

## RESEARCH ARTICLE

# Latency for facultative expression of male-typical courtship behaviour by female bluehead wrasses depends on social rank: the ‘priming/gating’ hypothesis

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## ABSTRACT

Although socially controlled sex transformation in fishes is well established, the underlying mechanisms are not well understood. Particularly enigmatic is behavioural transformation, in which fish can rapidly switch from exhibiting female to male-typical courtship behaviours following removal of ‘supermales’. Bluehead wrasses are a model system for investigating environmental control of sex determination, particularly the social control of sex transformation. Here, we show that the onset of this behavioural transformation was delayed in females that occupied low-ranking positions in the female dominance hierarchy. We also establish that expression of male-typical courtship behaviours in competent initial-phase (IP) females is facultative and gated by the presence of terminal-phase (TP) males. Dominant females displayed reliable TP male-typical courtship behaviours within approximately 2 days of the removal of a TP male; immediately following reintroduction of the TP male, however, females reverted back to female-typical behaviours. These results demonstrate a remarkable plasticity of sexual behaviour and support a ‘priming/gating’ hypothesis for the control of behavioural transformation in bluehead wrasses.

**KEY WORDS:** Social control of sex determination, Behavioural transformation, Coral-reef fishes

## INTRODUCTION

Sex determination in many organisms follows predictable patterns based on genetic inheritance and generally results in stable ratios of females and males. Gender identity and gender-specific behaviour, however, result from hormonal influences on the sexual differentiation of the brain (Cooke et al., 1998). Genetically male or female individuals can show atypical gender identity if sexual differentiation of the brain is counter to sex chromosome identity; for example, the masculinizing effects of androgens on the fetal brain strongly influence the development of male-specific behaviour (Collaer and Hines, 1995; Lee et al., 2017). Moreover, in many species, all aspects of sex determination are under environmental regulation (Janzen and Phillips, 2006; Kato et al.,

2011; Crews et al., 1994). Remarkably, in some fishes, sexual differentiation is under social control and plastic even in adults (Godwin, 2009). An excellent example of this type of environmental sex determination is seen in the bluehead wrasse *Thalassoma bifasciatum* (Warner and Swearer, 1991).

Bluehead wrasses are protogynous sequential hermaphrodites that undergo gonadal and behavioural transformation in a socially controlled manner (Warner and Swearer, 1991; Godwin, 2009). Two colouration phenotypes exist in this species: male or female initial-phase (IP) fish and terminal-phase (TP) males (Fig. 1); the relative proportion of IP males is greatest in large populations, and all IP fish on small patch reefs are female. A single TP male typically defends a territory and displays elaborate courtship behaviour towards IP females. Following removal of the TP male, a capable IP fish can transform into a new TP male. In this transformation (i.e. ‘sex reversal’), remarkably, a dominant IP female can show TP-typical courtship behaviour (Perry and Grober, 2003) within a day after removal of the TP male (Robertson, 1972; Warner and Swearer, 1991). The transformation to the TP male colouration pattern (e.g. blue head, Fig. 1) requires at least 8–10 days and appears to be androgen dependent (Semsar and Godwin, 2003; Kramer et al., 1988). The rapid behavioural transformation, however, can occur in the absence of gonadal androgens (Godwin et al., 1996; Semsar and Godwin, 2003).

Behavioural transformation in bluehead wrasses may reflect a rapid, irreversible physiological change triggered by loss of the TP male. Alternatively, the presence of a territorial TP fish may inhibit the expression of male-typical courtship behaviours in IP females that are already physiologically competent to assume the male role. In this ‘priming/gating hypothesis’, we predict that IP fish become more competent to transform as they ascend the social hierarchy. The positions of IP fish in the hierarchy are related to their relative sizes (Warner and Swearer, 1991; Warner and Schultz, 1992), and established through aggressive interactions (e.g. dominant fish chase subordinate fish away from food sources). In this model, the physiological changes that underlie behavioural gender plasticity occur with a longer time course than is generally postulated. Once competent, i.e. ‘primed’, the expression of male-typical courtship behaviour by an IP female can be toggled off or on depending on whether a territorial TP is present or absent, respectively. This model for behavioural ‘transformation’ is conceptually similar to classical ethological examples of sensorimotor gating (Pearson, 1993). For example, flight behaviour in insects is gated on or off depending on whether the feet are in contact with the substrate. The gating model could be extended to account for the presence of ‘non-territorial’ phenotypes in bluehead wrasses (as proposed by Perry and Grober, 2003) and social regulation of expression of aggressive signalling in cichlids (Fernald and Hirata, 1977; Maruska and Fernald, 2010; Desjardins et al., 2012).

