 58.18%; PC2: 27.32%). From the PCA variables factor plot,
293 nesting male reproductive success appears to be negatively correlated with the size of the egg
294 clutch ($z = -3.309$, $p = 0.0009$), which instead is linked to sneakers reproductive success (Fig.
295 3), as supported by the GLM analysis ($z = 3.56$, $p = 0.0004$). Moreover, the density of

296 individuals in the colony and the clutch sizes show a negative relationship ($z = -4.445$, $p <$
297 0.0001), whereas the density did not affect the sneakers' reproductive success ($t = -1.696$, $p =$
298 0.134). All analyses were repeated excluding nest L, characterized by the smallest egg clutch,
299 the higher density and the lowest number of sneakers. Without this nest, the relationships
300 explained by the factor map (PC1+ PC2 = 71%) were maintained but density and number of
301 sneakers were no longer significant, while the results of all the other tests were the same.

302 In the factor map, the total number of parents per nest was associated with the number
303 of females and both showed some level of inverse relationship with the number of defence
304 behaviours performed by nesting males. Pearson correlation between the number of parents
305 and the number of females per nest was strongly significant ($r = 0.96$; $p < 0.0001$) confirming
306 that the variability in number of parents among nests was due to the variation in number of
307 females. The rate of defence behaviours showed to have a significant, negative effect on the
308 number of females contributing to each nest ($z = -2.685$, $p = 0.007$). No correlation was found
309 between the number of females and clutch size ($r = 0.54$; $p = 0.13$).

310 The AIC test suggested that adding the study area as a covariate improved only the
311 model testing for the effect of the size of the egg clutch on sneakers reproductive success, hence
312 we removed it from the other two models. In any case, there was no significant effect of the
313 study area in all the models.

314

315

DISCUSSION

316 In both *C. chromis* colonies from Palermo and Zingaro, cuckoldry was found to be
317 pervasive in every single nest analysed. This is one of few studies (Munehara & Takenaka,
318 2000; Alonzo & Heckman, 2010) that report such trends in natural marine fish populations:
319 despite the very high variability in multiple paternities across species characterised by male

320 nest defence, Coleman & Jones (2011) reported an average of 35% of nests per population
321 being fertilized by multiple males, while in the current study 100% of the nests were cuckolded.

322 The nesting male was assumed to be the individual that fertilised the majority of eggs
323 within a nest in agreement with previous studies (Coleman & Jones, 2011). In species for which
324 parentage studies are available, the proportion of eggs sired by males other than the nesting
325 one was almost always below 30% (e.g., 12.4% for molly miller, *Scartella cristata*,
326 Mackiewicz *et al.*, 2005; 15% for pumpkinseed sunfish, *Lepomis gibbosus*, Rios-Cardenas &
327 Webster, 2008; 28% in the ocellated wrasse, *Symphodus ocellatus*, Alonzo & Heckman, 2010).
328 However, *C. chromis* sneakers stand out from the “typical” reproductive success patterns
329 observed in species characterised by male nest defence. On average, 51% of the eggs were
330 sired by sneakers, with a record value of 73% observed in nest C (where eggs were fertilized
331 by seven different sneakers). Similar values (an average of 48% of eggs sired by sneakers) have
332 been found recently in the plainfin midshipman fish, *Porichthys notatus* (Cogliati *et al.*, 2013).
333 In this species though, nest takeovers have been described, which lower the paternity estimates
334 for the original nest owner (when takeovers are taken into account, the actual sneaker
335 contribution to nest decrease to 37%). The high sneaker success in the two population of *C.*
336 *chromis* under study is not surprising, given the high number of sneakers parasitizing each
337 nest in the two colonies: with the exception of nest L, the number of sires contributing to each
338 nest ranged from six to seven (Table I).

