Heritability and adaptive significance of the number of egg-dummies in the cichlid fish *Astatotilapia burtoni*

Topi K. Lehtonen\(^1,2\) and Axel Meyer\(^1,\ast\)

\(^1\)Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, 78457 Konstanz, Germany

\(^2\)Section of Ecology, Department of Biology, University of Turku, 20014 Turku, Finland

Cichlid fishes are a textbook example of rapid speciation and exuberant diversity—this applies especially to haplochromines, a lineage with approximately 1800 species. Haplochrome males uniquely possess oval, bright spots on their anal fin, called ‘egg-spots’ or ‘egg-dummies’. These are presumed to be an evolutionary key innovation that contributed to the tribe’s evolutionary success. Egg-spots have been proposed to mimic the ovum of the mouthbrooding females of the corresponding species, contribute to fertilization success and even facilitate species recognition. Interestingly, egg-spot number varies extensively not only between species, but also within some populations. This high degree of intraspecific variation may appear to be counterintuitive since selection might be expected to act to stabilize traits that are correlated with fitness measures. We addressed this ‘paradox’ experimentally, and found that in the haplochromine cichlid *Astatotilapia burtoni*, the number of egg-spots was related to male age, body condition and dominance status. Intriguingly, the egg-spot number also had a high heritable component (narrow sense heritability of 0.5). These results suggest that the function of egg-spots might have less to do with fertilization success or species recognition, but rather relate to mate choice and/or male–male competition, helping to explain the high variability in this important trait.

**Keywords:** body condition; dominance hierarchy; narrow sense heritability; intraspecific variation; key innovation; signal value

1. INTRODUCTION

A famously high number of species, and unsurpassed rates of evolution, have made cichlid fishes of the great East African lakes one of the most remarkable examples of adaptive radiation [1–4]. The great majority of these species (approximately 1800 of the 2500 species in the family Cichlidae) belong to a single lineage, the haplochromine cichlids [5]. The anal fin of a sexually mature haplochrome male carries bright yellow or orange ovoid spots, which have been suggested to mimic real eggs of the species, and have therefore been called egg-dummies [6]. Females of some species also have spots, but these are less pronounced and on average lower in number. Interestingly, the less diverse lineages of cichlids do not possess similar egg-spots [7], and indeed, possession of egg-dummies may have promoted speciation within the group [5,8] and have been considered to be a key innovation that has contributed to the evolutionary success of haplochromines [5,7]. As a consequence of the particular mating system of female mouthbrooding and the concomitant evolution of egg-spots, these fish are limited by the number of eggs that fit into a female’s mouth (typically only 20–40), which might have contributed to smaller populations sizes and hence faster rates of evolution [5,7].

In haplochromines, fertilization typically takes place in the mouth of the female after she has nipped at the male’s egg-dummies, which are located near the male’s genital opening (figure 1; see the electronic supplementary material, video clip; see also [7]). Hence, egg-spots have been suggested to enhance fertilization success and facilitate spawning ([6], but see [9]). A few other functions for the spots have also been proposed (e.g. [8,10]), such as certainty of paternity and species recognition. Indeed, arrangement and number of egg-spots differs between haplochromine species [7,10–12]. Interestingly, in some species the number of egg-spots is not fixed, but varies among individuals of a population. For example, in a natural breeding colony of *A. burtoni*, the number of egg-spots ranged between five and nine [13]. Similarly, sibling males of a Lake Malawi haplochrome species (*Labeotropheus*) were reported to have from two to six egg-spots [14]. This type of variation calls for an explanation, as traits important for successful fertilization, species recognition or fitness, in general, are expected to be under stabilizing selection, which should erode heritable genetic variation behind these traits (e.g. [15]). However, so far the sources for this variation in egg-spots, an evolutionary key trait [5], have not been investigated.

We examined within-species variation in the number of egg-spots in the haplochromine cichlid *A. burtoni*, a widely used model system in behavioural endocrinology and genomics [16–22]. The species lives in ponds and rivers around Lake Tanganyika, East Africa. As
2. MATERIAL AND METHODS