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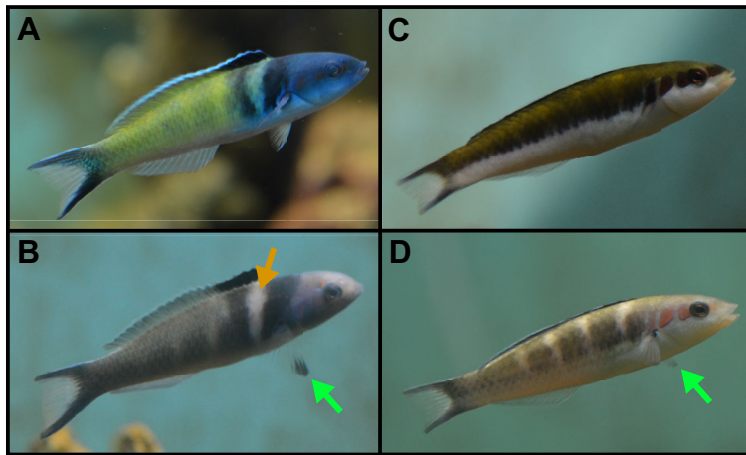
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**Fig. 1. Bluehead wrasses (*Thalassoma bifasciatum*).**

(A,B) Terminal phase; (C,D) initial phase. (B,D) Colouration typical of fish engaged in male-typical courtship behaviour; pectoral fins darkened during courtship display (green arrows), and a 'saddle' (orange arrow) appears.

To evaluate this priming/gating hypothesis, we conducted behavioural studies using artificial patch-reef environments that met the minimum physical requirements for reproductive behaviour, as determined from field studies (Warner, 1990). In these 'simulated patch-reef' aquaria, we were able to control the social structure of a population of bluehead wrasses and record the behaviour of individuals over extended periods; controlled behavioural studies of this nature would be difficult to achieve in the field. We hypothesized that fish at different levels of the dominance hierarchy will differ in their transformational competency. Dominant fish should be physiologically competent to rapidly transform, as reported in the literature. Fish lower on the hierarchy, however, should require longer to express behavioral transformation once the gate 'opens', i.e. all higher status fish are removed. According to the gating hypothesis, the dominant IP fish should reassume typical female behaviour after the TP male is returned to the tank. This hypothesis may challenge the prevailing view that all aspects of sex transformation are irreversible.

## MATERIALS AND METHODS

### Animal care

Bluehead wrasses [*Thalassoma bifasciatum* (Bloch 1791)] were caught at reefs off of Key Largo in the Florida Keys (Caribbean Tropicals, Inc., Tavernier, FL, USA), and shipped overnight, usually within 3 days of capture. The sex of each IP fish was determined by inspecting the external genitalia. The genital papilla is more prominent in IP males than in IP females, and the papilla in each sex is caudal to a large genital pore (Warner and Swearer, 1991). Upon arrival, all IP fish were always housed with a TP male to prevent premature sex transformation. Fish were measured for length and organized into groups of five to seven IP females with each fish being at least 4 mm different in length from the others in the same tank. To ensure that fish had not already begun transforming, the largest IP females in the population were not used in our experiments. IP fish rank was defined by dominance status, with first-order being the most dominant IP fish, second-order being the second most dominant fish and so on. The dominance status of each IP fish in the hierarchy was determined by assessing aggressive and territorial behaviours in each experiment and noting the yellow colouration of submissive displays from subordinate IP fish; dominance status was particularly evident during defence of food sources. Dominant individuals chased smaller, subordinate fish away from food sources and certain coral structures. Subordinate fish, in contrast, avoid dominant fish,

particularly during bouts of feeding. All IP fish included in these experiments were female. Experiments were conducted between 2009 and 2018.

Experiments were conducted using two 800-gallon aquaria (1.2×1.8 m floor, 1.2 m deep) with a natural light:dark cycle for Salt Lake City, UT, USA. Artificial light was used to supplement natural sunlight on days when natural sunlight was less than 12 h. Bluehead wrasses are not seasonal breeders, and seasonal variations were not observed in natural settings (Semsar and Godwin, 2004). Salt water was made by mixing Instant Ocean Sea Salt (Spectrum Brands, Blacksburg, VA, USA) with deionized water. pH, calcium, nitrates/nitrites, ammonia and phosphates were measured with the Reef Lab aquarium test kit (Red Sea, Houston, TX, USA). Water temperature was maintained at 24±2°C year-round. One of the two tanks could be fitted with an opaque or clear (Plexiglas) partition, dividing it into two 400-gallon compartments. Both aquaria have large glass windows (0.61×0.61 m) to allow for observation of fish behaviours. Fish were fed frozen *Mysis* shrimp (Hikari Bio-Pure, Wayward, CA, USA) or live brine shrimp (collected from the Great Salt Lake) daily. No other fish species were housed with the bluehead wrasses.

