The ART of mating: alternative reproductive tactics and mating success in a nest-guarding fish

Mascolino, S, Benvenuto, C, Gubili, C, Sacchi, C, Boufana, B and Mariani, S

http://dx.doi.org/10.1111/jfb.13130

<table>
<thead>
<tr>
<th>Title</th>
<th>The ART of mating: alternative reproductive tactics and mating success in a nest-guarding fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors</td>
<td>Mascolino, S, Benvenuto, C, Gubili, C, Sacchi, C, Boufana, B and Mariani, S</td>
</tr>
<tr>
<td>Type</td>
<td>Article</td>
</tr>
<tr>
<td>URL</td>
<td>This version is available at: <a href="http://usir.salford.ac.uk/id/eprint/39890/">http://usir.salford.ac.uk/id/eprint/39890/</a></td>
</tr>
<tr>
<td>Published Date</td>
<td>2016</td>
</tr>
</tbody>
</table>

USIR is a digital collection of the research output of the University of Salford. Where copyright permits, full text material held in the repository is made freely available online and can be read, downloaded and copied for non-commercial private study or research purposes. Please check the manuscript for any further copyright restrictions.

For more information, including our policy and submission procedure, please contact the Repository Team at: usir@salford.ac.uk.
The ART of mating: alternative reproductive tactics and mating success in a nest-guarding fish

S. MASCOLINO†, C. BENVENUTO‡*, C. GUBILI‡, C. SACCHI†, B. BOUFANA‡§, S. MARIANI‡

† UCD School of Biology and Environmental Science, University College Dublin, Belfield, Dublin 4, Ireland (srg.mascolino@gmail.com; carlotta.sacchi@ucd.ie)
‡ Ecosystems and Environment Research Centre, School of Environment & Life Sciences, University of Salford, Salford, M5 4WT, UK (c.benvenuto@salford.ac.uk; c.gubili@googlemail.com; bboufana@yahoo.com; s.mariani@salford.ac.uk)
§Current address: European Union Reference Laboratory for Parasites; Department of Infectious, Parasitic and Immuno-mediated Diseases - Italian National Institute of Health, Rome, Italy.

*Author to whom correspondence should be addressed. Tel.: +44(0)161 2955141; email: c.benvenuto@salford.ac.uk

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

Key words: *Chromis chromis*; microsatellites; mtDNA; parentage assignment; sneakiers.
INTRODUCTION

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

While comprehensive knowledge of these mating strategies is available from a behavioural point of view, less is known about the actual relative reproductive success occurring as a consequence of coexisting ARTs (Garant et al., 2001; Reichard et al., 2004; Cogliati et al., 2013). Knowledge of the relative fitness among different tactics can help to clarify if coexisting ARTs represent a plastic/conditional strategy (dependent mainly from environmental conditions, resulting in unequal male fitness) or a genetic polymorphism (maintained by negative frequency dependent selection, resulting in overall similar male fitness) or, as recently proposed, a mixture of these two modalities, called a conditional alternative strategy (Neff & Svensson, 2013; Cogliati et al., 2014). Fitness equilibrium between ARTs can be maintained by a complex interaction of multiple variables, including individual
traits, population features and environmental characteristics. According to literature, most of
the species with male nest-defence tactics exhibit a certain level of cuckoldry (i.e., part of their
egg clutch is fertilised by other males; Coleman & Jones, 2011). Yet, the relative number of
eggs fertilised by males other than the nesting one is rather limited (Mackiewicz et al., 2005;
Rios-Cardenas & Webster, 2008; Alonzo & Heckman, 2010). Furthermore, the rate of multiple
maternity for these species is very high, indicating that polygyny is fairly common in these
species, but the average number of dams contributing to a single nest is low (three females on
average; Coleman & Jones, 2011).