339 Multiple maternity is commonly observed in species characterised by male-nest
340 defence (Coleman & Jones, 2001 and references therein), and in this study the level of
341 polygyny was particularly high: up to 15 dams per nest were found, double the highest number
342 reported to date for the molly miller (Mackiewicz *et al.*, 2005). Nesting males are expected to
343 be polygamous; once the “reproductive resource” (i.e., the nest) is secured, their fitness is
344 mostly limited by the number of females they manage to mate with. Females, on the other hand,

345 are normally limited by the amount of eggs they can produce and are expected to exert strong
346 sexual selection by carefully choosing where to lay their eggs. Nests already containing eggs
347 may be favoured (Pruett-Jones, 1992; Brennan *et al.*, 2008) as males are less likely to desert
348 full nests (Jennions & Polakow, 2001). In this study, two females were found to lay their eggs
349 in two different nests during the same reproductive bout. A similar scenario has been rarely
350 reported (Taborsky *et al.*, 1987; Jones *et al.*, 1998). This strategy might be employed by
351 females to minimize the risk of predation on their offspring or to select males with different
352 qualities (Alonzo & Warner, 2000).

353 The size of the egg clutch was positively correlated with the reproductive success of
354 sneakers. This means that, contrary to expectations (Carrico *et al.*, 2014), the relative
355 reproductive success of the nesting male decreases when many eggs are present in his nest.
356 Indeed, a large clutch of eggs is very likely to be the result of multiple egg deposition events
357 by one or more females. Considering that the window of opportunity for a sneaker to achieve
358 parasitic fertilisation is during female spawning (as eggs are fertilised straight away; Picciulin
359 *et al.*, 2004), more egg deposition events will result in higher chances to sneak. Thus, a larger
360 clutch size possibly results in higher reproductive success for both types of males but lower
361 relative reproductive success for the cuckolded nesting males.

362 Colonial nesting with the associated high density of individuals per breeding colony
363 may favour parasitic spawning (Reichard *et al.*, 2004) and high levels of cuckoldry. Relatively
364 low reproductive success by nesting males have already been observed in the freshwater fish
365 *Variabilichromis morii* (Sefc *et al.*, 2008). Literature suggests that density of individuals plays
366 a major role in parentage patterns due to high encounter rates among conspecifics (Kokko &
367 Rankin 2006) making it easier for nesting males to attract females. Conversely, high numbers
368 of individuals are often associated with high numbers of sneakers, augmenting the chances to
369 be cuckolded (Soucy & Travis, 2003). In *C. chromis* though, clutch size seems to be negatively

370 correlated with the density of individuals in the colonies. Nesting males switch from courtship
371 to parental care when the trade-off between trying to receive more egg deposition and avoiding
372 to be cuckolded turns in favour of the latter (Kanoh, 2000). The threshold of this trade-off is
373 not fixed, and it is likely to shift in response to demographic and environmental variability.
374 Under high densities, it is possible that males perceive a higher risk of cuckoldry and shift more
375 quickly to parental care. A similar situation has been reported in *S. ocellatus*, where nesting
376 males surrounded by many sneakers can give up temporarily courtship activities by preventing
377 any female to spawn in their nest to avoid competition (Alonzo & Warner, 1999). To do so
378 effectively, males need to be able to recognise sneakers and distinguish them from females. In
379 most species a certain dimorphism between the sexes exists (e.g., in colouration and/or size)
380 and nesting males might be distinguishable from sneakers (Gross & Charnov, 1980; Taborsky
381 *et al.*, 1987). *Chromis chromis* appears to be morphologically monomorphic, making
382 individual behaviour (including acoustic behaviour) the most reliable information in
383 distinguishing males from females. Sneakers do not show significant differences in terms of
384 size when compared to nesting males (Picciulin *et al.*, 2004). They have however, been
385 reported to exhibit submissive behaviours, typical of females, when attempting to intrude in
386 the nests of guarding males (Verginella *et al.*, 1999). This behavioural adaptation, combined
387 with the lack of evident dimorphism between males exhibiting different ARTs, may explain
388 the high levels of cuckoldry (especially when densities are not high) and the relatively low
389 reproductive success of the nesting males observed. Higher density of individuals might inform
390 nesting males about the risk of cuckoldry.