### Behavioural observations

Behavioural observations (visual observations and video recordings) were made from inside booths constructed from PVC piping and

**Table 1. Male-typical courtship behaviour of bluehead wrasses**

Behaviour	Description
Courtship colouration	Blue head lightens, turns 'opalescent'. Tips of pectoral fins darken.
Glide	The courting fish swims with or toward a subordinate initial phase (IP) fish, raises dorsal fin, extends pectoral fins, slight body axis tilt.
Vibration	Swims directly above subordinate, aligning bodies vertically stacked, with tight shivering-like movement.
Fluttering	Flutters pectoral fins quickly, hummingbird-like.
Circling	Swims in tight circles above or around subordinate fish to signal intention of spawning.
Mock spawn	Swims vertically into water column, then quickly back down (no gametes are released).
Spawn	Same motion as a mock spawn but IP fish joins, vertically swimming to release gametes at apex of spawning 'rush'.

black plastic that surrounded the tank windows. Following a minimum acclimation period of 3–4 weeks, courtship behaviours of the dominant IP fish were scored in each experiment as described in Table 1. Previous studies have described each of these behaviours (Warner and Swearer, 1991; Dawkins and Guilford, 1993, 1994). Courtship colouration (Dawkins and Guilford, 1993) was noted in each experiment but not used as a quantifiable measure of sex transformation (Fig. 1, Table 1). Animal behaviours were scored by K.L., S.M.P. or an undergraduate supervised by K.L. or S.M.P. A video camera (Panasonic HC-V130) recorded behaviours during times when students were not present; videos were scored by the same students supervised by K.L. and S.M.P. Videos were recorded at 1920×1080 pixel resolution, and all animal behaviours and colorations can be accurately observed in video files. Fish reliably courted during a particular period each day; however, this time window varied across individuals. Initially, behaviour was monitored during the entire time that fish were active; after the courtship period was identified, behaviour was scored and/or video recorded primarily during that time period. Animal experiments were limited to as many replicates as needed in order to evaluate statistical significance per the University of Utah's Institutional Animal Care and Use Committee policies (14-07009 and 17-06009).

### Priming experiments

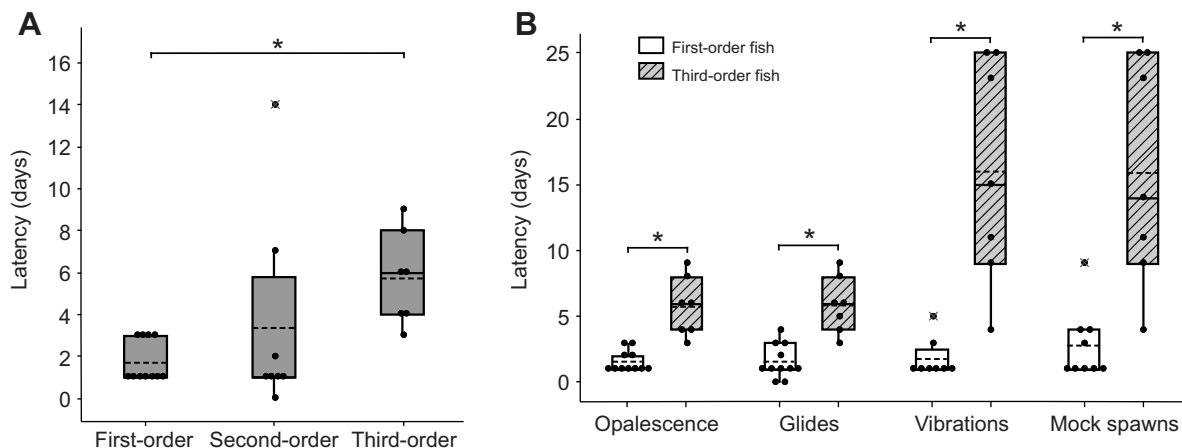
In priming experiments, five to seven IP females were housed with a TP male. Following the acclimation and 3-day baseline recording periods, the TP male was removed. Once courtship behaviour was observed in the first-order IP fish, it and the second-order fish were removed. The third-order fish was monitored until it displayed male-typical courtship behaviour. Latency and frequency of courtship behaviours were recorded. Latency was defined as the number of days between removal of all higher ranked fish and onset of courtship behaviours. We categorized the courtship behaviours into two types: 'early courtship' behaviours and 'late courtship' behaviours. Early courtship behaviours included glides and opalescent colourations. Late courtship behaviours are more

complex motor behaviours, including mock spawns and vibrations. Other late courtship behaviours such as pectoral fin fluttering, circling and paired spawn rush behaviours were also observed but were too infrequent to be included in statistical analyses. A total of 17 experiments were conducted to collect data from 11 first-order fish, eight second-order fish and seven third-order fish. Data from three of the 17 experiments were taken from removal and reintroduction experiments.

### Removal and reintroduction ('gating') experiments

During the removal and reintroduction experiments, the behaviour of the focal IP fish was scored during three sequential stages: (1) baseline period (range=0.5–8 days; median=3 days) prior to TP removal, (2) after the TP male was removed from the tank (range=8–42 days) and (3) following re-introduction of the TP male to the home tank (range=2–12 days; median=3.5 days). During 'baseline' periods of our initial experiments, behaviours of IP fish were recorded for up to 8 days prior to removing the TP male. Results confirmed that, as has been established in field studies (Robertson, 1972; Warner and Swearer, 1991), IP females do not show the spectrum of male-typical courtship behaviours if a TP male is present. In subsequent experiments, therefore, the durations of baseline recordings were progressively reduced. Similarly, based on initial experiments, an observation period of 2 days after TP reintroduction was sufficient to determine whether expression of male-typical courtship behaviours was suppressed; nevertheless, data were scored over a median of 3.5 days across all experiments. The period of time that the TP male was absent varied as a function of the latencies of expression of male-typical courtship behaviours across IP females (Fig. 2A). In each removal and reintroduction experiment, the number of courtship behaviours displayed by dominant IP females per minute during a 30–60 min peak courtship activity ('peak time') period was averaged. No more than 2 days elapsed between successive data recording sessions.

