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Sperm and milt characteristics and male *v.* female gametic investment in the Caribbean reef fish, *Thalassoma bifasciatum*

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Individual sperm cells produced by two male morphs of the bluehead wrasse *Thalassoma bifasciatum* did not differ in size (i.e. cell volume). Initial phase (IP) males (high sperm competition) had a 60% higher sperm concentration in their milt than did terminal phase (TP) males (low sperm competition), which may reflect differences in how accurately the two male morphs need to allocate sperm to their spawns. The energy density of milt was about 16% lower than that of eggs. Estimates of gametic energy investment based on (a) the difference in testis weights between the beginning and the end of the spawning period and (b) the number of sperm released in natural spawns (determined in other studies), suggested that, on a daily basis, IP males invest about 65% of that of females. Estimates based on stripping milt from IP males at the beginning and the end of the spawning period, however, indicated that their daily energy investment in gamete production is about 10% of that of females. Gametic investment by TP males is lower than that by both IP males and females.

Key words: sperm competition; sperm and milt characteristics; sperm; eggs; gametic energy investment; coral reef fish.

INTRODUCTION

Sperm competition occurs when ejaculates of two or more males compete to fertilize a given set of eggs (Parker, 1970). Under sperm competition, the male releasing more sperm should fertilize a larger proportion of the eggs relative to other individuals. The number of sperm and the amount of milt a male can produce is likely to be related to testis size, and relative testis weight is correlated positively with intesity of sperm competition in male morphs employing different mating strategies among members of several fish families (e.g. Labridae: Robertson & Choat, 1974; Warner & Robertson, 1978; Scaridae: Choat & Robertson, 1975; Robertson & Warner, 1978; Acanthuridae: Robertson, 1985; Salmonidae: Gage *et al.*, 1995; reviews in Petersen & Warner, 1998; Taborsky, 1998). Multi-species comparisons that controlled for phylogenetic effects have confirmed this relationship at higher taxonomic levels (primates: Harcourt *et al.*, 1981; butterflies: Gage, 1994; bats: Hosken, 1997; birds: Møller & Briskie, 1995; and fishes: Stockley *et al.*, 1997).

Evidence of effects of sperm competition on sperm and milt characteristics other than milt volume and sperm number is less clear (Petersen & Warner, 1998;

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Taborsky, 1998). Sperm length is related positively (e.g. butterflies: Gage, 1994) negatively (fishes: Stockley *et al*., 1997) or not at all (bats: Hosken, 1997) to interspecific variation in sperm competition. Recent studies in nematodes provide evidence for increased sperm size under sperm competition (LaMunyon & Ward, 1998, 1999). Gage *et al*. (1995) found no relation between sperm length of different male morphs of Atlantic salmon *Salmo salar* L., that experience different levels of sperm competition, but sperm motility and sperm longevity were higher in males experiencing high levels.

In this study it was examined whether characteristics of sperm and milt produced by the two male morphs of an externally fertilizing coral reef fish are affected by the different mating strategies and their consequently high and very low levels of sperm competition. Levels of energy investment in gametes by the two male morphs and females were also assessed, since, on large reefs, females and the male morph experiencing higher levels of sperm competition have similar growth and mortality rates, and similar relative gonad size. This suggested that both their current energy expenditures on reproduction and their prospects for future reproductive success were similar (Warner *et al*., 1975; Warner, 1984).

MATERIALS AND METHODS

STUDY SPECIES

The bluehead wrasse *Thalassoma bifasciatum* (Bloch) is a protogynous hermaphrodite, i.e. females can change sex to become males. It has two adult colour phases: the initial phase (IP) includes females and small males, and the terminal phase (TP) includes only large males. TP males can develop from IP males and from females (Reinboth, 1973). In Caribbean Panama, mating takes place daily throughout the year (Warner *et al*., 1975). Fertilization is external, eggs are pelagic and there is no parental care. Whereas males can spawn many times a day, a female releases her day's egg production in one spawning act (Warner *et al*., 1975).

During the spawning period, TP males are territorial and defend spawning sites at the downcurrent reef edge and pair-spawn with single females. TP males experience sperm competition only if an individual IP male joins a spawning pair just as they release their gametes (sneaking occurs in 3–5% of all pair-spawns by TP males, Warner & Robertson, 1978). IP males are non-territorial, and form spawning aggregations of dozens to hundreds of males, where typically five to 15 IP males group-spawn with single females (>98% of their spawns, Warner *et al*., 1975; Warner & Robertson, 1978).

The average total number of sperm released in a group-spawn is 50–100 times higher than that in a pair-spawn, and an individual IP male on average releases about six to 15 times more cells during a group-spawn than a TP male during a pair-spawn (Shapiro *et al*., 1994; Warner *et al*., 1995). This is reflected in the fact that IP male testes are three to four times heavier in absolute terms than those of TP males (Warner & Robertson, 1978), and may be due to the high levels of sperm competition experienced by IP males (Robertson & Choat, 1974; Choat & Robertson, 1975; Warner & Robertson, 1978).