The experiments were conducted in the animal care facility at the University of Konstanz, Germany, under a 12 L: 12 D cycle and water temperature of 25 ± 2 °C. The fish were fed daily with commercial fish foods if not otherwise noted. Juveniles were also fed with newly hatched brine shrimps (Artemia). Under these conditions, A. burtoni reaches sexual maturity and starts to breed at the age of approximately four months, with even the smallest and most subdominant individuals maturing in six months (T. K. Lehtonen & A. Meyer, 2008, personal observations), having attained a total length of 6–6.5 cm ([26]; T. K. Lehtonen & A. Meyer, 2008, personal observations). In the wild, the exact population structure seems to depend on the habitat [26,27], but most breeding populations are likely to consist of young individuals due to heavy natural predation and fishery [26,27]. Under these conditions, males do not seem to survive long enough to exceed the total length of 10–12 cm [26], although in captivity the species can ultimately attain a larger size (15 cm or even larger) and live for several years. However, many individuals start to show signs of senescence (ragged fins, deformations, increased mortality and decreased rate of breeding) even before the age of 2 years (T. K. Lehtonen & A. Meyer, 2008, personal observations). Accordingly, we used young, mature males to assess the following five explanations for the high within-population variation in the number of egg-spots in male A. burtoni.

(a) Explanation 1: body size

To assess whether there is a relationship between male size and the number or size of his egg-spots, we photographed four different groups of males: (1) 64 males of the age of 7.5 months (the second generation from ‘explanation 5’, see below), (2) 12 relatively young, mature males (age 10.5 months), (3) another of group of males of the same age (10.5 months, n = 15) and (4) 12 older, large males (16 months). At least two photographs of each male were taken on a grid paper that was covered with a smooth, transparent plastic sheet. Two photographs of each male were later analysed using Sigma Scan Pro 5.0 (SPSS Inc.) software, and average values from these measurements were used for statistical analyses. Where relevant, this same photography procedure was also used in other experiments, described below. We counted as an egg-spot a bright, ovoid-shaped spot on the male’s anal fin with a rim of lighter coloration around it (figure 1, see also [7]).

(b) Explanation 2: changes over time

Different stocking conditions (most importantly tank size, competitive regime and social background) of the different (age) groups of ‘explanation 1’ did not allow formal tests over these groups. Hence, in order to investigate whether the number (or size) of egg-spots changes over time (and hence presumably with age), males of the groups 2–4 were photographed again after a period of time. Specifically, we kept the males of group 2 singly for two months in tanks measuring 50 × 25 × 30 cm (length × width × height), the males of group 3 in contact with 2–4 conspecifics in tanks measuring 60 × 40 × 35 cm for 1–2 months, and the males of group 4 singly for three months in the 50 × 25 × 30 cm tanks.

(c) Explanation 3: condition dependence

In this experiment, we examined whether the number or size of egg-spots depends on body condition of the male. Immature juveniles that did not yet possess egg-spots (age under three months) were divided into two treatments: food restricted individuals (n = 18) were given food on 3 days a week, whereas control individuals were fed daily (n = 18). The same number (1–5) of individuals from any one family was used for both treatments. All male offspring (n = 10 in both treatments) were photographed at the age of 7.5 months. Besides comparing egg-spot number and size directly between the treatments, we also ran a general linear model (GLM) on egg-spot number, with total length as a
continuous factor and treatment as a categorical factor. After the interaction between variables ‘total length’ and ‘treatment’ was found to be non-significant, we removed it from the model and proceeded with assessing the main effects.

(d) Explanation 4: dominance hierarchy

Earlier work on A. burtoni has shown that dominant, territorial males have higher circulating plasma concentrations of androgens than subdominant, non-territorial males [17], but males are nevertheless able to switch between the two territorial stages within minutes depending on the immediate social environment [16]. We had two different set-ups to test for any relationship between male dominance and the number or size of his egg-spots. First, the most dominant male in 14 different family groups (see ‘explanation 5’, below) was identified (as distinguished by coloration and interactions among the individuals in the tank) and compared with the rest of the males of that family. In one case we took an average over three males that appeared to be equally dominant. The sample size for this measurement was 13 because one of the 14 families had only a single male. Secondly, in a separate experiment, two visually size-matched (for length and condition) males (age 16 months) were placed in a tank measuring 100 × 40 × 40 cm. The tank contained a single 20 cm long and 8 cm wide clay arch as a shelter. Behaviour of the males was monitored to assess their relative dominance positions and also to abort the replicate before males were able to cause unnecessary stress or any physical damage to each other. In all 20 replicates, only one of the two males claimed the shelter and the same male was also behaviourally dominant in behavioural interactions observed between the two males. The males were photographed after the experiment.