391 Females must carefully pick their mates to maximise their fitness (Steinwender *et al.*,
392 2012). Defence related behaviours were found to negatively affect the number of females
393 laying eggs in the nests. Extremely dominant individuals may not always be the best choice
394 (Qvarnström & Forsgren, 1998): an excessive amount of energy or time spent in aggressive

395 interactions might cause the nesting male to neglect other important activities related to
396 parental care, such as egg fanning (Verginella *et al.*, 1999). Moreover, visually conspicuous
397 behaviours, including defence related ones, might increase the chance to be spotted by
398 predators (Daly, 1978; Crowley *et al.*, 1991; Dill *et al.*, 1999). For a potential dam an extremely
399 aggressive male might not be the best carer for her eggs and likely more exposed to predators.
400 Alternatively, the negative relationship found between the rate of defence behaviours and the
401 number of dams, could be determined by nesting males decision: when the perceived risk of
402 cuckoldry is high (i.e., high density of individuals), nesting males switch early to parental care.
403 This is achieved by actively chasing away any further female approaching the nest (Alonzo &
404 Warner, 1999).

405 Overall, this study provides new information on the mating system and, more
406 specifically, the reproductive patterns of the nest-guarding *C. chromis*. Our findings shed new
407 light on the role and consequences of ARTs in this nesting species: 1) cuckoldry and
408 promiscuity were found to be widespread; 2) the number of males and females contributing to
409 the offspring of each nest are the highest reported in literature (Coleman & Jones, 2011); 3) the
410 reproductive success of nesting males is among the lowest reported for territorial fish (average
411 49%, lower than 52% recorded by Cogliati *et al.*, 2013 in the plainfin midshipman fish, where
412 nest takeovers occur; see also reviews by Avise *et al.*, 2002; Coleman & Jones, 2011). The
413 complex interactions between factors determining the relative success of ARTs require a
414 multidisciplinary approach ranging from molecular analyses to behavioural observations and
415 from focal individuals to populations. Knowledge about these interactions becomes extremely
416 important when predicting fitness patterns in a changing environment that is increasingly
417 affected by human impacts.

418

419

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427

428 **Supporting Information**

429 Supporting Information may be found in the online version of this paper:

430 **Table SI** Relative proportion of eggs fathered by each male contributing to each nest, and
431 number of female mates (in brackets); NM: nesting male; SNK: sneaker. Subscripts on the Nest
432 category define sampling location: Z from Zingaro and P from Palermo.

433

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620 Table I. Variables used to define relationships in alternative reproductive tactics and mating success of *Chromis chromis*. Variables include:
 621 number of offspring analysed (N), number of parents (N_P), number of sneakers (N_{SNK}), number of males (N_M), number of females (N_F), proportion
 622 of offspring sired by the nesting male (NM_{off}), area of the egg clutch expressed in cm², number of defence behaviour per hour and average density
 623 of individuals at the time of sampling. Subscripts on the Nest category define sampling location; Z from Zingaro and P from Palermo.

624

Nest	N	N_P	N_{SNK}	N_M	N_F	NM_{off}	Clutch Size	Defence	Density
C _Z	26	19	7	8	11	0.27	299	31	36.33
D _P	36	23	7	8	15	0.47	204	7	28.58
F _P	32	12	6	7	5	0.38	208	43	13.33
G _Z	31	15	7	8	7	0.35	153	19	69.33
H _Z	37	18	6	7	11	0.68	117	0	76.05
I _P	42	13	7	8	5	0.67	171	22	9.33
J _Z	29	13	7	8	5	0.41	180	22	36.33
K _P	34	21	6	7	14	0.44	242	7	28.58
L _Z	8	5	2	3	2	0.75	75	23	175.67

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630 Table II. Summary statistics from microsatellites and mtDNA analyses for each nest: sample size for microsatellite (N, mtDNA in brackets),
631 unbiased expected heterozygosity (H_e), observed heterozygosity (H_o), average number of alleles (N_A), allelic richness (A_R), deviation from Hardy-
632 Weinberg equilibrium (F_{IS} , starred if significant), number of breeders (N_b , 95% C.I. in brackets), number of haplotypes (n), haplotype diversity
633 (h), nucleotide diversity (π). Subscripts on the Nest category define sampling location: Z from Zingaro and P from Palermo.
634