COLLECTION OF FISH

The study was done from September 1994 to April 1995 on two reefs near the San Blas Field Station of the Smithsonian Tropical Research Institute (STRI), in Kuna Yala on the Caribbean coast of Panama. Both reefs carry thousands of individuals of this species (pers. obs.). Males were collected before, during and after the spawning period to assess the dynamics of sperm production. Prespawn males were captured 2 h before the time at which the spawning period had started on the previous day (i.e. between 0900 and 1000 hours) while feeding on plankton at the reef edge. Spawn males were collected at spawning sites within half an hour of the first observed group-spawn. Spawning started

between 1115 and 1225 hours and lasted for 1·5–4·5 h (pers. obs.). Postspawn males were caught at the feeding sites after spawning activity had ceased. Fish were collected using an urchin baited lift net. Further handling was done at the field station (<5 min from study sites), where fish were maintained in a net floating in the sea.

COLLECTION OF MILT

Fish were examined within 40 min after capture unless otherwise stated. Males were stripped after anaesthesia with quinaldine (in 70% ethanol) in sea water. Contamination of samples by sea water was avoided by drying the belly before stripping. Gentle pressure was applied on the abdomen, and milt collected into a plastic pipette tip placed over the genital papilla. Milt volume was determined by collection in micro-haematocrit capillary tubes (to the nearest µl). Samples containing obvious amounts of urine were rejected, but small amounts may have gone undetected. TP males probably produce more urine and less milt than IP males (see Results). Hence problems with urine contaminating the samples could be stronger in TP males. It was considered that urine could be detected if it was more than about 5% of the sample.

SPERM CONCENTRATION IN MILT AND SPERM SIZE

Milt samples from spawn males were used to determine sperm concentrations and sperm size (i.e. volume of individual sperm cells). TP males collected had territories, and IP males came from actively spawning aggregation at known group-spawn sites. Fish were collected by lift net or micro-spear. After stripping, a known volume of milt was diluted in tap water and fixed with a mixture of rose bengal dye and formalin (final concentration of 4% formalin). Tap water was used to avoid coagulation. When sufficient milt was collected dilutions were replicated ($n=3$). Sperm concentrations and sperm sizes were measured using a MultiSizer II electronic particle sizer. Samples were re-suspended, and two sub-samples were prepared by dilution in isotonic saline (Isoton II). Mean sperm volume is *c.* 3 μm^3 and a 50-µm orifice tube with a resolution between neighbouring size channels of *c.* 0·025 μm^3 was used (<1% of mean sperm volume). For each sub-sample 10 000–250 000 cells were measured. Dilution in tap water may have increased sperm size due to hydration, and water may again have been lost to the saline during subsequent dilution. Sperm size stabilized between 15 and 25 min after this second dilution, and measurements were made during this time window. Hence information on absolute size of sperm cannot be given, but it was assumed that sperm from IP and TP males did not respond differently to the treatment and that differences in relative size of sperm were able to be assessed. Sperm concentrations in milt samples were determined from prespawn and postspawn IP males to examine possible temporal variation in sperm concentration during the day (samples treated as above).

IP MALE GAMETIC INVESTMENT

Standard length (L_S) of fish was routinely measured (to the nearest mm). In order to estimate body dry weight (W) from L_S the relationship was estimated between L_S and W of 42 spawn IP males. After their milt had been stripped, fish were kept in individual plastic containers for 8 h, to give them time to empty their guts. Then they were killed with an overdose of quinaldine, frozen, dried to 60° C for 48 h, and weighed (to the nearest mg). The weight–length relationship was estimated using the regression equation, $W=aL_S^b$.

To estimate male gametic investment, both the energy content of sperm cells and the number produced per day were determined. To determine energy content, known volumes of spawn IP male milt were diluted in 1 ml of distilled water. Samples ($n=23$) were freeze-dried, weighed (to the nearest 1 µg) and combusted in a Phillipson Oxygen Microbomb Calorimeter (Gentry Instruments Inc.). Analysis required at least 50 µl of milt per sample and milt of multiple males (two to 16 males) was pooled when necessary. Large samples were split into sub-samples. Then mean energy content per unit volume milt, mean energy content per sperm cell, mean dry weight per unit volume of milt, mean energy content per unit dry weight of milt and mean dry weight of a sperm cell were

estimated (Table I). Seminal fluid of teleost fishes contains organic matter (Piironen & Hyyärinen, 1983). Therefore the seminal fluid was removed by centrifugation in eight samples of IP male milt, and these samples checked for a lower energy content per unit volume milt than untreated samples.