After baseline measurements were made, the TP male and, in some cases, the dominant IP and second-order females were



**Fig. 2. Latencies for behavioural transformation are longer for subordinate initial-phase (IP) female bluehead wrasses relative to those of dominant IP females.** (A) Latencies of onset of male-typical courtship behaviours for (left to right) first-order ( $n=11$ ), second-order ( $n=8$ ) and third-order ( $n=7$ ) IP fish following removal of the terminal-phase male and all larger IP fish for the latter two groups; third-order fish showed longer latencies before onset of male-typical courtship behaviours (Mann–Whitney  $U$ -test, two-tailed,  $P=0.00067$ ). (B) Latencies of onset of specific types of male-typical courtship behaviours for first-order (open bars) and third-order (shaded bars) IP fish following removal of the terminal-phase male and, for the third-order IP females, all larger IP fish. First-order fish showed shorter latencies compared with third-order fish in all noted behaviours: opalescence ( $n=11,7$ , respectively,  $P=0.0005$ ), glides ( $n=11,7$ , respectively,  $P=0.0011$ ), vibrations ( $n=8,7$ , respectively,  $P=0.0015$ ) and mock spawns ( $n=9,7$ , respectively,  $P=0.0021$ ). Each data point represents an individual fish; outliers are denoted with an 'x'. Solid horizontal line indicates the median value and dotted line indicates the mean value. The edges of the boxes represent first and third quartiles. Asterisks denote statistically significant differences ( $*P<0.05$ ).



removed. In each of the reintroduction experiments ( $n=7$ ), the TP male was returned to the tank once the focal IP displayed reliable TP male-typical courtship behaviours (i.e. courtship behaviour that occurred during the same daily time interval, or 'peak time', much like typical midday courtship behaviour seen in TP males on reefs; Warner and Hoffman, 1980), for at least six scored days.

Nine removal and reintroduction experiments were performed, but two of these cases were excluded from our analyses because the dominant IP fish did not reliably show courtship behaviour on each day of observation. Consistent with the results of the other experiments, however, these two IP fish did not show male-typical courtship after the TP male was returned to the tank.

### Partition experiments

To determine whether visual (and possibly chemical) communication results in gating, experiments ( $n=2$ ) were conducted in a clear Plexiglas partition separated the tank into two compartments; one compartment housed the reintroduced TP male, the other contained the group of IP fish. This partition provided visual communication and flow of water but no physical interaction between the TP and IP fish. The TP male was returned to the tank after the dominant IP fish displayed reliable (daily) courtship behaviour. The small sample size of these partition trials does not permit statistical analysis, and can only serve as a possible framework for future studies.

To evaluate whether the dominant IP fish actively associated with the TP male, videos were recorded throughout the day to measure the time the IP female spent (1) associating with the TP fish near the partition (2) near the partition but alone, and (3) away from the partition. Videos chosen were during times when the IP fish were active, and within 1.5 h of the 'peak time' of courtship display by the TP male. Fifteen-minute sections of videos were scored starting the day after the TP was introduced to the adjacent tank, and every 3 days thereafter, until the TP was removed. Sections of these videos in which the IP or TP swam out of view, e.g. swam underneath the coral or out of camera view, were excluded from analyses.

