PROTOGYNOUS HERMAPHRODITISM IN A TEMPERATE REEF FISH, THE BLACKEYE GOBY, Coryphopterus nicholsii (Pisces: Gobiidae)

by

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Date **March 7, 1997**
In this thesis, I examine the occurrence of protogynous hermaphroditism in the blackeye goby, *Coryphopterus nicholsii* (Pisces, Gobiidae) at Bamfield, Vancouver Island, Canada. The female biased sex ratios, size distributions of female and male *C. nicholsii*, and histological data all confirm that the species is protogynous. Histological data strongly suggest two patterns of sexual ontogeny, with males resulting from either pre- or post-maturational sex change. Sex change is not common during, and immediately after the breeding season.

The social organization of *C. nicholsii* is based upon the year-round defense of a territory, primarily by large individuals of both sexes. A territory always includes one or more shelter rocks. These are used as refuges by both sexes, and as nest sites by males during the breeding season. The defence of a shelter rock is important in reproduction, in the reduction of predation, and the avoidance of adverse environmental conditions. Suitable refuge and nest site rocks are a limited resource and intraspecific competition determines which individuals gain access to them. Since the outcome of competition for suitable shelter rocks is strongly size-specific, large males monopolize spawnings.

Large males receive a disproportionate share of the matings: males smaller than 7.0 cm were never seen nesting in the field. Yet, approximately 30% of males are smaller than 7.0 cm, indicating that many females change sex at smaller sizes. This early sex change confers a growth advantage: small, non-nesting males grow faster than similarly-sized mature females. As a result, small males may obtain nesting male status more rapidly than females that change sex later in life. The presence of early sex changers may also be related to an alternative mating tactic, sneak spawning.

Sex change is correlated with a reduction in whole-body concentration of 17β-estradiol, and an increase in 11-ketotestosterone concentration. Both steroids occur naturally in female and male gobies. Administration of 11-ketotestosterone, its precursor 11-ketoadrenosterone, and an
aromatase inhibitor (Fadrazole) resulted in complete sex change in mature females. These results strongly suggest that both 17β-estradiol and 11-ketotestosterone are involved in mediating sex change in this goby.

These results are discussed with regards to the geographical disparity of protogynous hermaphroditism in teleosts. Protogyny occurs much more frequently on tropical coral reefs than on temperate rocky reefs. I propose that a difference in the occurrence of certain mating systems in tropical and temperate waters partly explains this disparity.
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CHAPTER ONE

General Introduction

Sequential hermaphroditism is the transformation of an individual from one functional sex into the other (Policansky, 1982; Sadovy and Shapiro, 1987). Two patterns of sequential hermaphroditism can be distinguished: protogyny, which refers to female-to-male sex change, and protandry, which applies to male-to-female sex change. To distinguish occasional pathological cases of sequential hermaphroditism from natural ones, a species is only considered sequentially hermaphroditic if a substantial proportion of individuals in a population breed first as one sex and subsequently as the other (Sadovy and Shapiro, 1987).

Sequential hermaphroditism occurs both in plants and animals (Policansky, 1982). In vertebrates, sex change occurs predominantly among the bony fishes, the teleosts. Only one case of protogynous sex change has been documented in the amphibia, Hyperolius viridiflavus (Grafe and Linsenmair, 1989), while none is reported from birds, reptiles or mammals. In teleosts, protogyny occurs more commonly than protandry (Policansky, 1982; Warner, 1984b; Francis, 1992). The occurrence of both gonochorism (= separate sexes) and sequential hermaphroditism within individual teleost families, and of both protogyny and protandry in others, suggests that sequential hermaphroditism has evolved independently several times in the group (Francis, 1992).

In teleosts, there is a strong disparity to the geographical distribution of sequential hermaphroditism. Sex change occurs much more frequently among marine groups than among their freshwater equivalents (Policansky, 1982), and amongst these marine groups, most commonly on tropical coral reefs (Warner, 1984b). Protogyny, for example, is common among tropical reef families, such as wrasses (Labridae), parrotfish (Scaridae), sea basses (Serranidae), porgies (Sparidae), and gobies (Gobiidae) (Reinboth, 1970; Robertson and Warner, 1978; Warner and Robertson, 1978; Policansky, 1982; Thresher, 1984). In contrast, only eight
protogynous species are known from temperate, marine waters. These are the wrasses *Semicossyphus pulcher* (= *Pimelometopon pulchrum*) (Warner, 1975b), *Labrus bergylta* (Dipper *et al.*, 1977), *L. ossifagus* (Dipper and Pullin, 1979), *Notolabrus* (= *Pseudolabrus*) *celidotus* (Jones, 1980), *N. tetricus*, *Pictilabrus laticlavius* (Rusell, 1988), *Pseudolabrus psittaculus* (Last *et al.*, 1983), and one goby *Coryphopterus nicholsii* (Cole, 1983). Hence, there exists a difference in the frequency of occurrence of protogyny in tropical and temperate waters. However, relatively little is known about the conditions influencing sex change in temperate waters, since most studies have focussed on tropical species. As a result, the underlying ultimate and proximate reasons behind this marked difference in frequency of protogyny in tropical and temperate waters remain unknown.

