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The information content of odour, colour and tactile cues in the mate choice of minnows

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Summary

Sexual displays often involve many different signal components, which may give information about the same or different mate qualities. We studied the information content of different signals in male minnows (*Phoxinus phoxinus*) and tested whether females are able to discriminate between males when only olfactory cues are present. We found that females preferred the odour of males with a more saturated (i.e., redder) belly, but only when the females had been in physical contact with the males before the experiments. Instead, when unfamiliar males were used, females did not discriminate between male odours and also the overall swimming activity (mate choice intensity) of the females was significantly lower. More ornamented males had lower number of *Philometra ovata* parasites (indicated by belly saturation) and *Neoechinorhynchus rutili* parasites (indicated by belly hue) than their less ornamented counterparts. We did not find experimental evidence for female odour preference being linked to belly hue and breeding tubercle number, but in the nature these traits were associated with the condition factor of the males. Taken together, our results suggest that belly colouration and breeding tubercles give honest information on several aspects of male quality. In addition females may learn the association between male colouration and their olfactory signals and utilize this information when visual signals are not present.

Keywords: condition, female choice, multiple cues, odour, parasite abundance, *Phoxinus phoxinus*, sexual ornament.

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1. Introduction

Female preference for exaggerated male secondary sexual characters is a common phenomenon in the animal kingdom. Indicator ('good gene') models of mate choice predict that female preferences have evolved because these ornamental traits indicate differences in male quality ([Zahavi, 1975](#); [Hamilton & Zuk, 1982](#); [Andersson, 1994](#); [Grether, 2000](#); [Eilertsen et al., 2009](#); [Huuskonen et al., 2009](#)). Red, orange and yellow ornaments of birds and fish usually consist of carotenoids ([Aquilera & Amat, 2007](#)). Since animals are unable to synthesize these pigments, they must acquire carotenoids from food ([Fox, 1976](#)). Thus, carotenoid-based colouration is energetically costly to obtain ([Kodric-Brown, 1998](#)), which is believed to make carotenoid ornaments reliable signals for the foraging ability or nutritional status of the individuals ([Hill et al., 2002](#); [McGraw & Ardia, 2003](#)).

Carotenoids are assumed to have antioxidant and immunostimulatory functions in the body ([Blount et al., 2003](#); [McGraw & Ardia, 2003](#); [Grether et al., 2004b](#); [Kolluru et al., 2006](#)). Therefore, males may face a trade-off in carotenoid allocation between maintaining health (immune function) and elaborate ornamentation ([Skarstein & Folstad, 1996](#); [Skarstein et al., 2001](#); [Blount et al., 2003](#)). Higher quality, i.e., healthier, males should require fewer carotenoids in their immune system and could, therefore, use more pigments to enhance their ornamental display ([Lozano, 1994](#); [Olsson & Owens, 1998](#); [Møller et al., 2000](#); [Hörak et al., 2004](#); see also [Kolluru et al., 2009](#)). [Hamilton & Zuk \(1982\)](#) proposed that sexually selected traits advertise heritable resistance to parasites and that by mating with elaborately ornamented males females could produce offspring with superior parasite resistance. [Wedekind & Folstad \(1994\)](#) hypothesized that the trade-off between immune function and sexual displays is mediated by testosterone, a hormone that enhances sexual signals but also weakens immunity. Recently, [Boonekamp et al. \(2008\)](#) showed that there might also be an interaction in the opposite direction: immune activity suppresses testosterone production (and, thus, ornamentation). As an indirect support for the latter hypothesis, some studies have shown that parasites are capable of depressing the expression of the carotenoid-based ornaments (e.g., [McGraw & Hill, 2000](#); [Hörak et al., 2004](#); [Baeta et al., 2008](#)) and that they may alter the carotenoid allocation process of their host ([Baeta et al., 2008](#)).

Numerous studies have demonstrated female mating preferences for visual ornamental traits of males (e.g., [Andersson, 1994](#); [Blount et al., 2003](#)).