(e) Explanation 5: heritable differences

To estimate narrow-sense heritability \((h^2)\) of the number and size of egg-spots, 14 males aged 10.5 months were each placed into one tank with three females. At least one of the three females was considered ready to spawn, and the role of the two other was to diffuse aggression. As soon as a female was mouthbrooding, the rest of them were returned to stock tanks and the male was used for the group 3 of ‘explanations 1 and 2’. When the juveniles started to swim freely, the female was removed from the tank. Each batch of juveniles (families) was raised in equal-sized partitions measuring 40 × 60 × 50 cm. One large tank (120 × 60 × 50 cm) contained three such enclosures, partitioned with opaque dividers. The enclosure for each batch of juveniles was assigned randomly. After three months, the maximum number of juveniles in each family was limited to 14 by randomly removing any number of individuals exceeding 14, to assure as homogenized conditions across the families as possible. At the age of 7.5 months, all remaining male offspring (1–14 per family, average 4.6) were photographed.

Narrow-sense heritability \((h^2)\) measures the proportion of phenotypic variance explained by additive genetic variance; a high value of \(h^2\) indicates a high degree of additive genetic influence. To yield an estimate for \(h^2\), and its standard error, the slope of father–male offspring regression analysis and its standard error were multiplied by 2 [28]. In the regression, each offspring value consisted of the mean over the family. All statistical tests were conducted using Systat 12.0 (SPSS Inc.) software.

3. RESULTS

(a) Explanation 1: body size

Despite the large range in the egg-spot number and total length among males of each group (table 1), these two variables correlated only within one of the four male groups (table 1). In none of the groups male total length and egg-spot size (area) correlated significantly \((p > 0.10\) in all cases).

(b) Explanation 2: changes over time

All values below are given as ‘mean ± standard error of the mean’, if not otherwise noted. In all cases, males developed 0–2 new egg-spots during the experimental period and none of them lost any. The change in average spot number was 0.75 ± 0.18 (Wilcoxon signed-rank test, \(z = 2.71, n = 12, p = 0.007\), 0.67 ± 0.21 (Wilcoxon signed-rank test, \(z = 2.43, n = 15, p = 0.015\) and from 0.42 ± 0.19 (Wilcoxon signed-rank test, \(z = 1.89, n = 12, p = 0.059\) for the groups 2, 3 and 4, respectively (figure 2). The corresponding changes in the size of the spots were 0.40 ± 0.14 mm² (paired \(t\)-test, \(t = 2.81, d.f. = 11, p = 0.017\), 0.005 ± 0.12 mm² (paired \(t\)-test, \(t = 0.40, d.f. = 14, p = 0.97\)) and −0.031 ± 0.12 mm² (paired \(t\)-test, \(t = 0.26, d.f. = 11, p = 0.80\)). Young males also grew during the study period (group 2: paired \(t\)-test, \(t = 8.24, d.f. = 11, p < 0.001\); group 3: paired \(t\)-test, \(t = 6.11, d.f. = 14, p < 0.001\), whereas there was only a marginally non-significant tendency for older males to gain length during the study period (group 4: paired \(t\)-test, \(t = 2.18, d.f. = 11, p = 0.052\)).

(c) Explanation 3: condition dependence

Our food restriction treatment was effective in manipulating condition: well-fed males were longer (two sample \(t\)-test, \(t = 3.16, d.f. = 18, p = 0.005\) and visually clearly more ‘bulky’ than food-restricted males of the same age. Furthermore, at the age of 7.5 months, well-fed males \((6.2 ± 0.9)\) had on average a larger number of egg-spots than food-restricted males \((4.9 ± 0.9)\) (two sample

Table 1. Total lengths, egg-spot numbers and correlations between these two traits in four experimental groups of A. burtoni males.