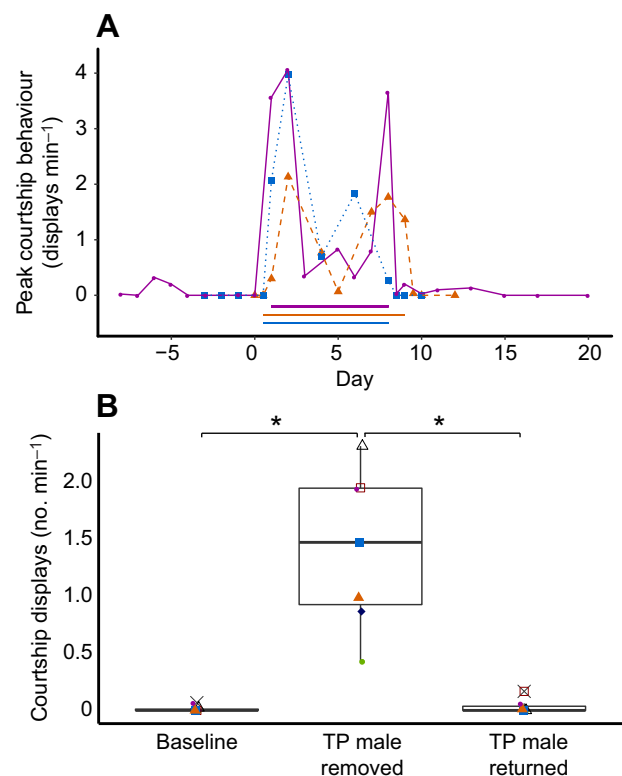
### Statistics

All statistical analyses were performed in R version 3.2.3 (<https://www.r-project.org/>). A two-tailed, Mann–Whitney  $U$ -test at  $\alpha=0.0167$  (after Bonferroni correction) was used to compare latencies (Fig. 2A) of onset of specific male-typical courtship behaviours in IP fish. Differences in the onsets of 'early courtship' and 'late courtship' behaviours between first-order and third-order IP fish were evaluated with a two-tailed, Wilcoxon signed-rank test at  $\alpha=0.05$  (Fig. 2B). For the removal and reintroduction trials, a one-tailed Wilcoxon signed-rank test ( $\alpha=0.05$ ; Fig. 3B) was used to test the effect of the TP male on IP fish courtship behaviour. Fig. 2 was generated via Minitab version 18.1 (Minitab Inc., State College, PA, USA), and Figs 3 and 4 were generated using R version 3.2.3.

## RESULTS

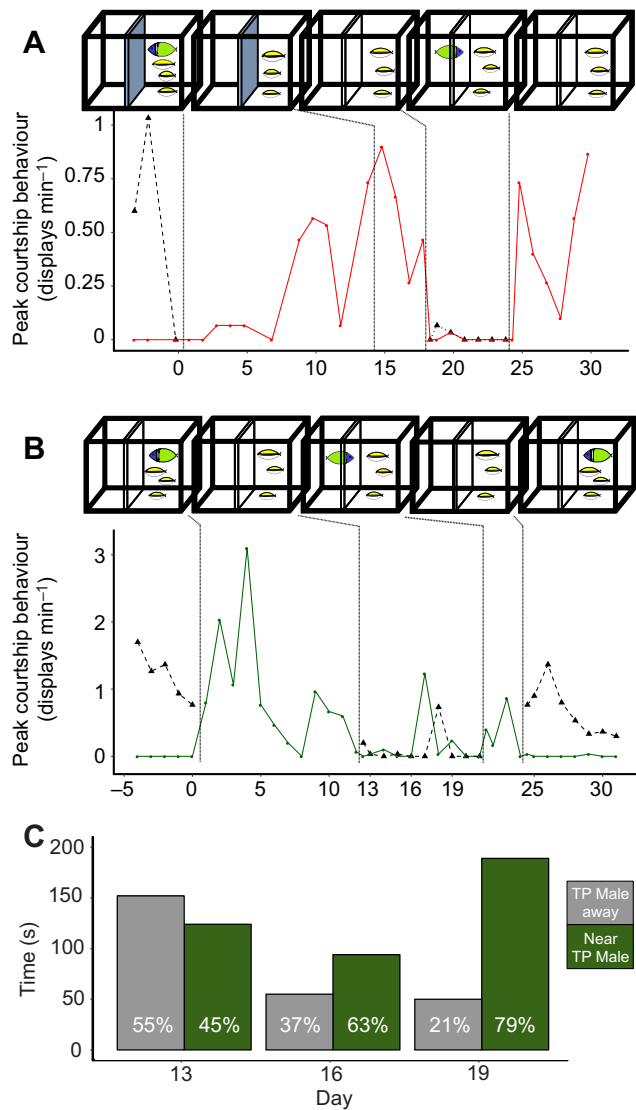
### Test of the priming hypothesis

Consistent with results from previous field experiments, all first-order (most dominate) IP female fish exhibited male-typical courtship behaviours within 1–3 days of removal of the TP male (median latency=1 day, range=1–3 days,  $n=11$ ; Fig. 2A). Third-order IP fish, however, showed longer latencies (median=6 days, range=3–9 days,  $n=7$ ) for courtship behavioural transformation (Mann–Whitney  $U$ -test,  $U=75$ ,  $P=0.00067$ ; Fig. 2A). Latencies for showing male-typical courtship behaviour did not differ between second-order IP fish ( $n=7$ ) and first- or third-order IP fish ( $P=0.96$  and  $P=0.12$ , respectively). The sizes of first-order, second-order



**Fig. 3. Presence of a terminal-phase (TP) male suppresses expression of male-typical courtship behaviour by competent initial-phase (IP) female bluehead wrasses.** (A) Male-typical courtship behaviour of individual IP females ( $n=3$ ) that showed rapid behavioural transformation versus days prior to (negative values) or after each TP male was removed from the tank (day 0). Horizontal lines below plots indicate the duration that the TP male was absent; the TP male was returned to the tank on the day indicated by the right-most end of each bar. (B) Daily 'peak' courtship behaviour was combined and averaged for each period ('baseline', 'TP male removed' and 'TP male returned') across IP fish ( $n=7$ ), including those in A and IP fish with longer latencies to exhibit courtship behaviour. While the TP male was removed, the dominant IP fish showed higher average courtship relative to baseline and after the TP male was returned (Wilcoxon signed-rank test, one-tailed,  $P=0.00781$  and  $P=0.00781$ , respectively). Baseline data taken prior to TP male removal include the average of all scored days for each of the seven IP fish. For the periods during which the TP male was removed or returned, plotted values comprise courtship behaviour over the periods of 2–3 days prior to or after reintroducing the TP male, respectively. Each box denotes median with highest and lowest values; outliers denoted as 'x'; colours and symbols from A match those in B. Asterisks denote statistically significant differences ( $*P<0.05$ ).