L_S and milt volume were determined for prespawn, spawn and postspawn IP males (n=120, 135 and 84) captured on 8 days over a period of 6 weeks. Fish were killed, testes were dissected out, freeze-dried, and testis dry weight (W_t) was measured (to the nearest 10 μg). To estimate total W_t the estimated dry weight of the stripped milt was added to the W_t. Number of sperm cells in the stripped milt was estimated by multiplying milt volume by the appropriate mean sperm concentration. Relative number of sperm present in the milt was calculated by dividing the above value by W. To compare total W_t between IP males the gonosomatic index (I_G=total $W_t/W \cdot 100$) was used. Three approaches were used to estimate daily gametic energy investment in males. (a) The differences were estimated in the numbers of sperm cells present in the stripped milt of spawn and postspawn males and these numbers were multiplied by the energy content per sperm cell (IP males only). (b) The reduction in total W_t from spawn to postspawn males was multiplied by the energy content per unit dry weight of milt (IP males only). (c) Data were used on levels of sperm release in natural group- and pair-spawns by *T. bifasciatum* at Puerto Rico and St Croix (from Shapiro *et al*., 1994 and Warner *et al*., 1995) and on daily spawning rates by IP and TP males in the study area (Warner *et al*., 1975) to estimate the number of sperm released by the males.

To estimate spawning activity on the days when IP males were collected, females were collected during the prespawn collections on the same reef as males, and kept until the end of the spawning period. Females that released hydrated eggs when stripped were counted as having a clutch. As no hydrated eggs could be stripped from females right after capture it seems reasonable to think that hydration takes place shortly before the spawning period, and that females with a hydrated clutch at the time of stripping would have spawned on that day. Also females do not release their eggs while kept in the net (Warner, 1985). The proportion of females with a clutch was used as an estimate of spawning activity on a given day.

I_G in prespawn and spawn males was expected to correlate positively with spawning activity, because both parameters are likely to be driven by food availability. Postspawn I_G levels may follow the same pattern, but it seems equally possible that fish spawn out the testis more on days with high spawning activity, hence, there was no clear expectation for postspawn IP males. Similarly, a positive correlation was expected between relative number of sperm present in the stripped milt of spawn males and spawning activity, because the males will expect much activity on such days. For postspawn males a negative correlation was expected, because sperm are likely to have been used up more completely on days with high spawning activity. There was no clear expectation for the prespawn males because it was not known when the fish prepare themselves for the spawning period.

FEMALE GAMETIC INVESTMENT

As part of another study (Robertson *et al*., 1999) daily egg production over a 90-day period was monitored on the study reefs in 1993 by catching females shortly before the daily spawning period (*c.* 50 females day^{-1}) and stripping them at the end of that period. The number of eggs produced by each female was estimated using the volume of her clutch and the relationship between the number of eggs contained in 50 clutches of a range of volumes (r^2=0·93, Robertson *et al*., 1999 for details). Average egg weights were obtained by washing, freezing, freeze-drying and weighing 50 eggs from each of 200 clutches. The average energy content (J mg^{-1}) of eggs was determined for 15 clutches. Here the estimate of average gametic investment per female per day takes into account the fact that on average only 75% of females spawned on any given day.

DATA ANALYSIS

The means of all MultiSizer measurements for each male were used as a datum for statistical analysis of sperm concentration and sperm size. Parametric tests were

TABLE I. Milt characteristics of initial phase (IP) and terminal phase (TP) males before (prespawn), during (spawn) and after (postspawn) the daily spawning period

Male phase	Collection time	Energy per unit volume of milt (J μl^{-1})	Energy per million sperm (J/10^6 cells)	Dry weight per volume of milt ($\mu g\ \mu l^{-1}$)	Dry weight of milt ($\mu g/10^6$ cells)	Energy per unit dry weight of milt (J mg^{-1})
IP	Prespawn	**2·5**	*0·079*	**126**	*3·9*	*20·2*
	Spawn	1·37	0·079	68	3·9	20·2
	Postspawn	**1·7**	*0·079*	**84**	*3·9*	*20·2*
TP	Spawn	**0·8**	*0·079*	**42**	*3·9*	*20·2*

Values in plain typeface were measured in this study. Values in italics are assumed to be independent of sperm concentration and therefore equal for the different collection times. The similarity in sperm size between the male phases suggests that these values can also be used for TP males. Values in bold are estimated using the appropriate sperm concentration values.

performed when data fulfilled the necessary assumptions. Data are reported as mean ± 1 S.E. Directed tests (Rice & Gaines, 1994*a*) were performed when there were clear predictions about the direction of examined differences. Ordered heterogeneity tests (Rice & Gaines, 1994*b*) were performed to test for correlations between the measures of male gametic investment and spawning activity.