However, animals sexual displays are often more complex and may involve many different visual, acoustic, chemical and tactile signal components (Candolin, 2003; see also Grether et al., 2004a). Different signals either may give information about different mate qualities (multiple messages hypothesis) or they may allow a more accurate mate quality assessment if each signal reflects the same quality trait (back-up signal hypothesis) (Møller & Pomiankowski, 1993; Candolin, 2003). The importance of chemical cues (pheromones) in animal communication is well demonstrated in a wide variety of taxa. Chemical cues function as mate attractors and may also give information about, for example, the dominance status (Moore et al., 1997), body size (Shine et al., 2003), degree of genetic relatedness (Smith, 1983; Mehliis et al., 2008), developmental stability (Thornhill, 1992) and health status (Penn & Potts, 1998; Johansson & Jones, 2007) of the individuals. On the other hand, odour cues also have an important role in individual recognition (e.g., Kodric-Brown & Strecker, 2001). Thus, it is often unclear whether such signals have independent signalling functions in female choice or whether they just help females to learn the association between male quality and their identity. Chemical signalling has been shown to be common also in various fish species, such as in fathead minnows, *Pimephales promelas* (Cole & Smith, 1992), Arctic charr, *Salvelinus alpinus* (Olsén et al., 1998), rainbow trout, *Oncorhynchus mykiss* (Yambe & Yamazaki, 2001) and goldfish, *Carassius auratus* (Kobayashi et al., 2002). However, the role of olfactory signalling in mate choice is still unclear for the vast majority of species (Burnard et al., 2008).

Secondary sexual characters of male minnows (*Phoxinus phoxinus*) include both conspicuous skin colours and breeding tubercles. Previous studies have demonstrated that these characters signal the dominance status of the male (Jacob et al., 2009; Kekäläinen et al., 2010) and that females usually release their eggs only when at least one large (dominant) male is present (Müller & Ward, 1995; Jacob et al., 2009). The red belly colouration of male minnows has been observed to correlate positively with the heterozygosity of the individuals (Müller & Ward, 1995). In addition, females have a clear behavioural preference towards colourful males when only visual cues are present (Kekäläinen et al., 2010).

Here, we studied the signalling content of olfactory cues and different secondary sexual ornamental traits (belly colouration and breeding tubercles) of male minnows. We conducted two separate experiments where females were

allowed to choose between the odours of two males when only olfactory cues were present. In the first experiment females were allowed to physically interact with the two males prior to experiments (familiar males), whereas in the latter trials fully unfamiliar males were used (no interaction period). The aim of our study was to investigate whether females have stronger behavioural preferences towards the odour of colourful males than the odour of their less ornamented rivals. Another aim was to test whether females are able to discriminate between male odours *per se* or whether they use odour cues mainly for individual recognition. In order to investigate the potential fitness benefits that females could gain by mating with highly ornamented males, we also tested whether male ornamentation could indicate the parasite infection status or condition and, thus, the potential genetic quality of the males.

2. Material and methods

2.1. *Experimental fish*

Sexually mature minnows were collected with net from the Kuusojä brook (62°48'N, 30°1'E) (males) and its tributary, Uuronpuro brook (62°50'N, 29°59'E) (females), in eastern Finland during the breeding season in May 2009 and 2010. Although both places are located within the same brook system, the distance between them (ca. 6 km) ensured that the females and males probably had no previous experience of each other. After being caught, the fish were transported to the laboratory at the University of Eastern Finland and housed in sex-specific 45-l aquaria with continuous water flow at 10°C water temperature under a natural photoperiod. The fish were automatically fed daily with commercial fish food (Biomar®, Aqualife, Hoersholm, Denmark). In order to activate breeding behaviour, the water temperature was gradually raised to 18°C after one week of maintenance, and a layer of gravel (20–40 mm in diameter) was then added to the bottom of the aquaria. Raised temperature and the insertion of gravel invariably triggered spawning behaviour and the appearance of the breeding colouration of the male minnows within a few minutes.