<table>
<thead>
<tr>
<th>male group</th>
<th>age (months)</th>
<th>total length (mm)</th>
<th>egg-spots (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± s.e.</td>
<td>range</td>
<td>mean ± s.e.</td>
</tr>
<tr>
<td>1</td>
<td>7.5</td>
<td>81 ± 0.7 70–100</td>
<td>7.2 ± 0.2 5–11</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>86 ± 1.2 78–95</td>
<td>6.4 ± 0.3 5–8</td>
</tr>
<tr>
<td>3</td>
<td>10.5</td>
<td>91 ± 1.3 80–98</td>
<td>9.5 ± 0.6 6–14</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>114 ± 1.3 106–121</td>
<td>10.3 ± 0.8 7–14</td>
</tr>
</tbody>
</table>
In the groups of individuals raised together until the age of 7.5 months, the most dominant males were always the males that exhibited dominant behaviour (paired t-test, $t = 0.51; p = 0.51$). Nevertheless, among visually size-matched male pairs, the male that exhibited dominant behaviour and acclaimed the single shelter (which as the only available structure typically provided the centre for the new territory) had more egg-spots (10.0 ± 0.8) than the subdominant male (7.9 ± 0.4) (paired t-test, $t = 2.57; d.f. = 19; p = 0.019$; figure 3). The average size of egg-spots was not different between dominant and subdominant males (dominant males: 2.1 ± 0.1 mm$^2$; subdominant males 2.1 ± 0.1 mm$^2$; paired t-test, $t = 0.284; d.f. = 19; p = 0.78$). The small size differences (0–6.5 mm in total length) between the males in a pair, due to imperfect visual matching, did not affect dominance (dominant male: 109 ± 1.4 mm; subdominant: 109 ± 1.5 mm; paired t-test, $t = 0.040; d.f. = 19; p = 0.97$).

For the two set-ups combined, dominant males were larger than subdominants in 24 cases out of 33 (binomial distribution: $p = 0.014$), had more egg-spots in 18 cases out of 25 (with eight cases of equal egg-spot number, binomial distribution: $p = 0.043$), and had larger average spot size in 17 cases out of 33 (binomial distribution: $p = 1.0$). Hence, it seems that when there are large size differences between males, body size is the most important determinant of dominance hierarchy, with egg-spot number being a better predictor among males of approximately similar size.

(e) **Explanation 5: heritable differences**

The number of egg-spots was significantly heritable ($h^2 = 0.50 ± 0.20$); males that had many egg-spots (at the age of 10.5 months) tended to have sons with many egg-spots (figure 4). The estimates for narrow sense heritability of egg-spot size (0.20 ± 0.51) and male total length (−0.32 ± 0.44) did not significantly differ from zero.

4. **DISCUSSION**

It has been reasoned that the evolution of the egg-spots and concomitant evolution of the maternal mouthbrooding mating system might have been a key innovation that permitted the evolution of extremely species-rich cichlid fish lineage, the haplochromines, which is composed of about 1800 species [5]. In this study, we set out to explore the sources of intra-population variation in this key trait. We found, among *A. burtoni* males of a certain age, mixed evidence regarding the relationship between the number of egg-dummies and body size. We propose that age (Henning & Meyer, 2009, unpublished manuscript) or social background of the males may determine whether such a link between the two traits exists. Heterogeneous male ages may also explain why an earlier study suggests the traits to correlate [11]. In any case, the results of the current study indicate that egg-spot number is not unequivocally determined by body size, especially among males of a certain age. Furthermore, males developed additional egg-spots over time and males in good condition had more spots than low condition males.

Taken together, these findings suggest that egg-spot number has potential to purport somewhat different information than male size per se, giving the female haplochromine cichlids the potential to use egg-spot number as a mate choice cue. By preferring males that have high-number of egg-spots, females could mate with older males (for their size) or males that are in good condition. In some systems, age correlates with a male’s survival skills and high immune resistance [29], and females would therefore benefit by choosing old males that pass
their good genes to their offspring. Similarly, mating with a male in good condition is generally thought to provide corresponding indirect (and sometimes direct) benefits [23,24,30]. Indeed, females of some haplochromine cichlid species have been reported to use egg-spot number as a mate choice cue [9,31,32], although this is not necessarily the case in A. burtoni (Henning & Meyer, 2009, unpublished manuscript) or some other haplochromine species [10]. Furthermore, condition dependence of a trait (here egg-spot number) as such may also help to explain how genetic variability in that trait persists [23,30], see the discussion below).

We also found that the number of egg-spot has relevance for the dominance hierarchy: although larger and heavier males generally tend to be more dominant ([10,33]; T. K. Lehtonen & A. Meyer, 2008, personal observations), among males of approximately the same size, individuals with many spots dominated those with a lower egg-spot number. However, in sister groups with high variation in body size, largest males, more often than those with the highest egg-spot number, were socially dominant. Nevertheless, our results suggest that besides potential relevance of egg-spot number to female choice, the trait is also important in male–male competition, predicting dominance status among males of the same size class. Due to a short lifespan and size assortative mortality [26], rival males are likely to be often of similar size in the wild. This is important also because if the egg-spots are ‘badges of status’ by honestly indicating a male’s position in dominance hierarchy (relative to his size) [25], the observed variation in the number of egg-dummies among males could be more readily understandable. In such a system, cheaters would be punished by their rivals [34–36].