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

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

**MATERIALS AND METHODS**

**STUDY AREA AND SAMPLE COLLECTION**

Two damselfish colonies were selected 5-10 m from the coasts of Palermo and Zingaro, Sicily, Italy (Fig. 1) in May 2011. Twenty-five spherical flowerpots (18 cm diameter; Knapp & Kovach, 1991) were placed at 7-12 m depth to provide males with artificial nests (minimising differences in nests’ quality and size; Fig. 1). Overall, twelve artificial nests (five from Palermo and seven from Zingaro) were successfully colonised by *C. chromis*. Colonized nests were monitored with video cameras during June and August 2011. At the end of the behavioural observations, artificial nests (which contained all the eggs attached to the pot surface on a single layer) were collected. Given their fast swimming behaviour, it was not possible to catch the nesting males. Once on the boat, the relative quantity of eggs present in
each nest was estimated (given their small size, it was not possible to count all the eggs). All egg clutches exhibited a diamond shape; minor and major axes were measured to calculate the surface area of each egg clutch (cm$^2$). All eggs were detached from the flowerpot using a scalpel and immediately stored in 100% ethanol and subsequently at -20°C.

DNA ISOLATION, PCR AMPLIFICATION, GENOTYPING AND SEQUENCING

Single eggs were separated under a stereomicroscope. DNA was isolated from a random subsample of 48 eggs from each nest (total N = 576) using a modified salt extraction protocol (Miller et al., 1988) and 376 eggs (30-48 per nest) were successfully amplified at seven microsatellite loci. Primers from four species of the Pomacentridae family were employed: 2AL2 (Abudefduf luridus; Carvalho et al., 2000), Cm_D006 (Chromis margaritifer; Underwood, 2009), Da360, Da542, Da589, Da590 (Dascyllus aruanus; Fauvelot et al., 2009) and SpTG53 (Stegastes partitus; Thiessen & Heath, 2007). Loci were amplified in two multiplex polymerase chain reactions (PCR). Each reaction was carried out in a total volume of 12 µl, using 6 µl of QIAGEN® Multiplex PCR kit, 4.4 µl of DNA template and 1.6 µl of primer mix. Fluorescently-labelled primers (FAM, VIC, NED and PET) were added with the following concentrations: Cm_D006 0.17 µM, 2AL2 0.17 µM, SpTG53 0.33 µM (multiplex 1); Da542 0.17 µM, Da589 0.17 µM, Da590 0.17 µM, Da360 0.08 µM (multiplex 2). Amplification conditions were as follows: 95°C for 15 min; 37 cycles of 94°C for 45 s, 52°C for 1 min, 72°C for 45 s and a final extension at 72°C for 45 min (multiplex 1); 95°C for 15 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 45 min (multiplex 2). PCR reactions were performed using a Biometra T3000 thermocycler. Allele sizes were determined on an ABI-3130xl Genetic Analyser (Applied Biosystems©) with an internal size standard (600 LIZ, Applied Biosystems©). Genotypic data were acquired using
GeneMapper 4.0 (Applied Biosystems©). Following standard practice, we re-amplified and re-scored genotypes for 10% (30 eggs) to check for consistency of genotype calling.

Furthermore, amplification of 355 bp of the mitochondrial control region (CR) was carried out on a subsample of eggs from each nest (total N = 222) using the primers CR-A and CR-E (Lee et al., 1995, Domingues et al., 2005) in a 25 µl reaction volume, according to the following protocol: NH₄ Buffer X1, dNTPs 800 µM (200 µM each), CR-A 0.3 µM, CR-E 0.3 µM, MgCl₂ 2.5 mM, Taq 2.5 U (BIOLINE), 1 µl template. Amplification conditions were as 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 were sequenced commercially (Beckman-Coulter Genomics). D-loop sequences were submitted to GenBank under the accession numbers KX442797-443014.

POPULATION GENETIC ANALYSES

Expected unbiased (Hₑ) and observed (Hₒ) heterozygosities and average number of alleles (Nₐ) per nest were calculated using the Microsatellite Toolkit add-in available for Microsoft Excel (Park, 2001). Allelic richness (Aₑ), departure from Hardy-Weinberg equilibrium (by calculating FₑIS and testing significance through 1320 permutations) and linkage disequilibrium were estimated using FSTAT 2.9.3.2 (Goudet, 1995).

The effective number of breeders (Nₑ) for each nest was initially investigated by computing the effective population size (Nₑ) for each nest, using the gametic disequilibrium method implemented in the software LDNe 1.31 (Waples & Do, 2008). Additionally, probability of individual identity, Pₑ(ID), was calculated for all seven loci with GIMLET (Valière, 2002); Pₑ(ID) is defined as the chance that two individuals drawn at random from the same population will share the same genotypic profile at multiple loci (Waits et al., 2001).