2.2. *Mate choice experiments with familiar males*

Prior to the mate choice trials, two sexually active, size-matched males (mean size \pm SD: 59.7 \pm 5.1 mm, 1.6 \pm 0.4 g) and one gravid female (mean

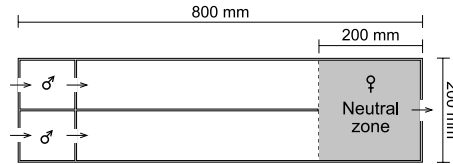


Figure 1. A schematic illustration of the fluvium used in the female mate choice experiments. The direction of water flow is indicated by arrows. Prior to the mate choice trials, the females were isolated in a neutral zone (grey area) of the fluvium for 3 min.

size \pm SD: 69.6 ± 9.1 mm, 2.9 ± 1.2 g) were placed into a glass aquarium (length \times height \times width: $300 \times 300 \times 225$ mm). Of the male pair, one had a highly saturated belly colouration, whereas the other had a much paler colouration. Three sides of the aquarium were covered with brown cardboard to prevent the effect of outside visual stimuli on the behaviour of the fish. The experimental aquarium was illuminated from above with fluorescent lamps. Otherwise the room was dark to minimize the bias effect of visual stimuli from the uncovered side of the aquarium. Before the tests began, 10 l of 18°C water and a layer of 20–40 mm gravel were added to the aquarium. The fish were then allowed to interact in the aquarium for 10 min. The aim of this procedure was to enable the female to gather information from the potential mates. Then, all of the fish were placed into the mate choice fluvium (Hirvonen et al., 2000) (Figure 1). Water flowed from male compartments (100 ml/min) through the fluvium so that the female was able to smell but not see the males. All visual and olfactory contacts between the males were prevented. Female behavioural preference towards the males' odours was followed for 10 min by measuring the time the female spent on each male's side in the fluvium. Before each experiment, the fish were allowed to habituate to the testing conditions for 3 min. During this time, the females were isolated in the neutral zone of the fluvium (Figure 1). After the habituating period, recording of female preference was started when the female was actively swimming. A total of 15 independent mate choice experiments were conducted. In order to eliminate the potential side effects of the testing conditions on fish behaviour, the positions of the saturated and pale males was changed between the left and right compartments after each mate choice trial. Thus, in approximately half ($N = 8$) of the trials the saturated male was in the left compartment and in the other half of the cases ($N = 7$) it was in the right compartment.

2.3. Mate choice experiments with unfamiliar males

To test whether females have behavioural preference towards the more colourful males *per se* or whether they only learn the association between male colouration and odour, we conducted mate choice trials ($N = 18$ experiments) also by using unfamiliar males. For this purpose we collected sexually mature minnows from the same two areas as described above again in May 2010. Females were again allowed to choose between the odours of bright and pale males, but all the trials were conducted without pre-experiment contact period. Female swimming activity (the total time female was actively moving, 0–600 s) in this group was compared to the control (familiar male) group, where females were allowed to interact with the males prior to experiment as described above.

2.4. Measurements and parasite analyses of the fish

After the experiments, all the fish were killed by an overdose of tricaine methanesulphonate (MS-222, Sigma, St. Louis, MO, USA). Both of the males were then photographed under standardized lighting conditions with a Sony DSC-F828 digital camera and the mean hue, saturation and lightness (HSL) values were calculated using a graphical user interface developed at the InFotonics Center, University of Eastern Finland. The colouration of each belly was measured in two standardized areas: a rectangle with the four corners defined by the origins of the pectoral fins and the end of the gill covers, and a rectangular area between the origin and tip of the left and right ventral fins. After the photographing all the fish were measured for total length and weight and their parasite abundances and breeding tubercle numbers were determined. Two parasite taxa were found from the males used in the mate choice trials: *Philometra ovata* (Nematoda) from the body cavity, and *Neoechinorhynchus rutili* (Acanthocephala) from the intestine. The condition factor of the males was determined using the equation: $K = 1000 \times (\text{fish weight (g)} / (\text{body total length (mm)}))^b$, where b is the slope of a regression of $\log_{10}(\text{weight})$ on $\log_{10}(\text{length})$ of the males (Bolger & Connolly, 1989). All of the experiments were performed according to the license of the Finnish Animal Experiment Board (ESLH-2008-03722/Ym-23).

