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Terminal Phase Males Stimulate Ovarian Function and Inhibit Sex Change in the Protogynous Wrasse *Thalassoma duperrey*

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ABSTRACT—Under experimental conditions, the probability of sex change in the protogynous wrasse *Thalassoma duperrey* is determined largely by an individual's relative size within a social group. Natural populations, however, contain two distinct male phenotypes that may also play a role in regulating sex change. To investigate potential effects of male phenotype, the ability to change sex, ovarian histology and serum estradiol-17β levels were examined in females maintained under controlled social settings. Large females housed with smaller or larger terminal phase males had significantly larger gonadosomatic indices than females housed singly, with other females or with smaller initial phase males. Similarly, ovaries of females housed with terminal phase males showed no histological evidence of sex change, whereas large females from other social groupings were in advanced stages of sex change. These results demonstrate terminal phase males inhibit sex change regardless of their size relative to the female. Furthermore, gonadosomatic indices, ovarian histology, and serum estradiol-17β levels of females housed with terminal phase males indicate normal ovarian function whereas ovaries of other treatment groups appear quiescent or are undergoing sex change. Consequently, terminal phase males may be required for normal ovarian development which may, in turn, inhibit sex change in *T. duperrey*.

Key words: sex change, male phenotype, gonadal function, social setting, teleost fish

INTRODUCTION

Sex determination is a primary event during reproductive development. In many teleost fishes, sex determination is extremely flexible as evidenced by the naturally occurring phenomenon of hermaphroditism. Simultaneous hermaphrodites (Fisher, 1987; Fisher and Hardison, 1987) often have mature ovotestes allowing the "male/female" to change quickly in response to environmental conditions such as spawning behaviors of potential mates. By contrast, the more common sequential hermaphrodites show extensive restructuring and redevelopment of the existing reproductive system (Nakamura *et al*., 1989; Cardwell and Liley, 1991) or a simultaneous regression and recrudescence of the respective portions of the gonad (Chang *et al*., 1995).

Thalassoma duperrey, the Hawaiian saddleback wrasse, is a diandric, protogynous hermaphrodite (Ross, 1982). Individuals mature initially as males or females. Under appropriate social conditions, male or female initial phase (IP) fish become terminal phase (TP) males. Whereas IP males change their reproductive role, females actually change their functional sex. In addition to several changes in behavioral and secondary sexual characteristics, the gonad is significantly restructured. The testes of IP males become significantly smaller with numerous, large Leydig cells (Hourigan *et al*., 1991). Ovaries, which have no detectable testicular tissue prior to sex change, develop into fully functional testes.

Early investigations of the social conditions favoring sex change in *T. duperrey* identified relative body size as the most important factor determining the probability of a female changing sex. When experimentally paired, smaller individuals including IP males stimulate large females to change sex (Ross *et al*., 1983). Larger individuals presumably inhibit the smaller IP fish from becoming TP males. Relative size within a larger social group also determines the probability of sex change in *T. duperrey*. A threshold ratio of small (stimulating) individuals to larger (inhibiting) individuals must be reached before a female undergoes sex change (Ross *et al*., 1990). Based on this experimental evidence, Ross *et al.*

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(1990) proposed the "relative-size" hypothesis for the regulation of sex change in *T. duperrey.*

Natural populations of *T. duperrey*, however, contain two, distinct male phenotypes (Ross, 1982; Hourigan *et al*., 1991). IP males mature initially as small males with large testes, group spawn or cuckold TP males. TP males, derived from females via sex change or IP males via role change, are larger, more brightly colored, and have much smaller testes with more numerous, larger Leydig cells than IP males. TP males also have much higher circulating concentrations of androgens, particularly the dominant fish androgen 11-ketotestosterone. In contrast to IP males, TP males defend spawning sites and spawn with individual females. While it is generally assumed that these differences relate to the alternative mating tactics of the males, little is known regarding their importance, if any, in the control of sex change and/or female function in *T. duperrey*.