RESULTS

SPERM CONCENTRATION IN MILT AND SPERM SIZE

Average sperm concentration in the milt of spawn males was *c.* 60% higher in IP males ($17{\cdot}3 \pm 0{\cdot}86 \times 10^6$ sperm μl^{-1}, $n=52$) than in TP males [$10{\cdot}7 \pm 1{\cdot}23 \times 10^6$ sperm μl^{-1}, $n=24$; one-way analysis of variance (ANOVA), $F_{1,74}=19{\cdot}1$, $P<0{\cdot}0001$]. Comparison of average sperm concentration in the milt of IP males collected before ($32{\cdot}2 \pm 2{\cdot}38 \times 10^6$ sperm μl^{-1}, $n=11$), during (above) and after ($21{\cdot}5 \pm 2{\cdot}68 \times 10^6$ sperm μl^{-1}, $n=8$) the daily spawning period detected a temporal effect ($F_{2,68}=23{\cdot}0$, $P<0{\cdot}001$). Multiple comparisons (Tukey–Kramer HSD, $q_{0{\cdot}05,68,3}=3{\cdot}40$) revealed a drop in sperm concentration from prespawn to spawn collections ($q=9{\cdot}55$, $P<0{\cdot}0001$), and a difference between prespawn and postspawn collections ($q=4{\cdot}91$, $P<0{\cdot}005$), but not between spawn and postspawn collections ($q=2{\cdot}34$, $P>0{\cdot}2$). Mean L_S of IP males differed with collection time (one-way ANOVA, $F_{2,66}=3{\cdot}46$, $P=0{\cdot}037$, prespawn: $55{\cdot}40 \pm 2{\cdot}24$ mm; spawn: $58{\cdot}96 \pm 1{\cdot}16$ mm; postspawn: $51{\cdot}53 \pm 3{\cdot}17$ mm). However, there was no correlation between male L_S and sperm concentration within either collection (Spearman, prespawn: $r_s=-0{\cdot}39$, $P>0{\cdot}25$; spawn: $r_s=0{\cdot}10$, $P>0{\cdot}45$; postspawn: $r_s=-0{\cdot}14$, $P>0{\cdot}7$).

There was no difference in average volumes of individual sperm cells produced by spawn IP and TP males ($3{\cdot}28 \pm 0{\cdot}04$ and $3{\cdot}34 \pm 0{\cdot}06$ μm^3, respectively; $F_{2,68}=0{\cdot}83$, $P>0{\cdot}35$).

IP AND TP MALE GAMETIC INVESTMENT

L_S of IP males captured for the weight–length relationship ranged 33–86 mm and W 0·181–2·88 g. The regression equation [$W=(4{\cdot}857 \times 10^{-6})\ L_S^{2{\cdot}988}$, $r^2=0{\cdot}991$, $n=42$] allowed us to convert L_S to W.

There was no significant difference between mean energy content per unit volume of uncentrifuged and centrifuged milt (uncentrifuged, $1{\cdot}37 \pm 0{\cdot}04$ J μl^{-1}, $n=23$; centrifuged, $1{\cdot}30 \pm 0{\cdot}07$ J μl^{-1}, $n=8$; $t=0{\cdot}86$, $P>0{\cdot}35$), indicating that energy is located primarily in the sperm cells. The following estimates use uncentrifuged samples only. Mean energy content per unit of milt dry weight was 20·2 J mg^{-1} ($\pm 0{\cdot}4$, $n=23$), energy content of milt was 0·079 J per million sperm cells, and average dry weight of milt equals 3·9 µg per million sperm cells ($\pm 0{\cdot}1$, $n=13$) and 68 µg μl^{-1} of milt ($\pm 2{\cdot}6$, Table I).

Mean I_G was significantly affected by collection time but not by L_S (analysis of covariance, ANCOVA, effect of collection: $F_{2,335}=27{\cdot}9$, $P<0{\cdot}0001$; effect of L_S: $F_{1,335}=0{\cdot}2$, $P>0{\cdot}6$, Fig. 1, Table II). Multiple comparisons (Tukey–Kramer HSD, $q_{0{\cdot}05,336,3}=2{\cdot}35$) showed an increase in I_G from prespawn to spawn collections ($q=8{\cdot}1$, $P<0{\cdot}0001$), a decrease from spawn to postspawn collections ($q=10{\cdot}2$, $P<0{\cdot}0001$), but no significant difference between prespawn and postspawn collections ($q=2{\cdot}85$, $P=0{\cdot}13$, Fig. 1). A similar pattern was evident in the mean relative number of sperm cells present in the stripped milt of those

TABLE II. Mean (± 1 S.E.) milt volume, number of sperm present in that volume, number of sperm present in that volume relative to body dry weight (W), total testis dry weight (total W_t) and gonosomatic index (I_G) for IP males collected before (prespawn), during (spawn) and after (postspawn) the daily spawning period