2.5. Breeding colouration and parasite abundance in the field

In order to study the signalling content of male ornamentation in naturally spawning individuals, 54 sexually active males were collected by net from

the same natural breeding areas as the males used in the mate choice experiments (see above). In order to achieve a reliable estimation of true breeding colouration only spawning-ready males (i.e., individuals collected directly from the spawning area) were used. Immediately after being caught, the males were photographed under standardized lighting conditions and their colouration was later analyzed as described above. The fish were transported to the laboratory at the University of Eastern Finland where they were measured for total length and weight, the number of breeding tubercles on their heads was counted and the parasite abundance in their body cavities and eyes was determined. In addition to *P. ovata*, *N. rutili* (see above) also *Diplostomum* sp. (Digenea) eye parasites were found from the field. Condition factor of the males was determined as described above.

2.6. Statistical analyses

The residence time differences of the females as well as differences in individual and gonad sizes between male pairs were studied using paired *t*-tests. The effect of different parasite species abundances on male colouration (saturation, hue) was studied using ordered-heterogeneity tests (field samples) (Rice & Gaines, 1994a,b; [Wedekind et al., 2001](#)) and with Wilcoxon signed-rank tests (mate choice trials). Ordered-heterogeneity (OH) tests combine the *p*-value from any of the variance heterogeneity tests (one-way ANOVA in the present study) and Spearman's rank correlation coefficient (r_s) to produce a broad-application test statistic $r_s p_c$ where p_c is the complement of the *p*-value from the heterogeneity test. In OH tests variation among groups (here parasite abundance classes) has both a heterogeneity component (*p*-value from a heterogeneity test) and an ordering component (measured by Spearman's rank correlation). In the present study we expected that parasite abundances decrease with increasing colour saturation and redness (hue). Due to low prevalence of parasites in some cases also distribution-free permutation (randomization) tests (with 10 000 randomizations) were used to estimate the parasite abundance differences between saturated and pale males. Pearson's product moment correlations were used to study the strength of association between male ornamentation (saturation, hue and the number of breeding tubercles) and their condition factor. Prior to analyses natural-logarithm transformation was applied for condition factor scores to satisfy the requirement of bivariate normal distribution. The difference in

swimming activity of the females between familiar and unfamiliar male experiments was studied using Mann–Whitney U -test. Following [Moran \(2003\)](#), we did not use Bonferroni corrections in our tests. Statistical analyses were performed with the SPSS 16.0 statistical package (SPSS, Chicago, IL, USA) and with free statistical software, AV-Biostatistics (permutation tests).

3. Results

3.1. Female odour preference for familiar males

Females in 11 out of 15 trials spent more time on the side of the male that had a more saturated belly than on the side of the paler male (paired t -test, $t_{14} = 2.603$, $p = 0.021$, Figure 2A). No differences were found in female preference time with respect to the belly hue or number of breeding tubercles of the males (paired t -test, $t_{14} = -0.239$ and -1.035 , $p = 0.814$ and $p = 0.318$, respectively). The size (total length and body mass) and gonad weight did not significantly differ between male pairs (paired t -tests, $p > 0.5$, in all cases). Only four males were infected with *P. ovata*. According to permutation (randomization) test, *P. ovata* numbers did not differ between saturated and pale males ($p = 1.0$). Similarly, the abundance of *N. rutili* did not significantly differ between males (Wilcoxon signed-rank test, $Z_{14} = -0.431$, $p = 0.666$). None of the males were infected with *Diplostomum* sp. Parasite abundances were not significantly associated with