In the study by Ross *et al*. (1983), IP males were clearly

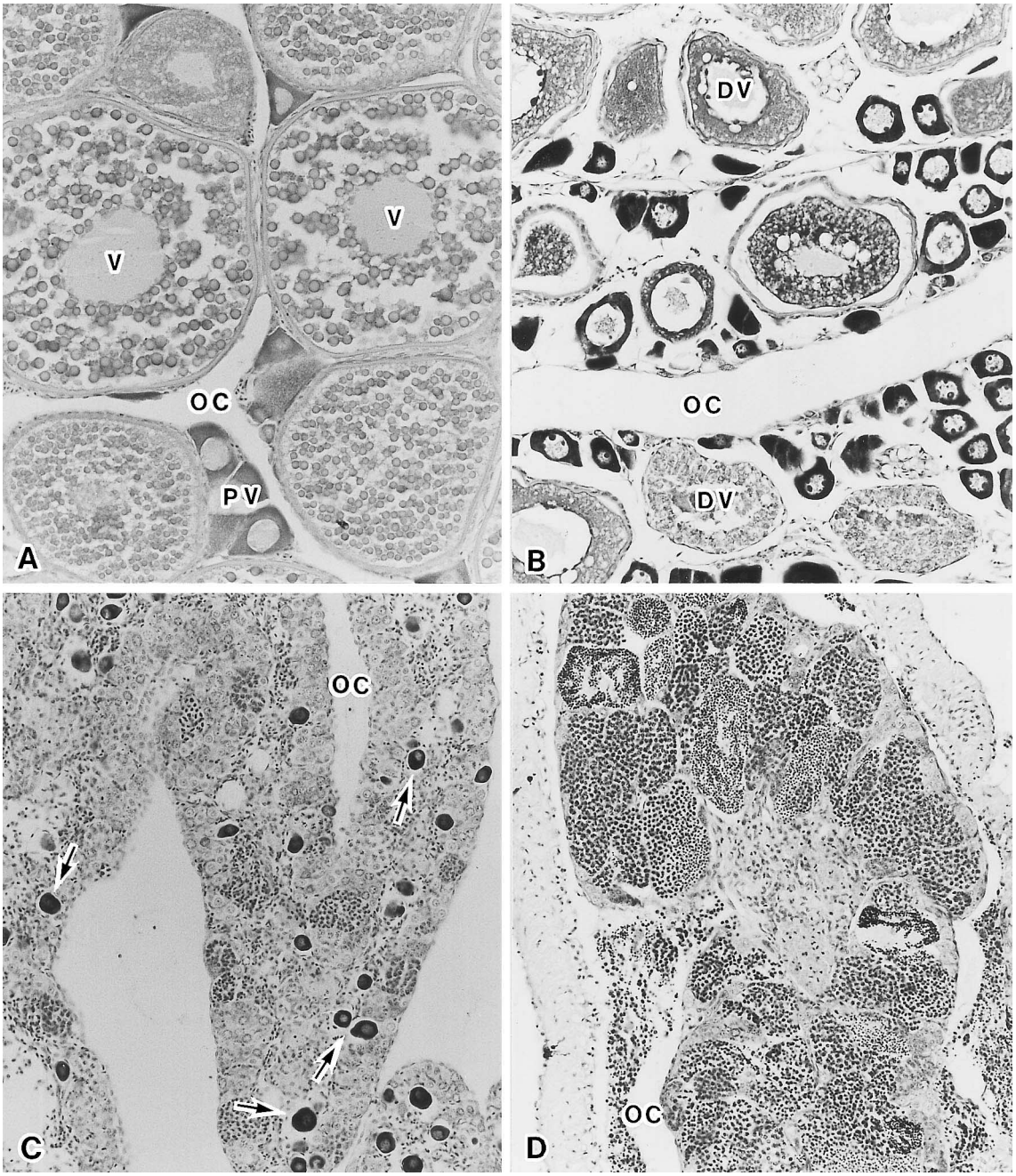

Fig. 1. Photomicrographs of the histological progresssion of sex change in *T. duperrey*. A, Stage 1 ovary with vitellogenic (V) and previtellogenic (PV) follicles. B, Stage 2 transitional gonad with degenerating vitellogenic follicles (DV). C, Stage 4 transitional gonad with proliferating spermatogonia and spermatogenic crypts. Arrows indicate degenerating pre-vitellogenic follicles. D, Stage 5 transitional gonad with all stages of spermatogenesis. The ovarian cavity (OC) is visible in all sections. A-D, X 240.

shown to stimulate sex change of IP females, but experimental design made assessment of the effect of TP males on sex change difficult. A tactile barrier separating a small TP male and female from a test pair of females may have prevented the largest female from perceiving unique behavioral and physiological attributes of the small TP male. Furthermore, the presence of numerous small, stimulatory fish may have compensated for any inhibitory cues from the TP male.