GIMLET allows to calculate both Pₑ(ID)sib (the Pₑ(ID) of a population where siblings are found and included, Evett & Weir, 1998) and Pₑ(ID)unbiased (the Pₑ(ID) after sample size corrections;
Paetkau et al., 1998); the observed $P_{(ID)}$ lays between $P_{(ID)_{sib}}$ and $P_{(ID)_{unbiased}}$ and is estimated by computing the proportion of all possible pairs of individuals that have identical genotypes.

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

RELATEDNESS ANALYSIS AND PARENTAGE ASSIGNMENT

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

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

BEHAVIOURAL OBSERVATIONS

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

DATA EXPLORATION AND STATISTICAL ANALYSES

The clutch size in each nest can be assumed to be a proxy of the success of the nesting male (Carriço et al., 2014), but this can be hampered by high presence of sneakers (Reichard et al., 2004) and/or by high density of individuals in the colony (Mück et al., 2013). Thus, nesting males should increase the number of defence behaviours against sneakers to maximize...
their fertilization rate. This defence behaviour could become even more predominant as number
of females visiting the nest increases (eggs can be deposited in a nest by a single or multiple
females). Starting with these assumptions and using the available variables estimated for each
nest (number of parents, number of sneakers, number of females, egg clutch size, proportion
of eggs fathered by the nesting male and defence behaviours; Table I), a principal component
analysis (PCA) was run. Although the dataset did not strictly meet all PCA assumptions
(Budaev, 2010), the variables factor map generated was used to visualise and gather a better
understanding of the complex relationships among the variables of this system. Such
relationships were also tested by fitting generalised linear models (GLM) and linear models, to
confirm their significance.

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

Given that the main source of variability in the number of parents per nest was the
number of females (see results below and Table I), a Pearson correlation was run between the
number of females and the total number of parents per nest. A GLM (Poisson distribution and
log link function) was fitted using the number of females per nest as the response variable and
the number of defence-related behaviour per hour as the explanatory variable.
All GLM were tested with and without the study area as a covariate to take into account general differences between the two areas; the two models for each test were compared and the best one was chosen according the Akaike information criterion (AIC; Burnham, 2011). Data exploration and statistical analyses were performed using R statistical software (R 2.13; R Development Core Team, 2011).

RESULTS

Overall, 376 offspring were successfully amplified at all seven microsatellite loci (data is available upon request). Microsatellite markers showed no evidence of linkage-disequilibrium. $H_0$, on average, was not significantly different from $H_e$ (0.77 and 0.75 respectively). Average number of alleles per nest was 8.08, with nest E showing the lowest and nest K showing the highest number of alleles and allelic richness respectively (Table II). The average number of breeders per nest, estimated by $N_b$, was 13.97, ranging from 2.3 (nest L) to 29.7 (nest K). Overall $P_{(ID)sibs}$ and $P_{(ID)unbiased}$ were respectively $4.130 \times 10^{-4}$ and $2.588 \times 10^{-13}$ indicating that two individuals had less than 0.04% probability of sharing the same multilocus genotypic profile (Table II).

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

Of the original 376 eggs, 360 (173 from Palermo and 187 from Zingaro) showed a probability of 100% to be either full- or half-sib with at least another offspring and thus, they were used to infer sibship within each damselfish colony. Moreover, $P_{(ID)sibs}$ and
271 $P_{\text{ID}}$ unbiased indicated a negligible probability that two individuals could share the same multilocus genotypic profile.

273 Overall, 201 offspring sequenced for mitochondrial CR (95 from Palermo and 106 from Zingaro) were used to assign sex to the parents previously inferred by COLONY. It was possible to extract clear information for nine of the twelve original nests. It was not possible to define a clear pattern for the remaining three, because either the information provided by mtDNA was not sufficient to discriminate between males and females (all offspring shared the same haplotype, nests A and E), or provided unrealistic scenarios (both parents for each given offspring showed more than one haplotype, e.g. nest B, in contrast with the assumption that females transmit the same mitotype to all of their offspring). However, even in these instances, 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 each nest, on average 49% (range 27-75%) of the egg clutch; we assumed that male to be the nesting one (Table I). The weighted average number of sneakers per nest was 6.48 (range 2-7); on average, each sneaker fathered 7% of the egg clutch (Supplementary Table SI). The weighted average number of females whose eggs were fertilised by nesting males was 6.76 (range 2-13), while each sneaker on average fertilised the eggs of 1.74 (range 1-8) females (Supplementary Table SI). Female polygamy was also observed: 2 females (out of the 72 that contributed to our sample) were found to have laid their eggs in two different nests, D and K, during the same reproductive bout.