Nest	Microsatellites						Mitochondrial			
	N	H_e	H_o	N_A	A_R	F_{IS}	N_b	n	h	π
A _Z	44(20)	0.7026	0.7208	8.57	5.40	-0.026	10.4 (7.8 - 13.5)	1	0	0
B _Z	14(14)	0.7993	0.7551	7.57	6.88	0.057	17.6 (9.6 - 44.5)	5	0.659	0.00822
C _Z	27(20)	0.7687	0.6349	7.57	5.94	0.177*	15 (10.3 - 22.9)	3	0.689	0.01047
D _P	40(20)	0.7633	0.7607	11.14	6.77	0.003	19.6 (15.4 - 25.3)	6	0.721	0.01284
E _P	29(20)	0.7043	0.7980	4.86	4.15	-0.136*	28.7 (13.8 - 110.6)	1	0	0
F _P	33(20)	0.7278	0.7403	5.86	5.11	-0.017	7.2 (3.9 - 10.7)	3	0.611	0.00961
G _Z	32(16)	0.7744	0.7321	8.14	6.13	0.055	3.7 (3.1 - 5.3)	2	0.5	0.00423
H _Z	38(20)	0.7690	0.8421	9.57	6.54	-0.097*	11.1 (8.6 - 14.1)	5	0.768	0.00722
I _P	43(20)	0.7612	0.9003	7.43	5.44	-0.185*	7.6 (5.1 - 10.3)	3	0.563	0.01631
J _Z	29(19)	0.7131	0.7931	7.43	5.35	-0.114*	14.8 (9.8 - 23.6)	1	0	0
K _P	37(20)	0.7593	0.7799	13.43	7.66	-0.027	29.7 (22.6 - 40.7)	11	0.874	0.01924
L _Z	10(9)	0.7398	0.8286	5.43	5.43	-0.127*	2.3 (1.6 - 4.8)	2	0.556	0.01095

635

636

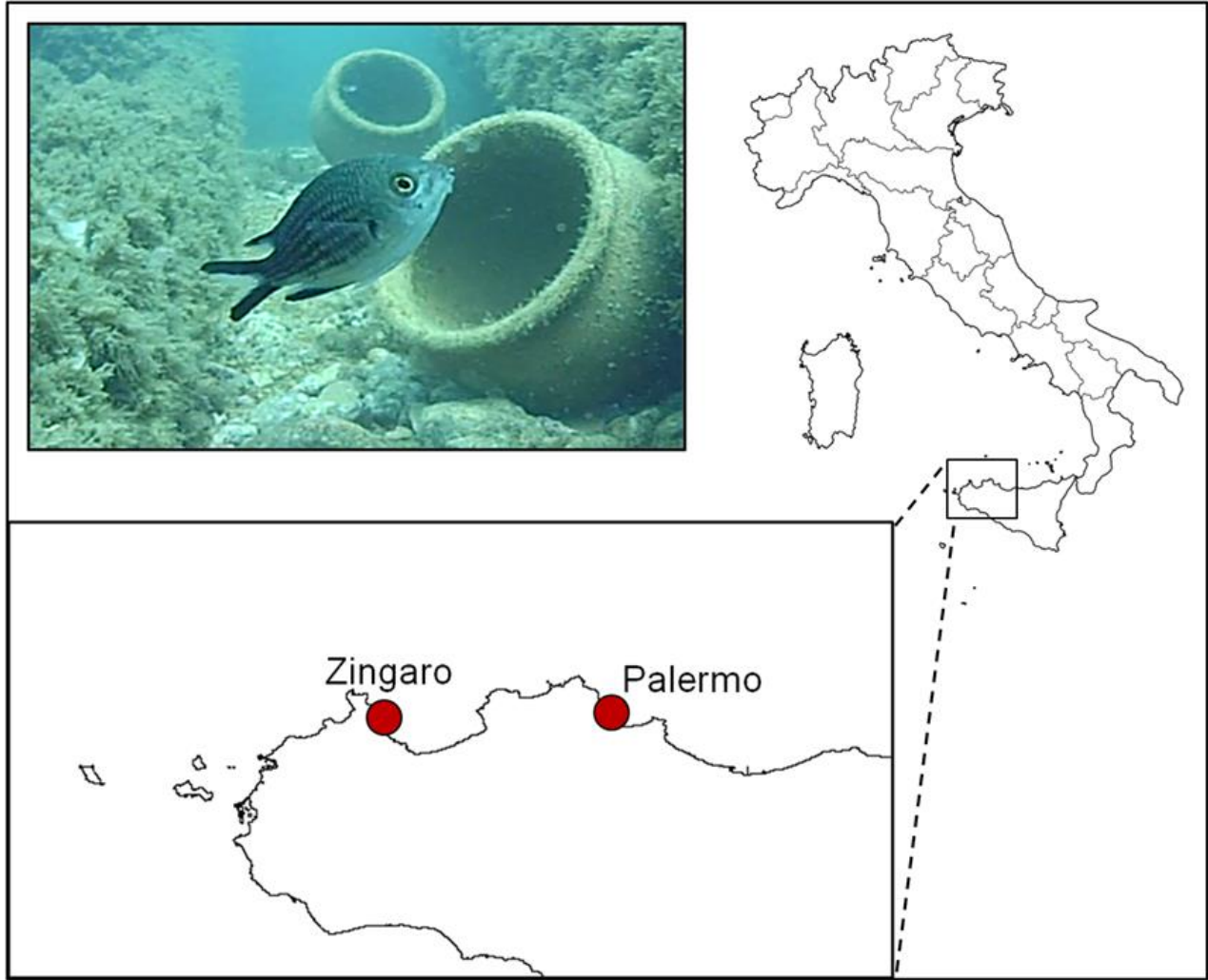
637 FIG. 1. Sampling localities (red circles) for *Chromis chromis* along the Sicilian coast. In the inset, a focal male is guarding an artificial nest