To further investigate potential effects of the TP phenotype on the control of sex change, various social groups were maintained to determine the largest female's ability to initiate and/or complete sex change. In this study, failure of a large female to change sex when housed with a smaller TP male indicates that the TP phenotype effectively inhibits sex change regardless of the TP male's relative size. Comparison of criteria for ovarian development of females from different experimental groups also provides insight into possible effects of male phenotype on female reproductive function and/or mechanisms that inhibit sex change.

METHODS AND METHODS

Animals

To account for normal, lunar variation in ovarian development, *T. duperrey* were collected by hook and line from Kaneohe Bay (Oahu, Hawaii) within 2 days of the full moon, a period of high spawning activity. Sex was determined by applying light abdominal pressure to elicit gamete release. Animals that did not release sperm were assumed to be female. TP males were distinguished from IP males on the basis of body size, coloration, and the relative amount of milt released. Subsequent histological analysis confirmed the reliability of this method for determining sex (98% correct, C.E.M.). All animals were treated humanely and in accordance with University of Hawaii and Federal guidelines.

Experimental Conditions

After length measurements were taken, the following social groups were established in floating Vexar (Internet, Inc., Minneapolis, MN) cages ($1m^3$) in the Hawaii Institute of Marine Biology lagoon:

A) Isolated: isolated female;
B) Small TP: large female, small TP male, small female;
C) Large TP: large TP male, large female, small female;
D) Small IP: large female, small IP male, small female;
E) Female: large female, 2 small females.

Within each social group the largest individual was a minimum of 5 mm larger than the other fish. To insure the presence of at least one stimulatory cue, a small female was included in all groups except isolated females. For purposes of comparison, the largest female of each experimental group was defined as the predicted sex changer, i.e. the individual developing a testis in place of an ovary, based upon the prevailing hypothesis (relative size). All other females were classified as non-sex changing, stimulus fish. Animals were able to forage on algae and invertebrates growing on the cages. In addition, diet was supplemented with chopped squid to satiation 4 times per week.

Sample collection

After one lunar cycle individuals were sacrificed by immersion in a lethal concentration of MS-222 (Sigma, St. Louis, MO). Following measurements of standard length and body weight, blood samples were obtained and allowed to clot for approximately 4 hr on ice. Following centrifugation, serum samples were frozen for subsequent estradiol-17β (E2) analysis. Gonads were removed and weighed. Gonads were then fixed in Bouin's fixative for approximately 14 hr and stored in 70% EtOH.

Histological analysis

After dehydration and paraffin embedding, gonads were sectioned at 7 μm. Standard histological slides were prepared using hematoxylin and eosin to identify evidence of sex change. Although gonadal restructuring is a gradual process, sex change in *T. duperrey* has been divided into six distinct stages (Nakamura *et al.*, 1989). Stages 1-3 represent the degeneration of the ovary with the appearance of a few spermatogonia in Stage 3 (Fig. 1A, B). Stages 4-6 represent the development of the testis and onset of spermatogenesis (Fig. 1C, D). Individual gonads were considered to be undergoing sex change if spermatogonia were present. Late Stage 4 / early Stage 5 gonads were considered to be TP testes as spermatogenesis had already begun. Gonads without evidence of testicular development / spermatogonia were classified as non-sex-changing ovaries.

Estradiol-17β Analysis

Serum samples were assayed in duplicate using a commercially-available E2 ELISA (Cayman Chemical, Ann Arbor MI) according to manufacturerís protocol. Following validation of parallelism on non-extracted, pooled serum samples (1x=83.7pg/ml; 2x=168.5 pg/ml; 4x=389.7pg/ml), samples were assayed directly. Intra- and inter-assay variations were 4.8% and 5.1%, respectively. All data are presented as pg of E2/ ml of sera.