291 The first two principal components of the PCA explained more than 85% of the variance of the dataset (PC1: 58.18%; PC2: 27.32%). From the PCA variables factor plot, nesting male reproductive success appears to be negatively correlated with the size of the egg clutch ($z = -3.309$, $p = 0.0009$), which instead is linked to sneakers reproductive success (Fig. 3), as supported by the GLM analysis ($z = 3.56$, $p = 0.0004$). Moreover, the density of
individuals in the colony and the clutch sizes show a negative relationship \((z = -4.445, p < 0.0001)\), whereas the density did not affect the sneakers’ reproductive success \((t = -1.696, p = 0.134)\). All analyses were repeated excluding nest L, characterized by the smallest egg clutch, the higher density and the lowest number of sneakers. Without this nest, the relationships explained by the factor map \((PC1 + PC2 = 71\%)\) were maintained but density and number of sneakers were no longer significant, while the results of all the other tests were the same.

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

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

**DISCUSSION**

In both *C. chromis* colonies from Palermo and Zingaro, cuckoldry was found to be pervasive in every single nest analysed. This is one of few studies (Munehara & Takenaka, 2000; Alonzo & Heckman, 2010) that report such trends in natural marine fish populations: despite the very high variability in multiple paternities across species characterised by male
nest defence, Coleman & Jones (2011) reported an average of 35% of nests per population being fertilized by multiple males, while in the current study 100% of the nests were cuckolded.

The nesting male was assumed to be the individual that fertilised the majority of eggs within a nest in agreement with previous studies (Coleman & Jones, 2011). In species for which parentage studies are available, the proportion of eggs sired by males other than the nesting one was almost always below 30% (e.g., 12.4% for molly miller, Scartella cristata, Mackiewicz et al., 2005; 15% for pumpkinseed sunfish, Lepomis gibbosus, Rios-Cardenas & Webster, 2008; 28% in the ocellated wrasse, Symphodus ocellatus, Alonzo & Heckman, 2010).

However, C. chromis sneakers stand out from the “typical” reproductive success patterns observed in species characterised by male nest defence. On average, 51% of the eggs were sired by sneakers, with a record value of 73% observed in nest C (where eggs were fertilized by seven different sneakers). Similar values (an average of 48% of eggs sired by sneakers) have been found recently in the plainfin midshipman fish, Porichthys notatus (Cogliati et al., 2013).

In this species though, nest takeovers have been described, which lower the paternity estimates for the original nest owner (when takeovers are taken into account, the actual sneaker contribution to nest decrease to 37%). The high sneaker success in the two population of C. chromis under study is not surprising, given the high number of sneakers parasitizing each nest in the two colonies: with the exception of nest L, the number of sires contributing to each nest ranged from six to seven (Table I).

Multiple maternity is commonly observed in species characterised by male-nest defence (Coleman & Jones, 2001 and references therein), and in this study the level of polygyny was particularly high: up to 15 dams per nest were found, double the highest number reported to date for the molly miller (Mackiewicz et al., 2005). Nesting males are expected to be polygamous; once the “reproductive resource” (i.e., the nest) is secured, their fitness is mostly limited by the number of females they manage to mate with. Females, on the other hand,
are normally limited by the amount of eggs they can produce and are expected to exert strong
sexual selection by carefully choosing where to lay their eggs. Nests already containing eggs
may be favoured (Pruett-Jones, 1992; Brennan et al., 2008) as males are less likely to desert
full nests (Jennions & Polakow, 2001). In this study, two females were found to lay their eggs
in two different nests during the same reproductive bout. A similar scenario has been rarely
reported (Taborsky et al., 1987; Jones et al., 1998). This strategy might be employed by
females to minimize the risk of predation on their offspring or to select males with different
qualities (Alonzo & Warner, 2000).