and third-order IP females ranged from 7.7 to 10.3 cm (median=8.3 cm), 7.0 to 8.1 cm (median=7.4 cm) and 6.6 to 8.0 cm (median=6.8 cm), respectively. However, two of the largest third-order IP females were larger than the smallest first-order female. Latencies for third-order IP females were significantly greater than those of first-order fish for each of the four display types considered (Mann–Whitney  $U$ -tests; change to opalescent coloration: median=1 day, range=1–3 days versus median=6 days, range=3–9 days, respectively,  $U=76$ ,  $P=0.0005$ ,  $n=11,7$ ; glides: median=1 day, range=0–4 days versus median=6 days, range=3–9 days, respectively,  $U=74.5$ ,  $P=0.0011$ ,  $n=11,7$ ; vibrations: median=1 day, range=1–5 days versus median=15 days, range=4–25 days,  $U=55$ ,  $P=0.0015$ ,  $n=8,7$ ; and mock spawns: median=1 day, range=1–9 days versus median=14 days, range=4–25 days, respectively,  $U=60.5$ ,  $P=0.002$ ,  $n=9,7$ ; Fig. 2B). Latencies



**Fig. 4. Terminal-phase (TP) male bluehead wrasses are able to suppress expression of male-typical courtship behaviour by competent initial-phase (IP) females even when fish are separated by a transparent partition.** Plots of courtship behaviour of IP females (solid lines) during periods wherein the resident TP male and the IP female occupied the same water compartment, were separated by a clear partition, or the TP male was removed from the tank. A and B show results of separate experiments. Courtship behaviour during 'peak time' was recorded, as described previously, for both IP and TP (dashed lines) fish in each tank. Vertical lines denote times at which experimental conditions were changed, as indicated by the pictures that are shown above the plots; the clear partition was placed in the tank on day 13 (A). (C) Time (s) that the IP female (from B) was at the partition boundary and associating with the TP male (green bars) or alone, i.e. TP male not at partition (gray bars) for days 13, 16 and 19.

of onsets of opalescent colouration and glides (early courtship behaviours) were highly correlated, as were onsets of vibrations and mock spawns (late courtship behaviours) ( $r^2=0.923$  and  $r^2=0.981$ , respectively). Onsets of opalescence and glides were only moderately predictive of the onsets of vibrations and mock spawns ( $r^2=0.442$ ,  $P=0.0013$ ). In first-order IP females, late courtship behaviours were observed 1.5 days ( $n=8$ ) after the onset of early courtship behaviour. In contrast, the latency differential was significantly greater for third-order IP females (10.0 days; Mann–

Whitney  $U$ -test, two-tailed,  $U=8$ ,  $P=0.0222$ ). Circling, fluttering of the pectoral fins, and paired spawn rush behaviours were not included because they were observed too infrequently for statistical analyses. Latencies of late-courtship behaviours for second-order fish were intermediate to those of first-order and third-order fish and were not significantly different from either group (Fig. 2A). Two of the seven fish in the third-order group did not display late courtship behaviour before the end of the experiment (25 days), and therefore, their late courtship behaviour was conservatively estimated as 25 days for statistical analysis.

#### Removal and reintroduction experiments

In the presence of a TP male, it appears that IP females in our patch-reef environments do not display TP male-typical courtship behaviours; baseline behavioural data enabled us to confirm this property for the focal IP female and further assess its position in the hierarchy. Results of the priming experiments suggested that IP females develop competency to display male-typical courtship behaviours as they ascend the social dominance hierarchy; the largest, most dominant females may, therefore, be physiologically competent to display male-typical courtship behaviours, but the presence of a TP fish suppresses their expression. To test this 'gating hypothesis', we conducted additional experiments in which we temporarily removed the TP male from its home tank and monitored the behaviour of the most dominant IP fish. Results of three of these experiments are shown in Fig. 3A. Consistent with results of priming experiments, in each case, the first-order IP female displayed male-typical courtship behaviour within 1–2 days of removal of the resident TP fish (Fig. 3A). During the period after TP removal, the focal IP fish showed male-typical courtship behaviours on each day that we monitored the fish; the frequency of these behaviours varied across days and subjects. We then returned the TP male to the tank while the IP fish was engaged in courtship behaviour. In each case, the IP female immediately stopped displaying male-typical courtship behaviour upon reintroduction of the TP male (Fig. 3A), assumed submissive (yellow) colouration, and began associating with the TP male. This reversion to female-typical behaviour persisted for as many days as the reintroduced TP male remained in the tank. Courtship behaviour was significantly elevated following TP male removal relative to TP male reintroduction (Wilcoxon signed-rank test, one-tailed,  $W=28$ ,  $P=0.00781$ ,  $n=7$ ; Fig. 3B).