Statistical Analysis

Gonadosomatic indices (GSI) were calculated as (gonad weight)×100/ body weight. Mean GSIs and E2 concentrations for all treatment groups were compared using analysis of variance (ANOVA). Log 10 transformations were performed to satisfy

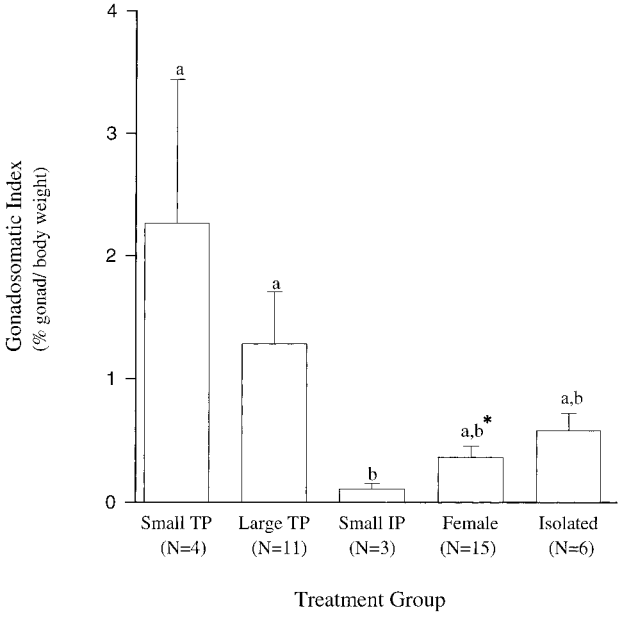

Fig. 2. Gonadosomatic indices of the predicted sex changer (largest female) of each social group. Bars represent the mean ± SEM of the number of individuals shown in parentheses. Different letters represent statistically different values ($p \leq 0.05$). The asterisk represents a statistical difference at $p \leq 0.1$.

ANOVA assumptions of normality and equal variance. Pair-wise analyses were completed using Bonferroni's t-test or Student-Newman-Keuls t-tests. Statistical significance was defined at $p \leq 0.05$.

RESULTS

Large females housed with either a smaller or larger TP male had significantly larger GSIs compared with the predicted sex changer (largest female) of the small IP male treatment group (Fig. 2). Similarly, GSIs of large females

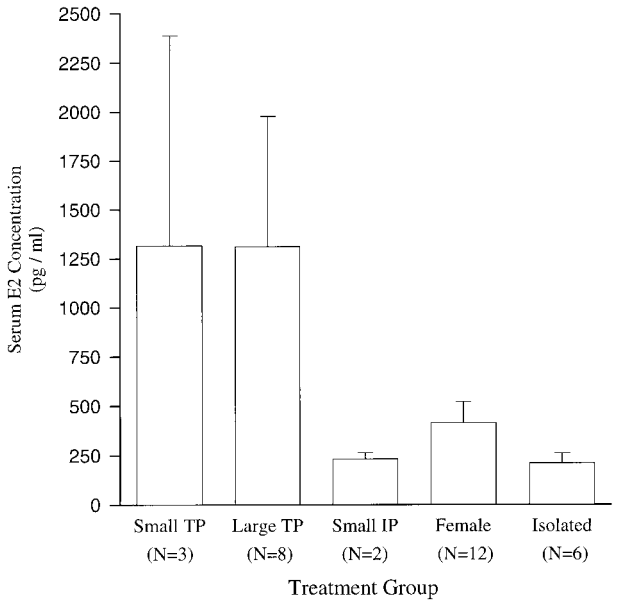

Fig. 3. Serum E2 concentrations of the predicted sex changer of each social group. Bars represent the mean ± SEM of the number of individuals shown in parentheses.