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

Colonial nesting with the associated high density of individuals per breeding colony
may favour parasitic spawning (Reichard et al., 2004) and high levels of cuckoldry. Relatively
low reproductive success by nesting males have already been observed in the freshwater fish
Variabilichromis morii (Sefc et al., 2008). Literature suggests that density of individuals plays
a major role in parentage patterns due to high encounter rates among conspecifics (Kokko &
Rankin 2006) making it easier for nesting males to attract females. Conversely, high numbers
of individuals are often associated with high numbers of sneakers, augmenting the chances to
be cuckolded (Soucy & Travis, 2003). In C. chromis though, clutch size seems to be negatively
correlated with the density of individuals in the colonies. Nesting males switch from courtship to parental care when the trade-off between trying to receive more egg deposition and avoiding to be cuckolded turns in favour of the latter (Kanoh, 2000). The threshold of this trade-off is not fixed, and it is likely to shift in response to demographic and environmental variability. Under high densities, it is possible that males perceive a higher risk of cuckoldry and shift more quickly to parental care. A similar situation has been reported in *S. ocellatus*, where nesting males surrounded by many sneakers can give up temporarily courtship activities by preventing any female to spawn in their nest to avoid competition (Alonzo & Warner, 1999). To do so effectively, males need to be able to recognise sneakers and distinguish them from females. In most species a certain dimorphism between the sexes exists (e.g., in colouration and/or size) and nesting males might be distinguishable from sneakers (Gross & Charnov, 1980; Taborsky *et al.*, 1987). *Chromis chromis* appears to be morphologically monomorphic, making individual behaviour (including acoustic behaviour) the most reliable information in distinguishing males from females. Sneakers do not show significant differences in terms of size when compared to nesting males (Picciulin *et al.*, 2004). They have however, been reported to exhibit submissive behaviours, typical of females, when attempting to intrude in the nests of guarding males (Veriginella *et al.*, 1999). This behavioural adaptation, combined with the lack of evident dimorphism between males exhibiting different ARTs, may explain the high levels of cuckoldry (especially when densities are not high) and the relatively low reproductive success of the nesting males observed. Higher density of individuals might inform nesting males about the risk of cuckoldry.

Females must carefully pick their mates to maximise their fitness (Steinwender *et al.*, 2012). Defence related behaviours were found to negatively affect the number of females laying eggs in the nests. Extremely dominant individuals may not always be the best choice (Qvarnström & Forsgren, 1998): an excessive amount of energy or time spent in aggressive
interactions might cause the nesting male to neglect other important activities related to parental care, such as egg fanning (Verginella et al., 1999). Moreover, visually conspicuous behaviours, including defence related ones, might increase the chance to be spotted by predators (Daly, 1978; Crowley et al., 1991; Dill et al., 1999). For a potential dam an extremely aggressive male might not be the best carer for her eggs and likely more exposed to predators. Alternatively, the negative relationship found between the rate of defence behaviours and the number of dams, could be determined by nesting males decision: when the perceived risk of cuckoldry is high (i.e., high density of individuals), nesting males switch early to parental care. This is achieved by actively chasing away any further female approaching the nest (Alonzo & Warner, 1999).

Overall, this study provides new information on the mating system and, more specifically, the reproductive patterns of the nest-guarding _C. chromis_. Our findings shed new light on the role and consequences of ARTs in this nesting species: 1) cuckoldry and promiscuity were found to be widespread; 2) the number of males and females contributing to the offspring of each nest are the highest reported in literature (Coleman & Jones, 2011); 3) the reproductive success of nesting males is among the lowest reported for territorial fish (average 49%, lower than 52% recorded by Cogliati et al., 2013 in the plainfin midshipman fish, where nest takeovers occur; see also reviews by Avise et al., 2002; Coleman & Jones, 2011). The complex interactions between factors determining the relative success of ARTs require a multidisciplinary approach ranging from molecular analyses to behavioural observations and from focal individuals to populations. Knowledge about these interactions becomes extremely important when predicting fitness patterns in a changing environment that is increasingly affected by human impacts.
We would like to thank: Jon Yearsley for statistical advice, Natalia Niceta, Jen Coughlan and
Siobhán Bradley, Kelly Hickey, Emma Lawlor, Meabh Nic Mhathúna, Claire Morton and
Martina O’Brien for their help in the field, Stephen Woodward for his help in the lab. We are
grateful to two anonymous reviewers for their constructive comments on a previous version of
this manuscript. This study was supported by the Irish Research Council (IRC) under the
Embark Initiative (RS/2010/2106). Ethical approval was obtained by UCD, University College
Dublin (AREC-P-10-33).