Size class L_S (mm)	Collection	n	Milt volume (µl)	No. sperm (10^6)	Relative no. sperm (10^6 mg^{-1} W)	Total W_t (mg)	I_G
30–39	Prespawn	18	1·56 ± 0·30	50·1 ± 9·8	206 ± 39·1	4·86 ± 0·65	1·98 ± 0·24
	Spawn	19	4·16 ± 0·91	71·9 ± 15·8	306 ± 60·5	4·90 ± 0·61	2·09 ± 0·22
	Postspawn	10	1·90 ± 0·50	40·9 ± 10·8	165 ± 43·7	3·71 ± 0·32	1·46 ± 0·12
40–49	Prespawn	43	3·63 ± 0·61	117 ± 19·6	262 ± 39·2	9.07 ± 0·77	2·10 ± 0·15
	Spawn	61	11·4 ± 0·80	197 ± 13·8	483 ± 32·6	11·2 ± 0·54	2·76 ± 0·11
	Postspawn	48	4·19 ± 0·74	90·0 ± 16·0	208 ± 33·8	7·26 ± 0·62	1·67 ± 0·12
50–59	Prespawn	36	6·61 ± 0·97	213 ± 31·2	292 ± 41·4	15·0 ± 1·03	2·08 ± 0·14
	Spawn	38	20·5 ± 1·76	354 ± 30·4	482 ± 40·8	20·1 ± 0·96	2·74 ± 0·13
	Postspawn	17	6·53 ± 1·71	140 ± 36·8	174 ± 46·0	16·6 ± 0·95	2·19 ± 0·13
60–69	Prespawn	23	6·17 ± 0·85	199 ± 27·5	172 ± 25·0	19·0 ± 1·77	1·60 ± 0·15
	Spawn	17	29·4 ± 3·86	509 ± 66·7	441 ± 60·6	24·7 ± 2·16	2·12 ± 0·18
	Postspawn	9	13·4 ± 4·69	289 ± 101	226 ± 76·7	20·1 ± 3·09	1·56 ± 0·22

Too few males >70 mm L_S were collected, possibly because IP males at this size start to change into TP males.

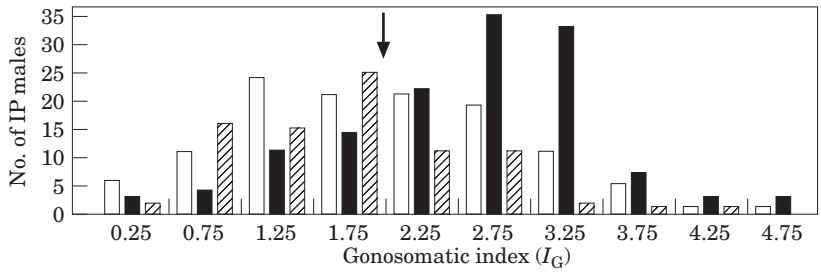

FIG. 1. Frequency distributions of gonosomatic index (I_G) of initial phase (IP) males collected before (prespawn males, □) during (spawn males, ■) and after (postspawn males, ▨) the daily spawning period. The arrow indicates the mean I_G of the prespawn IP males.

males (Kruskal–Wallis using χ^2-approximation, $\chi^2=73{\cdot}6$, d.f.$=2$, $P<0{\cdot}0001$). Multiple comparisons (Mann–Whitney tests using χ^2-approximation and a Bonferroni adjusted α-level of 0·016 to correct for multiple comparisons) revealed the same patterns as above (prespawn *v.* spawn, $\chi^2=46{\cdot}5$, d.f.$=1$, $P<0{\cdot}0001$; spawn *v.* postspawn, $\chi^2=55{\cdot}9$, d.f.$=1$, $P<0{\cdot}0001$; prespawn *v.* postspawn, $\chi^2=4{\cdot}19$, d.f.$=1$, $P=0{\cdot}04$). Mean L_S of these males did not differ significantly between collection times (Kruskal–Wallis, $\chi^2=3{\cdot}14$, d.f.$=2$, $P>0{\cdot}2$; prespawn: $50{\cdot}1 \pm 0{\cdot}8$ mm, $n=120$; spawn: $48{\cdot}4 \pm 0{\cdot}7$ mm, $n=135$, postspawn: $48{\cdot}1 \pm 0{\cdot}9$ mm, $n=84$).

Unfortunately it is not possible to collect prespawn and spawn IP males at the same location. Therefore it was checked whether the observed differences in I_G could stem from males with small testes not migrating to the spawning site. If so, the increase in I_G in spawn males may be due to the fact that no males with small testes were sampled at the spawning site. One would then, however, expect that the upper half of the spawn male I_G frequency distribution would not be different from the upper half of the prespawn I_G frequency distribution (Fig. 1). Thus a statistical difference between these halves of the I_G frequency distributions would suggest a real change in I_G over time. The mean I_G value of prespawn males (i.e. the mean value of the population that would include all fish) was used as the cut-off point for this comparison (arrow in Fig. 1). This test revealed a significant difference between prespawn and spawn males (Mann–Whitney test, $\chi^2=6{\cdot}2$, d.f.$=1$, $P<0{\cdot}05$) indicating that I_G of IP males actually changes over time.