A dominant A. burtoni male usually spreads his anal fin during courtship and male–male interactions, suggesting that the fish are likely to use the signal potential of egg-spots in the context of sexual selection. However, even if the high success of males with a large number of egg-spots was purely countable to correlates of the trait, some disadvantages associated with a high egg-spot number (or its correlates) should be expected, given the observed high within-population variation in the trait. These potential disadvantages include the above-mentioned social costs, physiological costs of producing or maintaining spots [23,37] and higher predation pressure on males with many bright spots [38–42]. For example, in small tropical fishes called swordtails (genus Xiphophorus), females prefer males with a brightly coloured and long extension of their caudal fin (i.e. ‘sword’), but these males are also preferably targeted by predatory fish [43,44]. However, we are not aware of any empirical tests on the potential costs of high egg-spot number in A. burtoni or other haplochromines.

Given that individual differences in egg-spot number can be related to passing of time, as well as a male’s dominance status (which may, in turn, change quickly and be under hormonal control, see [16,17]), the question arises whether these within-population differences are solely under environmental control and strongly affected by phenotypic plasticity. Interestingly, our results suggest that this is not the case, since the differences among males in egg-spot number have a significant heritable component. Specifically, a male with many spots tends to sire male offspring with a high egg-spot number, resulting in a high estimate for narrow sense heritability ($h^2 = 0.50$) of this conspicuous trait. This finding is notable because, obviously, traits can respond to selection only if they have a heritable component. In this respect, egg-spot number seems to be similar to the length of caudal fin extensions in male swordtails, a trait under sexual selection and clear genetic control [45]. More generally, although mating success and fecundity may cause more persistent directional evolution than selection through survival [46], and most traits associated with fitness are expected to have low heritability values [15,47,48], traits involved in sexual selection have recurrently been reported to have relatively high heritabilities (reviewed in [49]). For example, in collared flycatchers (Ficedula albicollis), the size of a male’s forehead patch had narrow sense heritability of $h^2 = 0.381$ [50]. In a population of guppies (Poecilia reticulata) the number of orange-coloured later spots, a trait relevant to female mate choice, had an estimated heritability as high as $h^2 = 0.62$ [51]. Regarding these comparisons, it is worth noting that our estimate ($h^2 = 0.50$) might have been somewhat influenced by the fact that, for logistical reasons, the male offspring needed to be photographed at a slightly younger age than their fathers; it is possible that the lower range of trait values in the former than the latter (figure 4) underestimated the strength of regression.

The large within-population variation in the number of egg-spots found in this study is also relevant regarding earlier research suggesting that the number of egg-spots plays a role in species recognition [8]. Specifically, the large range of egg-spot number in A. burtoni, and some other species [14], means that there is likely to be considerable overlap in the number of egg-spots among many species, albeit males of some species always have a lower and more consistent egg-spot number [5,7,12]. Our results may therefore call for re-evaluation of egg-spots as a species recognition cue, although we also note that it is still possible that the arrangement or appearance (such as size) of egg-spots is more species-specific.
than their number [5,7,8]. Accordingly, in the current study, we also assessed the average size of each male’s egg-spots. When males at the age of 10.5 months were isolated from other individuals, the size of spots slightly increased over time. We found no other consistent patterns concerning the size of the egg-spots in relation to passing of time, body size, dominance status or condition of males. Similarly, heritability of the average size of egg-spots was not significant. These findings are not surprising since new, emerging spots are initially small, and we also found that large spots sometimes ended up dividing into two smaller spots in older fish. Furthermore, one might expect that the final size of the spots could be under stabilizing selection, for example if they truly functioned as egg mimics [6] or if any sexual selection pressures on egg-spot size are counter-selected by natural selection [52,53]. However, neither hypothesis has been rigorously tested in cichlid fishes. Moreover, females do not necessarily prefer egg-spots that are of the same size as the ova they produce [8,12].

Here, we have provided insights into the proximate and ultimate factors that contribute to the variation in the number of egg-spots on anal fins of male haplochromine cichlid fish. Multiple, potentially interacting, mechanisms seem to be involved: dominant males in good condition are likely to have many egg-spots, the number of which increases over time. Interestingly, the differences in the egg-spot number are highly heritable. Hence, it seems that the primary function of egg-spot number could lie in sexual selection rather than species recognition or fertility assurance. Future work will also have to further investigate the developmental proximate mechanisms of this key trait with special evolutionary importance [5,7].

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