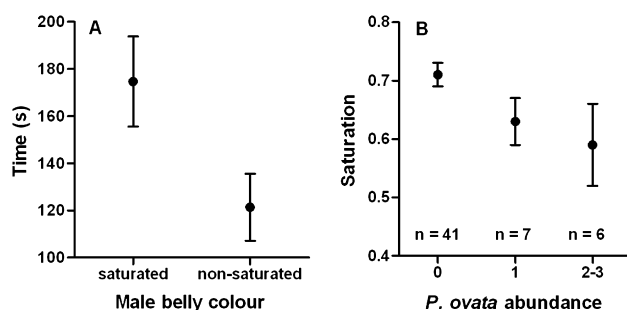


Figure 2. The mean \pm SE time spent by female minnows on the respective sides of the males with saturated and non-saturated belly colourations in the experimental fluvium during the 10 min lasting experiments ($N = 15$) (A) and the mean \pm SE saturation values of belly colouration for different *Philometra ovata* abundance classes in the male minnows collected from nature (B).

belly hue (*P. ovata*: permutation test, $p = 0.670$; *N. rutili*: Wilcoxon signed-rank test, $Z_{14} = -0.108$, $p = 0.914$). Saturation and hue of the belly as well as breeding tubercle number of the males were not significantly associated with male condition (Pearson, $r = 0.236$, $p = 0.209$ for saturation; $r = 0.006$, $p = 0.976$ for hue and $r = 0.203$, $p = 0.281$ for breeding tubercles, $N = 30$, in all cases).

3.2. Female odour preference for unfamiliar males

When females were not allowed to interact with the males prior to experiment, females showed no statistically significant preferences for belly saturation, hue or breeding tubercle number of the males (paired t -tests; $p > 0.2$, in all cases). In addition, swimming activity time of the females was significantly lower in unfamiliar male trials (mean \pm SE 138 ± 62 s) than when familiar males were used (416 ± 58 s) (Mann-Whitney U -test, $Z_{27} = -2.783$, $p = 0.005$), potentially indicating that females may actively choose between males only when they had met the males before. The size (total length and body mass) and gonad weight did not significantly differ between male pairs (paired t -tests, $p > 0.1$, in all cases). Only three males were infected with *P. ovata*. Parasite numbers did not significantly differ between saturated and pale males (permutation test, $p = 1.0$ and 0.648 , respectively). However, the abundance of *N. rutili* was significantly lower in redder (lower hue) males than in less red individuals (Wilcoxon signed-rank test, $Z_{17} = -2.071$, $p = 0.038$). *N. rutili* numbers did not differ between saturated and pale males (Wilcoxon signed-rank test, $Z_{17} = -1.215$, $p = 0.224$). None of the males were infected with *Diplostomum* sp. Saturation and hue of the belly as well as breeding tubercle number of the males were not significantly associated with male condition (Pearson, $r = 0.104$, $p = 0.545$ for saturation; $r = -0.026$, $p = 0.880$ for hue and $r = 0.268$, $p = 0.115$ for breeding tubercles, $N = 36$, in all cases).

3.3. Parasite abundance and breeding colouration of the males in the nature

The saturation of the male belly colouration decreased with increasing *P. ovata* numbers (abundance classes: 0, 1 or 2–3 parasites) (OH test, $r_{spc} = -0.979$, $p < 0.01$, Figure 2B). Abundance of *N. rutili* did not have significant effect on belly saturation (OH test, $r_{spc} = -0.02$, $p > 0.4$).