The difference in number of sperm cells in stripped milt of spawn and postspawn IP males suggests a mean daily investment of 11·3 J (Fig. 2). The estimate based on the decline in testis weight from spawn to postspawn IP males suggests a mean daily investment of 68·2 J by IP males (Fig. 2). Finally the estimate, which used literature data on the number of sperm released in natural group- and pair-spawns (IP males: $52{\cdot}8 \times 10^6$ sperm per male and spawn, Shapiro *et al.*, 1994; TP males: $3{\cdot}3 \times 10^6$ sperm per male and spawn, Warner *et al.*, 1995) and on daily spawning rates (IP males: 17·1 spawns per male and day; TP males: 43·9 spawns per male and day, Warner & Robertson, 1978), suggests a daily sperm release of 903×10^6 and 146×10^6 sperm per male and day, and a daily gametic investment of 71·3 and 11·5 J for IP and TP males, respectively (Fig. 2).

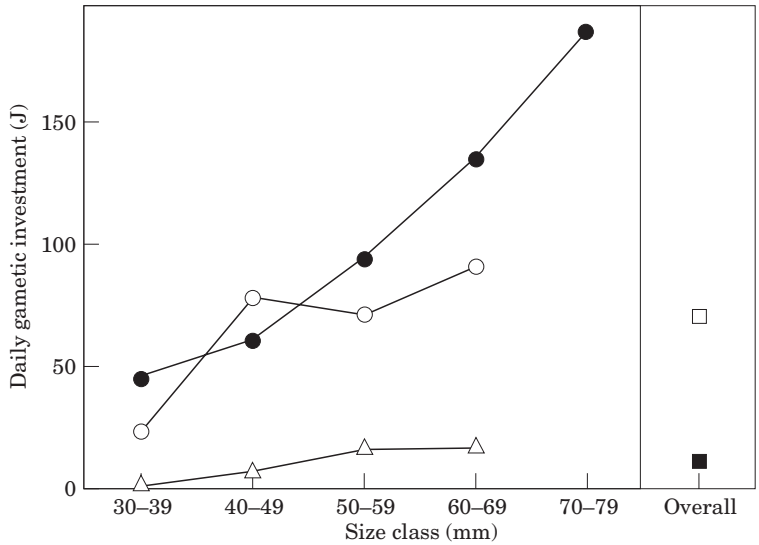

FIG. 2. Estimated daily gametic energy investment by initial phase (IP) males, terminal phase (TP) males and females. Estimates for different size classes of IP males are based on (a) the difference in the number of sperm present in the milt of spawn and postspawn IP males times the energy content of sperm cells (△) and (b) the mean loss of total testis weight from spawn to postspawn IP males times the energy content per dry weight of milt (○). Estimates for different size classes of females (●) are based on the mean number of eggs stripped times their mean energy content per egg. Estimates based on data from the literature on sperm release in natural group- and pair-spawns and the number of spawns per day times the energy content of sperm cells for IP males (□) and TP males (■).

To estimate spawning activity an average of 31·1 (± 5·0) females were collected per day (8 days), 55·3–97·1% of which had a clutch (76·4 ± 5·3%). As expected, positive correlations were found between I_G values of prespawn and spawn IP males, and the measure of spawning activity (prespawn, 8 days, one-way ANOVA, $F_{7,112}=4{\cdot}43$, $r_sP_c=0{\cdot}43$, $P_{dir}<0{\cdot}05$; spawn, 7 days, $F_{6,128}=4{\cdot}48$, $r_sP_c=0{\cdot}54$, $P_{dir}<0{\cdot}05$). There was no such correlation for postspawn males (6 days, $F_{5,78}=1{\cdot}39$, $r_sP_c=0{\cdot}06$, $P_{2\text{-tailed}}=0{\cdot}76$). The expected positive correlation between relative number of sperm cells and spawning activity was not found (Kruskal–Wallis, $\chi^2=33{\cdot}5$, d.f.=6, $r_sP_c=0{\cdot}27$, $P_{dir}>0{\cdot}34$). The expected negative correlation with sperm numbers in postspawn males, however, was confirmed ($\chi^2=8{\cdot}40$, d.f.=5, $r_sP_c=-0{\cdot}815$, $P_{dir}<0{\cdot}005$). A negative correlation was found for prespawn males ($\chi^2=20{\cdot}7$, d.f.=7, $r_sP_c=-0{\cdot}59$, $P_{2\text{-tailed}}=0{\cdot}02$).