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

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

References
Alonzo, S. H. & Heckman, K. L. (2010). The unexpected but understandable dynamics of
mating, paternity, and paternal care in the ocellated wrasse. *Proceedings of the Royal
Society of London B*, 277, 115-122.

Alonzo, S. H. & Warner, R. R. (2000). Dynamic games and field experiments examining intra-
and intersexual conflict: explaining counterintuitive mating behavior in a Mediterranean

wrasse males refuse present mates to increase future success. *Behavioral Ecology*, 10, 105-
111.


Table I. Variables used to define relationships in alternative reproductive tactics and mating success of *Chromis chromis*. Variables include: 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 of offspring sired by the nesting male (N_M_off), area of the egg clutch expressed in cm², number of defence behaviour per hour and average density of individuals at the time of sampling. Subscripts on the Nest category define sampling location; Z from Zingaro and P from Palermo.

<table>
<thead>
<tr>
<th>Nest</th>
<th>N</th>
<th>N_P</th>
<th>N_SNK</th>
<th>N_M</th>
<th>N_F</th>
<th>N_M_off</th>
<th>Clutch Size</th>
<th>Defence</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_Z</td>
<td>26</td>
<td>19</td>
<td>7</td>
<td>8</td>
<td>11</td>
<td>0.27</td>
<td>299</td>
<td>31</td>
<td>36.33</td>
</tr>
<tr>
<td>D_P</td>
<td>36</td>
<td>23</td>
<td>7</td>
<td>8</td>
<td>15</td>
<td>0.47</td>
<td>204</td>
<td>7</td>
<td>28.58</td>
</tr>
<tr>
<td>F_P</td>
<td>32</td>
<td>12</td>
<td>6</td>
<td>7</td>
<td>5</td>
<td>0.38</td>
<td>208</td>
<td>43</td>
<td>13.33</td>
</tr>
<tr>
<td>G_Z</td>
<td>31</td>
<td>15</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>0.35</td>
<td>153</td>
<td>19</td>
<td>69.33</td>
</tr>
<tr>
<td>H_Z</td>
<td>37</td>
<td>18</td>
<td>6</td>
<td>7</td>
<td>11</td>
<td>0.68</td>
<td>117</td>
<td>0</td>
<td>76.05</td>
</tr>
<tr>
<td>I_P</td>
<td>42</td>
<td>13</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>0.67</td>
<td>171</td>
<td>22</td>
<td>9.33</td>
</tr>
<tr>
<td>J_Z</td>
<td>29</td>
<td>13</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>0.41</td>
<td>180</td>
<td>22</td>
<td>36.33</td>
</tr>
<tr>
<td>K_P</td>
<td>34</td>
<td>21</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>0.44</td>
<td>242</td>
<td>7</td>
<td>28.58</td>
</tr>
<tr>
<td>L_Z</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0.75</td>
<td>75</td>
<td>23</td>
<td>175.67</td>
</tr>
</tbody>
</table>
Table II. Summary statistics from microsatellites and mtDNA analyses for each nest: sample size for microsatellite (N, mtDNA in brackets), unbiased expected heterozygosity ($H_e$), observed heterozygosity ($H_o$), average number of alleles ($N_A$), allelic richness ($A_R$), deviation from Hardy-Weinberg equilibrium ($F_{IS}$, starred if significant), number of breeders ($N_b$, 95% C.I. in brackets), number of haplotypes ($n$), haplotype diversity (h), nucleotide diversity ($\pi$). Subscripts on the Nest category define sampling location: Z from Zingaro and P from Palermo.