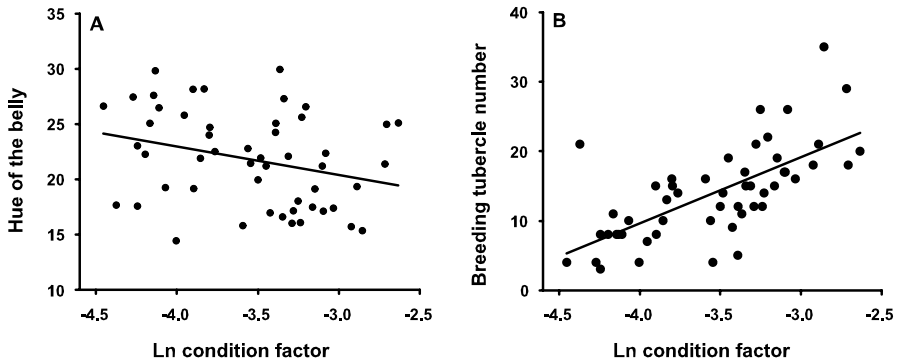


Figure 3. The associations between condition and belly hue (A) and between condition and the number of breeding tubercles (B) of male minnows collected from nature.

Only three out of the 54 analyzed males were infected with *Diplostomum* sp. According to permutation (randomization) test, *Diplostomum* numbers did not significantly differ between saturated and pale males ($p = 0.669$). *P. ovata* and *Diplostomum* sp. abundances were not associated with belly hue (*P. ovata*: OH test, $r_{spc} = -0.035$, $p > 0.4$; *Diplostomum* sp.: permutation test, $p = 0.747$). However, belly hue increased (i.e., redness decreased) with increasing *N. rutili* numbers (OH test, $r_{spc} = 0.546$, $p < 0.05$). Although the females did not show any significant preferences for redder (= lower hue) males or males with higher breeding tubercle counts, red belly colouration and high breeding tubercle number were associated with better condition of the male (Pearson, $r = -0.285$, $p = 0.037$, $N = 54$, for hue and $r = 0.514$, $p < 0.001$, $N = 54$ for breeding tubercle number) (Figure 3). The association between belly saturation and male condition was not significant (Pearson, $r = 0.003$, $p = 0.984$, $N = 54$).

4. Discussion

When female preference was tested in familiar males, we found that females preferred the odour of the males with a bright red, i.e., highly saturated, belly colouration. However, no significant preference for belly colouration was observed when unfamiliar males were used. In addition, the swimming activity of the females was significantly lower in the presence of unfamiliar male odours than when females were exposed to the odours of familiar males. In the field the saturation of the belly was negatively associated with

Philometra ovata abundance on the males. *P. ovata* impair the fitness of its host, for example, by causing parasitic castration, chronic inflammation and tissue necrosis ([Moravec, 2006](#); [Saraiva et al., 2008](#)). Furthermore, as the length of the adult *P. ovata* can be up to 9–12 cm, they often cause abdominal swelling, which decreases swimming ability and, thus, increases predation risk of the host individuals ([Saraiva et al., 2008](#)). The harmfulness of *P. ovata* suggests that the fitness cost of infection with this species is probably remarkable. Therefore, belly colouration may be an honest signal of the health status and overall quality of the males in the present minnow population.

Female mate choice is often based on multiple secondary sexual traits of the male and the magnitude of female preference may be positively related to the number of concurrent male traits ([Künzler & Bakker, 2001](#); [Robertson & Monteiro, 2005](#); [Costanzo & Monteiro, 2007](#)). For example, [Costanzo & Monteiro \(2007\)](#) showed that female *Bicyclus anynana* butterflies preferred to mate with males with both visual and olfactory cues present compared to males that only carried one of these signals. However, the females also mated with the latter male, which suggests that not all signal components are vital for mate discrimination. On the other hand, present results indicate that minnow females may not be capable or willingness of discriminating between males based only on olfactory cues. Thus, it seems likely that females use odour signals mainly for individual recognition. In other words, females probably learned the association between male colouration and odour during the 10-min pre-experimental contact period and used this information later when only odour signals were present. Even if odour cues and breeding colouration may not act as fully independent signals in the mate choice, the observed negative association between breeding colouration and parasite numbers (see above) suggests that odour cues may yield indirect information on male quality.