#### Partition experiments

We next investigated whether this gating process depends on direct interaction of IP and TP fish in the same reef space. In the first phase of these experiments, we removed the TP male and recorded the behaviour of the most dominant IP female for several days. As in the previous experiments, the most dominant IP female began displaying male-typical courtship behaviour within 3 days of removal of the TP male (Fig. 4A). We then placed a clear (Plexiglas) partition in the tank and reintroduced the TP male to the compartment that lacked the other fish. In each of the two trials, the IP female immediately stopped displaying TP male-typical courtship behaviours upon reintroduction of the TP to the adjacent compartment. During the subsequent 7 days that the TP male was present, one IP female showed only transient and infrequent male-typical courtship behaviour (Fig. 4A); male-typical courtship behaviour was again observed after the TP male was removed from the adjacent tank. In contrast, the second IP female displayed courtship behaviour 5 and 7 days after reintroduction of the TP fish to the adjacent tank (Fig. 4B). Interestingly, the TP male in this experiment displayed transient

courtship behaviour. Nevertheless, this IP fish associated with the TP male at the partition boundary (Fig. 4B), much like as was seen in the previous experiments ('gating'; Fig. 3) in which both fish occupied the same water space. Although not able to approach the other fish and thereby perform certain courtship manoeuvres, such as glides and circling behaviours, the TP male displayed courtship colouration during each of the days that it was in the adjacent tank. Initially, during time that the IP fish swam near the partition, 45% was spent in association with the TP male directly on the other side; approximately 55% of the time the TP male was not near the partition. Towards the end of the time the TP male was in the adjacent tank, the IP fish spent less time visiting the partition alone (21% of total partition-visit time) while spending the majority of the time at the partition swimming near the TP male (79%). Although the IP fish showed instances of TP male-typical courtship behaviour, this association between the IP and TP fish at the partition was similar to the behaviour displayed by the IP and TP fish after the TP male was returned to the original (home) tank (Fig. 4A,B).

## DISCUSSION

Previous studies of bluehead wrasses have shown that IP females can display male-typical courtship behaviour within 1 day (hours in some cases) of removal of TP males (Warner and Swearer, 1991; Godwin et al., 1996). Similarly, our first-order IP fish quickly showed male-typical behaviour after the TP male was removed. However, lower ranking fish took longer on average to begin displaying these courtship behaviours, particularly mock spawns and vibrations (Fig. 2B). To the best of our knowledge, these are the first reports of courtship behaviour of these fish in captive conditions. These results show that the sex transformation process appears to be slower than was previously assumed. Development of physiological competency to display male-typical courtship behaviour appears to begin prior to loss of the TP male and is related to the position of the IP fish in the dominance hierarchy. Although physiological experiments are needed before mechanisms can be identified, these results suggest that a 'priming process' is required to achieve competency for displaying TP male-typical courtship behaviour. Also, it would be useful to conduct future experiments in the field to test the priming hypothesis in that environment.

In our experiments, the dominant IP fish in a particular assemblage was also the largest. The question thus arises whether competency to undergo behavioural transformation might be related to absolute size versus hierarchical position. Our results fail to support this alternative explanation. Third-order IP females in some groups were larger, or almost as large, as first-order IP females in other groups. The two largest third-order females were 8.0 and 7.8 cm, while the three smallest first-order females were 7.7, 8.1 and 8.1 cm. Nevertheless, the three smallest first-order females showed short latencies for behavioural transformation, typical of first-order fish, and the two largest third-order IP females showed longer latencies, typical of third-order fish.

The expression of courtship behaviour by a dominant IP fish was suppressed upon the reintroduction of the TP male into the home tank, even when these fish were separated by a transparent partition (Fig. 4B); gating occurred even when the TP male was unable to occupy the same water space as the IP fish, and the dominant IP fish actively affiliated with the TP male. Thus, the presence of the TP male successfully suppressed expression of male-typical courtship behaviour by the dominant IP female, even when a transparent partition prevented the TP male from engaging in many of the normal courtship behaviours such as inspections, circling and spawns. This 'gating' could have been due to the

visual presence of the TP, the remaining courtship and colouration displays, or both.