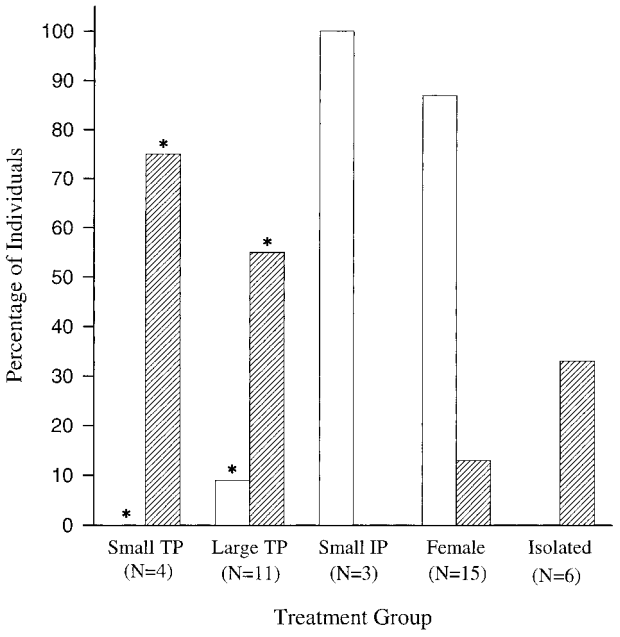

Fig. 4. The percentage of predicted sex changers' gonads from each social group showing histological evidence of sex change (i.e. detectable spermatogonia; open bars) or containing vitellogenic follicles (hatched bars). Sample size is shown in parentheses. Asterisks represent statistical differences at $p \leq 0.05$.

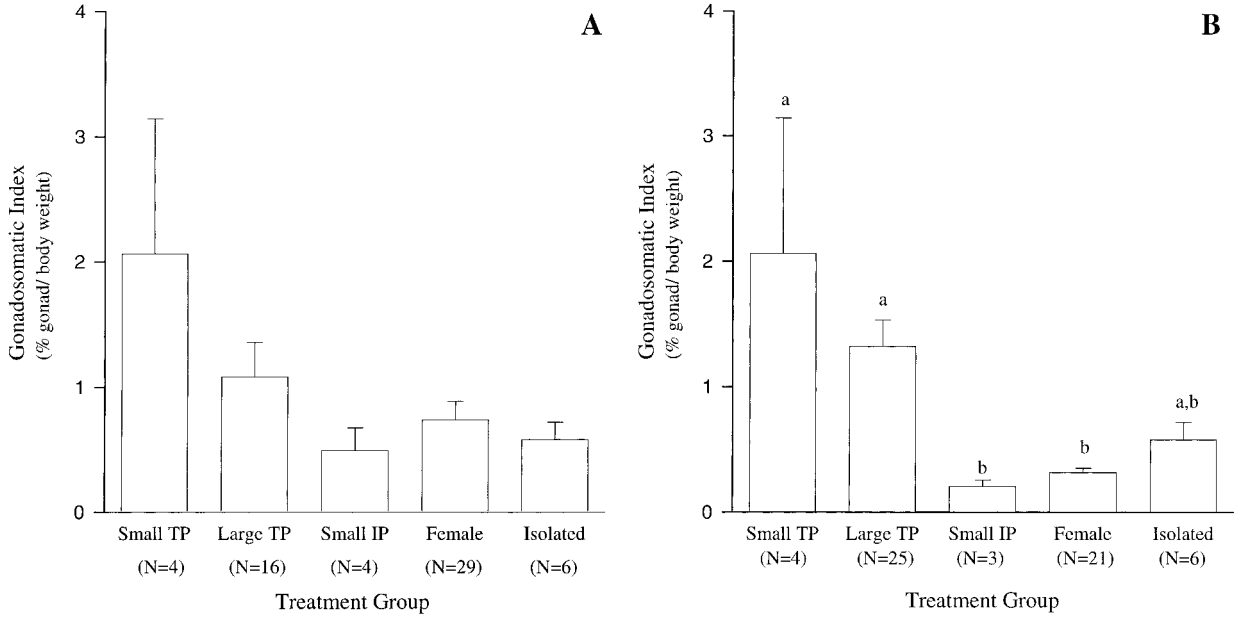

Fig. 5. Gonadosomatic indices of the small stimulus females from each treatment group before (A) or after (B) reclassification of individuals based on the completion or near completion of sex change. Bars represent the mean ± SEM of the number of individuals shown in parentheses. Different letters represent statistically different values ($p \leq 0.05$).

housed with TP males were also higher than those of the largest female of the all female group. Two large females from the all female group did not initiate sex change throughout the experiment, thus affecting significance levels ($p \leq 0.1$). When these individuals are removed, however, the differences in the GSIs of the TP treatments and the all female group are statistically significant ($p \leq 0.05$). In contrast, GSIs of large females housed with a small IP male were not different from the female group values. Isolated females had GSIs that were not significantly different from those of any other treatment.