FEMALE GAMETIC INVESTMENT

Females collected for the determination of daily energy investment to gametes were grouped into five size classes. Females released an average of 450 ± 16 ($n=395$), 610 ± 13 ($n=1166$), 940 ± 23 ($n=1050$), 1340 ± 42 ($n=700$), and 1860 ± 108 ($n=236$) eggs for each size class, respectively. Average egg dry weight was $4{\cdot}21 \pm 0{\cdot}27$ µg ($n=50$), mean energy content of eggs was $24{\cdot}07 \pm 0{\cdot}27$ J mg^{-1} ($n=15$), and mean energy content per egg was 0·101 J per egg. This allows calculation of daily gametic investment by females (Fig. 2) in relation to IP and TP male gametic investment.

DISCUSSION

SPERM CONCENTRATION IN MILT

The data show that mean sperm concentration in the stripped milt of IP males of *T. bifasciatum* is *c.* 60% higher than in TP males. Assuming this reflects actual differences in sperm concentrations in naturally released milt, there appear to be at least three possible non-exclusive explanations.

(1) Shapiro *et al.* (1994) found that the number of sperm released in natural spawns by both IP and TP males varied positively with female size, and suggested that males economized by releasing an optimal number of sperm necessary to fertilize most of clutch of the female. Warner *et al.* (1995) found that TP males released $3{\cdot}3 \times 10^6$ sperms per spawn on average. They proposed that TP males control sperm output, with the amount released being determined by (a) the relationship between sperm output and fertilization rate, which results in diminishing returns at high levels of sperm output, and (b) benefits associated with having sperm for additional spawns. The present data suggest that 3×10^6 sperm cells are contained in 0·3 µl of milt. Examination of the fertilization curve in fig. 1 of Warner *et al.* (1995) indicates that TP males may control sperm release with an accuracy of $\pm 10^6$ cells per spawn, which corresponds to controlling the release of a milt volume at *c.* $0{\cdot}3 \pm 0{\cdot}1$ µl. It is suggested that it may be easier for the fish to control accurately the release of batches of sperm in such small milt volumes if milt is relatively dilute. The results of Shapiro *et al.* (1994) regarding IP males may be due to IP males also adjusting the level of sperm release, or may stem from size assortative mating (van den Berghe & Warner, 1989), with the fish allocating a fixed amount of milt to each spawn. Despite a recent investigation into the morphology of the genital papilla of *T. bifasciatum* (Rasotto & Shapiro, 1998) it is to date unclear how sperm release is controlled. In an average group-spawn, IP males release about 50×10^6 cells (Shapiro *et al.*, 1994), which according to our data would correspond to 3 µl of milt. They may therefore not need to produce dilute milt to achieve the same relative degree of control over sperm release. Differences in the morphology of the reproductive tract between IP and TP males that attain low and high mating rates may improve our understanding of control of sperm release.

(2) Alternatively, it seems possible that the higher sperm concentration in the milt of IP males allows them to allocate large amounts of sperm to a spawn faster, which may be advantageous in the presence of intense sperm competition.

(3) A third alternative explanation for differences in sperm concentration between IP and TP male milt may be that the different mating modes require different milt characteristics (e.g. viscosity, Ruchon *et al.*, 1995; Marconato *et al.*, 1996) which may cause milt to dissolve and disperse at different rates. The body of water into which gametes are released is likely to have different properties between group- and pair-spawns simply due to the different amount of turbulent mixing caused by different numbers of fish turning around at the apex of the spawning rush. High rates of turbulent mixing cause quick dilution of gametes (Denny & Shibata, 1989) and concentrated semen may be able to counteract this.

The only other study that looked at differences in sperm concentration between male morphs of an externally fertilizing fish that experience high and low levels of sperm competition was performed on *Salmo salar* L. (Kazakov, 1981). Data reported there suggest a 20% lower sperm concentration in parr males which experience higher levels of sperm competition (*cf.* Taborsky, 1998) but Kazakov gave no statistics on that issue and the fish of the two male morphs had different origins.

SPERM SIZE

The data here indicate no differences in average sperm sizes (i.e. volume of sperm cells) of IP and TP males. There could, however, be differences in sperm characteristics between the phases that do not affect sperm volume. Gomendio & Roldan (1991) have found that polyandrous primate and rodent species have longer sperm than monandrous species, which they linked to differential sperm competition. Gage *et al.* (1995) found that more sperm were motile in milt of parr males of Atlantic salmon which are employed in sneaking and that these sperm lived longer. They did not, however, find evidence for differences in sperm length between the male morphs. Stockley *et al.* (1997) found a negative relationship between sperm length and sperm competition in fishes. Additionally the method used to determine sperm size in this study may be unsuitable to detect small differences in sperm volume. The question of whether there are differences between IP and TP males of *T. bifasciatum* in terms of sperm motility, longevity and morphometry that may represent adaptations to large, persistent differences in sperm competition remains to be investigated.