638

639 FIG. 2. Median-joining network of mtDNA haplotypes of *Chromis chromis*. The size of each circle corresponds to the relative haplotype
640 frequencies and black circles represent hypothetical intermediate haplotypes. Single mutational steps are assumed between haplotypes unless
641 specified. Nests D, E, F, I, K are from Zingaro; nests A, B, C, G, H, J, L are from Palermo.

642

643 FIG. 3. PCA variables factor map. Variables showed are: number of parents (N_P), number of sneakers (N_{SNK}), number of females (N_F), proportion
644 of offspring sired by the nesting male (NM_{off}), area of the egg clutch expressed in cm^2 , number of defence behaviour per hour and average density
645 of individuals at the time of sampling.



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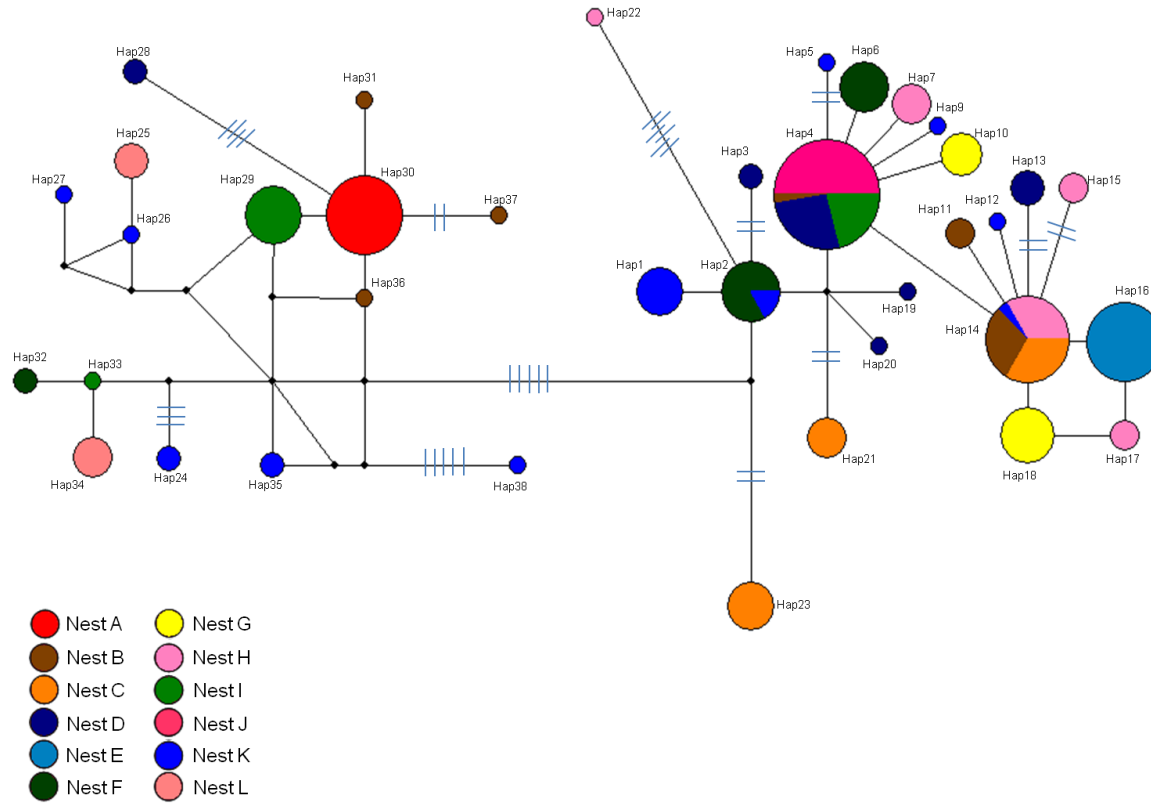
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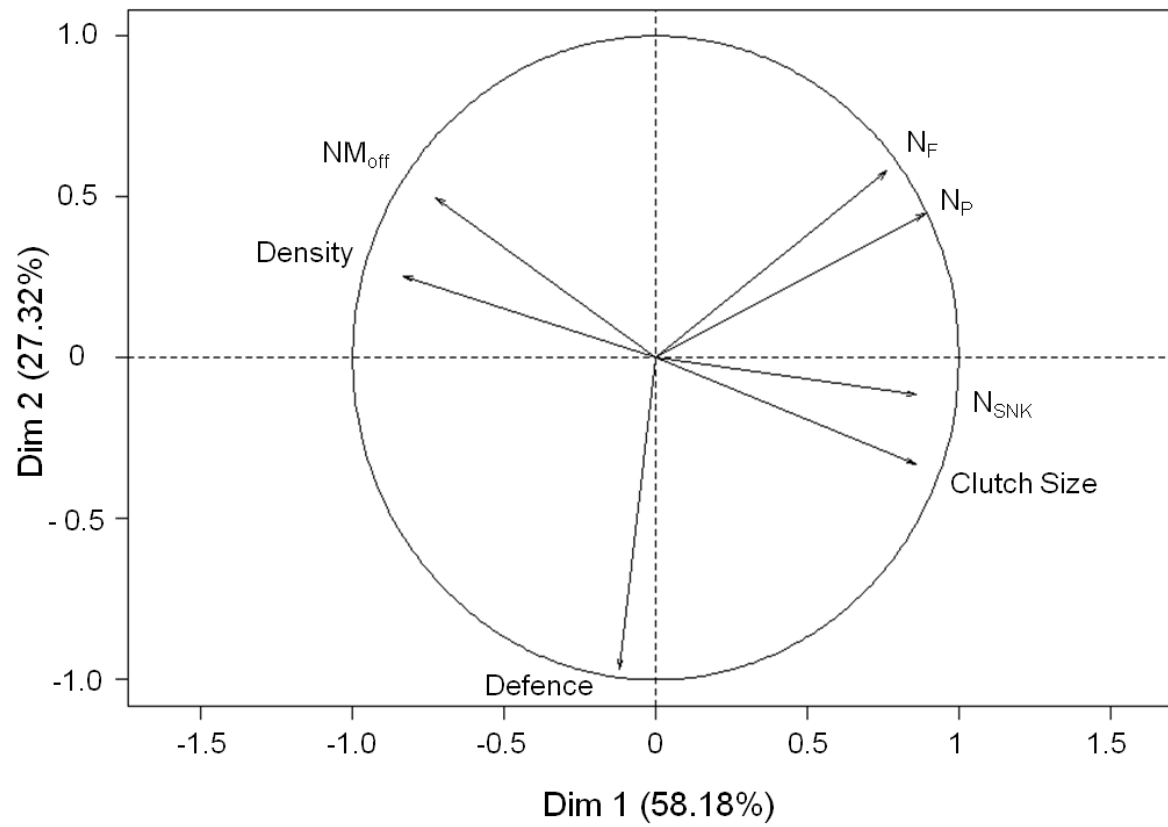
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