Serum E2 concentrations of the predicted sex changer in the TP male treatment groups varied considerably (Fig. 3). Although no definite correlations were drawn, the variations appear to be related to the stage of vitellogenesis and possible spawning as evidenced by the presence of post-ovulatory follicles in several of the ovaries. Nevertheless, mean serum E2 concentrations of the predicted sex changers from the TP treatment groups were much higher than the mean serum E2 concentrations of all other treatments.

Histological evidence of sex change was consistently seen in the predicted sex changer from the female and small IP male treatment groups ($p \leq 0.05$; Fig. 4). Four individuals from the female group and one individual from the small IP group had either completed sex change or were in advanced stages (Fig. 1C, D). Females housed with TP males generally had vitellogenic ovaries indistinguishable from normally developing ovaries (Fig. 1A; Fig. 4). A single individual from the large TP treatment was in Stage 5. Several of the large females housed with TP males had hydrated oocytes and/or post-ovulatory follicles present in the ovary indicating imminent or recent spawning, respectively. Although a few vitellogenic follicles were present, ovaries of isolated females were mainly comprised of previtellogenic follicles; however, spermatogonia were not found (Fig. 1A, B; Fig. 4).

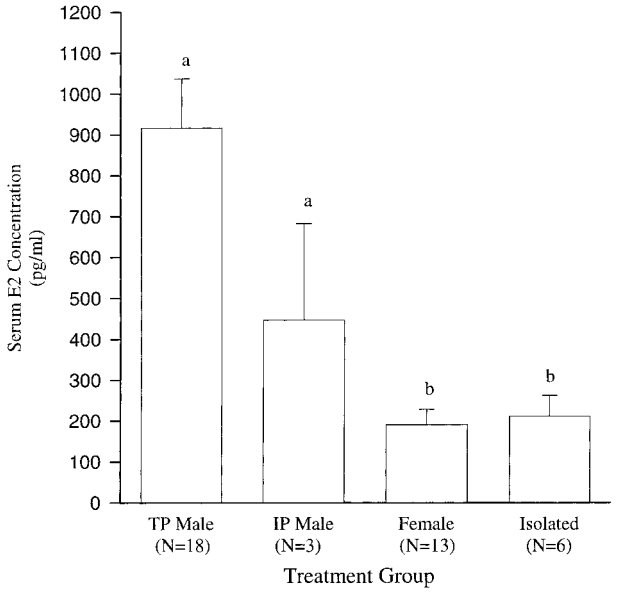

Fig. 6. Serum estradiol-17β (E2) concentrations of the small stimulus females of each social group after reclassification of individuals that completed sex change. Bars represent the mean ± SEM of the number of individuals shown in parentheses. Different letters represent statistically different values ($p \leq 0.05$).

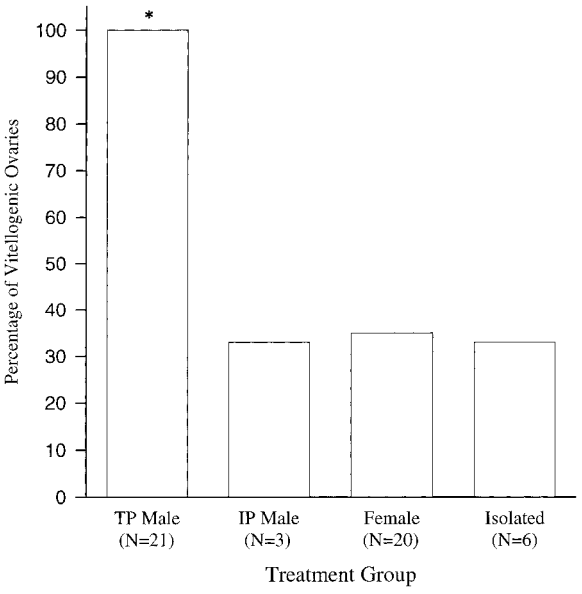

Fig. 7. The percentage of small stimulus females from each social group with vitellogenic follicles in the ovary. Sample size is shown in parentheses. The asterisk represents a statistical difference at $p \leq 0.05$.