<table>
<thead>
<tr>
<th>Nest</th>
<th>N</th>
<th>$H_e$</th>
<th>$H_o$</th>
<th>$N_A$</th>
<th>$A_R$</th>
<th>$F_{IS}$</th>
<th>$N_b$</th>
<th>n</th>
<th>h</th>
<th>$\pi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_Z</td>
<td>44(20)</td>
<td>0.7026</td>
<td>0.7208</td>
<td>8.57</td>
<td>5.40</td>
<td>-0.026</td>
<td>10.4 (7.8 - 13.5)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B_Z</td>
<td>14(14)</td>
<td>0.7993</td>
<td>0.7551</td>
<td>7.57</td>
<td>6.88</td>
<td>0.057</td>
<td>17.6 (9.6 - 44.5)</td>
<td>5</td>
<td>0.659</td>
<td>0.00822</td>
</tr>
<tr>
<td>C_Z</td>
<td>27(20)</td>
<td>0.7687</td>
<td>0.6349</td>
<td>7.57</td>
<td>5.94</td>
<td>0.177*</td>
<td>15 (10.3 – 22.9)</td>
<td>3</td>
<td>0.689</td>
<td>0.01047</td>
</tr>
<tr>
<td>D_P</td>
<td>40(20)</td>
<td>0.7633</td>
<td>0.7607</td>
<td>11.14</td>
<td>6.77</td>
<td>0.003</td>
<td>19.6 (15.4 – 25.3)</td>
<td>6</td>
<td>0.721</td>
<td>0.01284</td>
</tr>
<tr>
<td>E_P</td>
<td>29(20)</td>
<td>0.7043</td>
<td>0.7980</td>
<td>4.86</td>
<td>4.15</td>
<td>-0.136*</td>
<td>28.7 (13.8 – 110.6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F_P</td>
<td>33(20)</td>
<td>0.7278</td>
<td>0.7403</td>
<td>5.86</td>
<td>5.11</td>
<td>-0.017</td>
<td>7.2 (3.9 – 10.7)</td>
<td>3</td>
<td>0.611</td>
<td>0.00961</td>
</tr>
<tr>
<td>G_Z</td>
<td>32(16)</td>
<td>0.7744</td>
<td>0.7321</td>
<td>8.14</td>
<td>6.13</td>
<td>0.055</td>
<td>3.7 (3.1 – 5.3)</td>
<td>2</td>
<td>0.5</td>
<td>0.00423</td>
</tr>
<tr>
<td>H_Z</td>
<td>38(20)</td>
<td>0.7690</td>
<td>0.8421</td>
<td>9.57</td>
<td>6.54</td>
<td>-0.097*</td>
<td>11.1 (8.6 – 14.1)</td>
<td>5</td>
<td>0.768</td>
<td>0.00722</td>
</tr>
<tr>
<td>I_P</td>
<td>43(20)</td>
<td>0.7612</td>
<td>0.9003</td>
<td>7.43</td>
<td>5.44</td>
<td>-0.185*</td>
<td>7.6 (5.1 – 10.3)</td>
<td>3</td>
<td>0.563</td>
<td>0.01631</td>
</tr>
<tr>
<td>J_Z</td>
<td>29(19)</td>
<td>0.7131</td>
<td>0.7931</td>
<td>7.43</td>
<td>5.35</td>
<td>-0.114*</td>
<td>14.8 (9.8 – 23.6)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K_P</td>
<td>37(20)</td>
<td>0.7593</td>
<td>0.7799</td>
<td>13.43</td>
<td>7.66</td>
<td>-0.027</td>
<td>29.7 (22.6 – 40.7)</td>
<td>11</td>
<td>0.874</td>
<td>0.01924</td>
</tr>
<tr>
<td>L_Z</td>
<td>10(9)</td>
<td>0.7398</td>
<td>0.8286</td>
<td>5.43</td>
<td>5.43</td>
<td>-0.127*</td>
<td>2.3 (1.6 – 4.8)</td>
<td>2</td>
<td>0.556</td>
<td>0.01095</td>
</tr>
</tbody>
</table>
FIG. 1. Sampling localities (red circles) for *Chromis chromis* along the Sicilian coast. In the inset, a focal male is guarding an artificial nest.

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

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 of offspring sired by the nesting male (N_NM_off), area of the egg clutch expressed in cm^2, number of defence behaviour per hour and average density of individuals at the time of sampling.