Although the physiological basis of the putative priming process is unknown, a likely candidate is the arginine vasotocin system (AVT), the teleost homolog of arginine vasopressin (AVP). Arginine vasotocin and the AVT receptor paralogues (vasotocin 1a-type receptors V1a1 and V1a2) promote sensorimotor responses in zebrafish early in development (Iwasaki et al., 2013). Additionally, the AVT/AVP neuropeptides and receptors are found throughout the brain (Foran and Bass, 1999; Grober et al., 2002; Kline et al., 2011), important for integrating social stimuli, and reported to have a role in socially regulated reproductive behaviour (Semsar et al., 2001; Huffman et al., 2015; Loveland and Fernald, 2017). In the African cichlid *Astatotilapia burtoni*, male fish ascending from subordinate to dominant status show increased aggressive behaviour following administration of an AVT antagonist (Huffman et al., 2015). In bluehead wrasses, studies by Semsar, Godwin and colleagues suggest that arginine vasotocin and the V1a-type AVT receptors (AVTRs) are important for expression of male-typical courtship behaviour (Godwin et al., 2000; Semsar and Godwin, 2003). AVTR mRNA levels are elevated in dominant females that have begun displaying male-typical courtship behaviour (Lema et al., 2012), and changes in AVT expression can be independent of gonadal influences (Semsar and Godwin, 2003, 2004). An intriguing possibility, therefore, is that competency for exhibiting male-typical courtship behaviour may be socially induced through regulation of AVTR gene expression and AVT receptor density in areas of the brain that are important for social behaviour, e.g. the preoptic area.

While AVT appears important for transformation in bluehead wrasses, nonapeptides are known to bind to orthologous nonapeptide receptors (Darlison and Richter, 1999), a concept described as 'receptor promiscuity'. It is possible, therefore, that the effects of AVT may be mediated through more than just AVT receptors. For example, isotocin receptors (orthologue to mammalian oxytocin) bind both isotocin and AVT peptides (Darlison and Richter, 1999). In contrast, AVT receptors have very low affinity for isotocin in the teleost *Catostomus commersonii* (white sucker; Mahlmann et al., 1994; Hausmann et al., 1996), determined by *in vitro* receptor expression followed by physiological recordings. Considering receptor promiscuity between the AVT and isotocin peptides and receptors, it would be useful to determine whether isotocin plays a role in behavioral transformation in wrasses, i.e. promotes expression of courtship behavior typical of TP males, and, if so, whether it acts in part via binding to AVT receptors; Fluorescence resonance energy transfer (Kauk and Hoffmann, 2018) could be used to identify interactions between isotocin and AVT receptors.

It is generally assumed that the process of transformation from the IP to the TP phenotype is irreversible. This process could involve a rapid physiological change that enables IP females to exhibit male-typical courtship behaviour. Alternatively, dominant females may already be competent to display as males, but TP males suppress expression of this behaviour. Our results support the latter 'gating' hypothesis: TP males can gate expression of male-typical courtship behaviour by competent IP females. Importantly, IP fish that showed robust male-typical courtship behaviour following removal of the TP male reverted to female-like behaviour immediately after the TP male was returned to the tank. Fernald and colleagues have shown that subordinate male cichlids are competent to perform dominance behaviours, but dominant males normally suppress their expression of these displays (Kustan et al., 2011). Thus, reproductive behaviour in wrasses and cichlids appears to be governed by a gating process. The



two systems differ, however, with regard to behavioural competency; subordinate female wrasses are not immediately competent to display male-typical courtship behaviour, particularly advanced-stage behaviours such as vibrations and mock spawns.

Results of our partition experiments suggest that a TP male can nearly completely suppress expression of male-typical courtship behaviour in IP fish even if there is no direct interaction in the same water space. Maintenance of this suppression, however, may be, to some extent, dependent on the TP male exhibiting courtship colouration; the dominant female in one partition experiment (Fig. 4B) resumed displaying although a transiently displaying TP male was present (but physically separated from the IP female). Because of the small sample size, these results should be considered suggestive at this time. Additional experiments such as manipulations that eliminate male-typical courtship in TP fish, e.g. central nervous system lesions or chemical ablations, may help assess the significance of behavioural interaction versus visual presence alone in gating the expression of male-typical behaviour in dominant IP females.

Taken together, our findings support the ‘priming/gating’ hypothesis, which posits that as IP fish ascend the dominance hierarchy, they become progressively more physiologically competent to show TP male-typical courtship behaviour. The presence of a TP fish, however, inhibits behavioural expression of sex transformation. Removal of the TP fish releases the inhibition on the IP fish and allows expression of the transformation process. Therefore, these results challenge the presently held notion that sex transformation is a rapid process that begins only after removal of the TP fish. Rather, the rapid transformational phase appears to be only the last portion of a larger, slower process that is governed by the social interactions within a hierarchy.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: S.M.P., K.L., G.J.R.; Methodology: S.M.P., K.L., G.J.R.; Software: S.M.P., K.L., G.J.R.; Validation: S.M.P., K.L., G.J.R.; Formal analysis: S.M.P., K.L., R.S.B., G.J.R.; Investigation: S.M.P., K.L., G.J.R.; Resources: S.M.P., K.L., G.J.R.; Data curation: S.M.P., K.L., R.S.B., G.J.R.; Writing - original draft: S.M.P., K.L., R.S.B., G.J.R.; Writing - review & editing: S.M.P., K.L., G.J.R.; Visualization: S.M.P., K.L., G.J.R.; Supervision: S.M.P., K.L., G.J.R.; Project administration: S.M.P., K.L., G.J.R.; Funding acquisition: S.M.P., G.J.R.

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#### Data availability

Data are available on FigShare (Luong et al., 2018): <https://doi.org/10.6084/m9.figshare.6953231.v2>.

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