To identify potential effects of the male phenotype on ovarian function independent of sex change, GSIs and serum E2 concentrations of small stimuli fish were also compared. GSIs of stimuli fish housed with TP males were larger than those of stimuli fish of the female and small IP treatments as well as those of isolated females (Fig. 5A). The differences became statistically significant when the stimuli fish housed with individuals which had become functional TP males by the end of the experiment were reclassified as members of the large TP male treatment rather than the female and small IP male treatment groups (Fig. 5B). Serum E2 concentrations (Fig. 6) and the percentage of vitellogenic ovaries (Fig. 7) were statistically ($p \leq 0.05$) higher in stimuli fish housed with TP males than in stimuli fish from any other treatment group or isolated females.

DISCUSSION

The results of this study clearly indicate that male phenotype has important implications beyond alternative mating tactics in the diandric hermaphrodite *T. duperrey*. While females housed with TP males had functional ovaries, females housed with IP males or other females changed sex. These results suggest that the presence of TP males, but not IP males, inhibits sex change and stimulates female function. The "active" maintenance of ovarian function through the presence of TP males may, therefore, represent

a mechanism that inhibits sex change in *T. duperrey*.

T. duperrey ovaries, unlike those of many hermaphroditic species, have no distinguishable primordial testicular tissue prior to sex change (Nakamura *et al*., 1989). Once sex change has been initiated, ovarian degeneration and testicular development occur as essentially separate developmental events. Supported by the failure of isolated females to change sex despite ovarian regression, this temporal separation suggests that sex change may be under the control of mutually exclusive inhibitory and stimulatory mechanisms. Following removal of the inhibition, the animal requires a specific stimulatory cue to initiate testicular development and complete the process of sex change.

The "relative-size" hypothesis suggests that interactions with larger or smaller fish of any sexual phenotype inhibit or stimulate sex change, respectively. Given the importance of social cues in initiating physiological sex change in *T. duperrey* as well as the profound effects of social interactions on the reproductive biology of gonochoristic fishes (eg. Degani, 1993; Degani and Schreibman, 1993), closer examination of potential effects of male phenotypes on female reproduction was warranted. Interestingly, consideration of the male phenotype (IP or TP) with respect to sex change does not usually violate the "relative size" hypothesis. IP males and females have similar size distributions in the population (Ross, 1982). The wide range in size allows the presence of larger and smaller IP males relative to a female. In contrast, TP males are typically the largest individuals in local populations; however, overlap in size ranges of the entire population allowed the experimental separation of size from other characteristics of the TP phenotype in this study.

To assess the effects of various social groupings on the sex-changing capabilities of large females that were predicted by the "relative size" hypothesis to change sex, we compared gonadosomatic indices, serum E2 concentrations, and gonadal histology between treatments. The onset of sex change is characterized by a rapid decline in GSI and low serum E2 levels (Nakamura *et al*., 1989). As these parameters may also vary with the ovarian cycle and season (Hourigan *et al*., 1991; Hoffman, 1987), gonadal histology was utilized to confirm the progression of sex change. Based on all criteria, the predicted sex changer from the small TP male treatment had no evidence indicating sex change. Similar results were obtained in the large TP male treatment in which the largest female was not expected to change sex. The single individual that changed sex in the large TP treatment likely represents a minor limitation in the methodology for determining sex prior to establishing social groups. As the animals were collected directly from the reef, it is possible that some individuals would be undergoing sex change at the time of collection. It is impossible to account for this without sacrificing the animal; however, the likelihood of catching a transitional fish is too low to warrant further consideration. Nevertheless, these results clearly indicate that TP males inhibit sex change regardless of their relative size within a social group.