Both visual and olfactorial cues have been demonstrated to indicate, for example, the heterozygosity of the major histocompatibility complex (MHC) genes ([Reusch et al., 2001](#); [Jäger et al., 2007](#); see also [Thom et al., 2008](#)), which are responsible for resistance against pathogens and parasites (e.g., [Kekäläinen et al., 2009](#)). Thus, belly saturation and olfactory cues of male minnows may signal heritable differences in parasite resistance ability ([Penn & Potts, 1998](#); [Worden et al., 2000](#); [Rantala et al., 2002](#); [Blount et al., 2003](#)).

However, as the extravagance of carotenoid-derived ornaments is often positively associated with male androgen levels ([Folstad & Karter, 1992](#); [Hillgarth et al., 1997](#); [Blas et al., 2006](#); [Dijkstra et al., 2006](#); [Peters, 2007](#)), it is possible that the odours of male minnows may also simultaneously indicate some other aspects of male quality, such as social status ([Kortet & Hedrick, 2005](#); but also see [Moore et al., 2001](#)). These results clearly indicate that besides revealing individual identities, olfactory signals may potentially give detailed information from several aspects of individual quality. However, since it is often unclear whether this information is independent from other cues, further experimental studies in minnows and other taxa are needed to clarify the exact signalling function of olfactory cues.

The colouration of ornamental traits often differs in saturation (i.e., colour intensity: bright vs. dull), but they also differ in colour quality (hue: e.g., red, orange and yellow). Thus, the same colour ornaments in fact include multiple traits that may have different signalling functions ([Wedekind et al., 1998](#); [Grether et al., 2004a](#)). Such ornamental traits may give more detailed information to the females than a one-dimensional male trait would allow. In the present study, we found that along with saturation also the hue of the belly and the number of breeding tubercles were associated with the quality of the male: In the nature redder and highly tubercled males were in better condition and redder males also had lower number of *N. rutili* parasites than their less ornamented counterparts. In support of our results, a similar pattern was also observed in another cyprinid, the roach (*Rutilus rutilus*). Lateral breeding tubercle ornamentation of the male roach was associated with parasite resistance ([Taskinen & Kortet, 2002](#)) and front head breeding tubercles with condition ([Kortet & Taskinen, 2004](#)). Although in the present study female mate choice decisions were not associated with belly hue or breeding tubercle numbers, these condition-dependent traits could be expected to be important mate choice cues under more natural breeding conditions. In nature, female mate choice might be based on multiple cues, i.e., visual, olfactory and tactile/hydrotactile signals (breeding tubercles) simultaneously ([Candolin, 2003](#)), or the females might use different male traits in different years or in different mating situations (e.g., [Kokko et al., 2007](#); [Chaine & Lyon, 2008](#); [Lehtonen et al., 2009](#); see also [Wedekind, 1992](#)). In fact, such fluctuating female preferences may be one of the main factors explaining the evolution and maintenance of multiple sexual signals ([Candolin 2004](#); [Chaine & Lyon, 2008](#)).

In conclusion, the different colour traits (hue and saturation) of male minnows probably indicate different aspects of male quality. Saturation of the male belly colouration may indicate the infection status of potentially harmful *P. ovata* parasites, whereas the hue of the same ornamental trait (as well as number of breeding tubercles) might indicate male condition. Furthermore belly hue may signal resistance against another parasite species, *N. rutili*. The females were able to discriminate between the odours of pale and saturated males, but only when females had been in physical contact with the males before the trials. Thus, olfactory cues may not have fully independent signalling function from the visual cues in the female choice, but may be mainly used as signals in individual recognition. In any case, these results show that the colour and breeding tubercle ornamentation, as well as olfactory cues, could all confer direct or indirect information about male quality. Therefore, under natural conditions, the mating decisions of female minnows may rest on multiple cues or females may use different cues in different situations or mating seasons.

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