LEVELS OF GAMETIC INVESTMENT AMONG THE SEXES

Energy content per unit dry weight of eggs was higher than that of sperm. This is to be expected because fish eggs generally contain a lot of lipids which have a higher energy density than carbohydrates and proteins (Weatherley & Gill, 1987). Also, sperm may have become activated during dilution in tap water and may have lost some energy due to flagellar movement.

The significant increase in I_G from prespawn to spawn IP males indicates allocation of inorganic or organic material to the testis during this period. This was accompanied by a significant decrease in sperm concentration in the milt of IP males and by a significant increase in relative number of sperm cells present in stripped milt over the same period.

The decline in I_G from spawn to postspawn collections may indicate the amount of testicular material used during the spawning period. The significant drop in number of sperm cells present in the milt over that period showed the same pattern. The higher sperm concentration in prespawn over postspawn males indicates, that these males had not yet reached the state they were in before the spawning period.

One of the approaches to estimate daily gametic investment by IP males compared the number of sperm strippable at the beginning and the end of the spawning period (Fig. 2). This approach assumed that most milt present in the testis at the time of stripping was extracted by stripping. It also assumed that no additional milt became available during the spawning period. The increase in I_G from prespawn to spawn collections, suggests high metabolic activity in the

testis. Possibly additional sperm are not actually produced, but rather detached from the sperm producing tissues in the testis. Working with IP males of *T. bifasciatum*, M. Sheehy (pers. comm.) was able to express additional milt towards the end of the spawning period from individually marked IP males that had already been stripped at the beginning of the spawning period on the same day, indicating that milt stores can be refilled during the spawning period. The approach finally assumed that spawn males captured had just started to spawn and postspawn males had not yet started to produce sperm to be used the following day. This may not be true because fish were collected within half an hour of the first observed group-spawn. As IP males spend only a fraction of the spawning period in sexual activity (Warner, 1984) they may have released milt already at time of collection. Equally, postspawn males may be already in the process of refilling their sperm stores. So there are a number of indications that this approach could well underestimate daily gametic investment by IP males.

The approach using changes in total W_t over the reproductive period (Fig. 2) assumed that no allocation of organic or inorganic material to the testis takes place after the onset of spawning. It is not known whether this is the case, but if so, the loss in testis weight from spawn to postspawn collections would be underestimated. Hence the estimate of IP male gametic investments is probably conservative in that regard. If the testis had started to recover already and add dry weight before the postspawn males were collected, gametic investment would have been underestimated further.

The approach using literature data on sperm release in natural group- and pair-spawns (Fig. 2) may be problematic because data were used from studies at different locations (Panama, Puerto Rico and St Croix) and different times.

The fact that the expected positive correlations were found between I_G of prespawn and spawn IP males and the proportion of females having a clutch on a given day indicated that I_G, and hence also total W_t, was a reliable measure for reproductive activity. No significant correlation was found between relative number of sperm cells present in the milt of spawn IP males and spawning activity. This lends further support to the notion, that the milt volume stripped from spawn IP males actually does not contain all the sperm cells that will be used on that day.

The relative number of sperm cells present in the milt of postspawn IP males correlated negatively with spawning activity, indicating (a) that sperm are used more completely on days with high spawning activity and (b) that the testis was still in a state of recovery when the postspawn males were collected.

The close agreement between the estimates of daily energy investment in IP males based on testis weight loss and sperm release in natural group spawns suggests that they may be more realistic than the estimate based on sperm in the stripped milt.

Data from populations on similarly large reefs in the study area indicate that IP males and females have similar growth and mortality rates (Warner, 1984). Based on the available data it appears that gametic investment by IP males is *c.* 35% less than that by females (Fig. 2). This apparent discrepancy could be due to two factors: (1) IP males spend more time at the spawning ground than do females (Warner, 1984), and may spawn on a daily basis (in contrast to the females that spawn on only 75% of the days). They therefore have to do the

journey from feeding to spawning sites more often than females. A recent study (Warner, 1995) showed that travel times can be up to 50 min one way on a very large reef and that feeding does not occur *en route*. The greater time spent at the spawning ground and in transit may reduce energy intake and decrease the amount of energy available for gamete production by IP males compared with females. (2) The estimates of sperm output by IP males (Shapiro *et al.*, 1994) were based on spawns by a small aggregation of IP males (*c.* 15 fish). On large reefs in the study area IP male aggregations often contain hundreds of males. Perhaps levels of sperm competition and levels of sperm release by individual males per spawn are higher in large aggregations.

At present this issue cannot be resolved. To do so, it will be necessary to collect detailed data on all aspects of the growth, mortality, spawning activity, gamete production of females and IP and TP males from a single population. Food availability may drive the levels of reproductive activity, as suggested by the finding that prespawn IP male I_G correlates with the proportion of females having a clutch on a given day. This opens up the possibility for experimental manipulation of food availability in order to study its effect on gametic investment in relation to sex.

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