In contrast, the predicted sex changers from the small IP male treatment had significantly smaller GSIs, lower E2 concentrations and histological evidence of sex change. This confirms that the TP phenotype, not the mere presence of a male, inhibits sex change. Similarly, the predicted sex changers from the female treatment with two exceptions also underwent sex change according to the criteria applied. The time required to initiate and/or complete sex change varies considerably and may depend on the degree of social stimulation (unpublished data). Consequently, the two individuals from this group that did not initiate sex change may have required more time than was allotted in the experimental design. Isolated females had intermediate GSIs and E2 levels but no histological evidence of sex change suggesting a reproductively inactive state, further supporting the critical nature of social cues for normal reproduction and/or sex change in *T. duperrey*. Taken together, these results indicate that, in the absence of TP males, the "relative size" hypothesis reliably predicts the social control of sex change; however, the TP phenotype is likely to play a predominant role under natural conditions.

The inhibition of sex change was paralleled by an effect of the TP phenotype on ovarian development. Large females housed with TP males had GSIs and serum E2 levels comparable to those of females sampled from the reef during the season of highest reproduction (Nakamura *et al*., 1989; Hoffman, 1987). The preponderance of vitellogenic follicles or evidence suggesting impending spawning argues for normal ovarian function in these individuals. In contrast, isolated females had GSIs and E2 concentrations similar to those of females sampled during the season of lowest reproduction. Ovaries of isolated females appeared histologically quiescent. These results suggest the TP phenotype stimulates ovarian development relative to isolated fish, but the highly social nature of *T. duperrey* cautions against drawing strong conclusions from these data alone.

Large females presumably initiate sex change as soon as they detect the absence of the inhibitory TP male and the presence of the stimulatory fish. Consequently, it is difficult to determine whether the differences seen between females housed with or without a TP male are a result of stimulation by the TP male, degeneration of the ovary which accompanies sex change, or a combination of both. The small, stimulatory fish included in our treatment groups, however, allow comparison of the criteria for ovarian development in social groups independent of sex change. Small females housed with TP males showed normal ovarian development as indicated by GSIs similar to those seen in large females from the same treatment groups and females from the reef. Small females housed with an IP male or female, however, had small GSIs similar to those of isolated females indicating arrested development. The difference between the TP treatments and other treatments became more profound when a few individuals that had completed or were in advanced stages of sex change were reclassified as TP males. Once again, the apparent dependence on social cues of reproduc-

tion in *T. duperrey* complicates the interpretation of this observation. Behavioral sex change precedes changes in secondary sex characteristics which occur prior to actual production of sperm in *T. duperrey* (Larson, 1999). Depending on which aspect of the TP phenotype affects other fish, small females could easily perceive a transitional fish as a TP male before gonadal sex change is complete. Theoretically, if cues from TP males are responsible for synchronizing gamete development, ovulation should coincide with the new male's ability to spawn. Therefore, only individuals with active spermatogenesis in the testis were reclassified. E2 concentrations and ovarian histology of females housed with TP males also indicate a normal ovarian cycle whereas the same criteria suggest a temporary period of quiescence in small females of other treatment groups. Combined with the differences between isolated females and females housed with TP males, these data illustrate that the TP phenotype is responsible for maintaining normal ovarian function.

The implications of the link between the TP phenotype and ovarian function and its effect on sex change are threefold. First, TP males increase the likelihood of successful pair spawning by insuring the presence of ripe females in their immediate proximity. This also prevents females from wasting valuable resources on ovarian development unless the preferred mate, the TP male, is present. Second, stimulation of ovarian function precludes sex change which ultimately increases a TP male's reproductive success by limiting competition. Finally, with respect to the physiology of sex change, mechanisms regulating ovarian development either directly or indirectly inhibit sex change. Consequently, the results of this study have not only added to our understanding of the social factors governing sex change, but have also provided a framework for detailed physiological investigations based on general ovarian function in teleosts. Building upon our findings, future research will focus on internal and external factors affecting normal ovarian function. Ultimately, we hope to elucidate the physiological correlates that transduce the social cues regulating sex change in *T. duperrey*.

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