In this thesis, I investigate the occurrence, the social and endocrine context, and adaptive significance of protogynous hermaphroditism in a temperate, marine teleost, the blackeye goby, *Coryphopterus nicholsii* (Pisces, Gobiidae). I contrast and compare this with protogynous hermaphroditism in tropical teleost species. My aim is to identify whether seasonal variation in temperature, daylength, and food availability, or correlated changes in life history patterns, accounts for the difference in occurrence of protogyny in temperate and tropical marine waters. The comparison is expected to improve our understanding of socio-ecological and endocrine mechanisms regulating sex change, and to contribute to revealing patterns involved in the evolution and maintenance of protogyny.

Gobies are members of the family Gobiidae, suborder Gobioidae, and are found in marine and fresh-waters of both tropical and temperate regions, with the exception of the pelagic zone (Breder and Rosen, 1966; Parker, 1982). A characteristic feature of most gobies is their fused pelvic fins, which form a ventral suction disk and with which they attach themselves to the substrate. The suborder Gobioidae includes 1300-1700 species, comprising close to 10% of all living teleost species (Miller, 1984). The Gobiidae is the largest family in the suborder, including over 800 species in 170 genera (Parker, 1982).
The sexes of some gobies can be distinguished by the shape of the genital papilla: in males, long and truncate, in females short and blunt (Miller, 1984; Thresher, 1984). Additional sexual dimorphism in some species includes the larger body size of older males, longer dorsal and anal fin-rays in males, and some colour differences (Miller, 1984; Thresher, 1984). However, the extent of sexual dimorphism within the Gobiidae family, and even within a single genus, varies considerably (Thresher, 1984).

Protogyny in the family Gobiidae was first documented by Lassig (1977), in the species *Paragobiodon echinocephalus* and *P. xanthosoma*. Later studies have confirmed sequential hermaphroditism in an additional 12 goby genera: *Coryphopterus* and *Gobiosoma* (Robertson and Justines, 1982), *Lythrypnus* (Cole, 1988), *Pleurosicya*, *Bryaninops* (Fishelson, 1989), and *Lophogobius*, *Fusigobius*, *Eviota*, *Trimma*, and *Priolepis* (Cole, 1990). In the *Coryphopterus* genus, protogyny has been confirmed in 10 of the 11 species; all show similar gonadal tissues (Cole and Shapiro, 1990). The condition of the one other *Coryphopterus* species is yet to be determined (Cole and Shapiro, 1990). Hence, protogyny is probably an ancestral condition in this genus (Cole and Shapiro, 1990). Whether sex change is an ancestral condition in the family Gobiidae is unknown.

In this thesis, I examine the occurrence of protogynous hermaphroditism in the goby, *Coryphopterus nicholsii*. This species lives on subtidal rocky reefs from central Baja California, Mexico, to northern British Columbia, Canada (Hart, 1973). Protogynous sex change in *C. nicholsii* was first observed in the laboratory, where it occurred year-round (Cole, 1983). The detection of four mature individuals with transitional gonads in the field confirmed the occurrence of sex change in the wild (Cole, 1983). These transitionals were captured both in breeding and non-breeding seasons (Cole, 1983). In the laboratory, sex change of mature female *C. nicholsii* takes a minimum of four weeks (Cole, 1983); the time for complete sex change in the field is unknown.
In an attempt to identify the conditions that promote sex change in *C. nicholsii*, I examine the abundance, size distribution and sex ratio of this species in breeding and non-breeding seasons in Chapter 3. I examined gonadal material and gonadosomatic indices to determine the breeding season, and to detect individuals in the process of sex change.

I describe *C. nicholsii*s social organization, including space use and intraspecific agonistic behaviour, in both the breeding and non-breeding seasons in Chapter 4. I ask whether there is a relationship between sex change and the social organization and mating system. Further, I examine the influence of intraspecific competition on the acquisition of territories by smaller individuals.

In Chapter 5, in an attempt to determine the ultimate causes of protogyny, I provide estimates of the potential reproductive output and growth rates of both female and male *C. nicholsii*. The results are discussed in light of the current hypotheses for the evolution of protogynous hermaphroditism.

In the last chapter, Chapter 6, I examine whole-body concentrations of testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (17β-E2) in female and male *C. nicholsii*. Hypotheses regarding causation of sex change, arising from correlations observed between sex and steroid levels, are subsequently tested in hormone administration studies.
CHAPTER TWO

General Materials and Methods

A) Study Organism

The goby genus Coryphopterus consists of 11 species, 10 of which are found in tropical waters of the western Atlantic and eastern Pacific. Coryphopterus nicholsii is the only temperate species in its genus. Protogyny has been confirmed in 10 of the 11 Coryphopterus species, including C. nicholsii; all show similar gonadal tissues (Cole and Shapiro, 1990). The condition of the one other Coryphopterus species is yet to be determined (Cole and Shapiro, 1990). Hence, protogyny is probably an ancestral condition in this genus (Cole and Shapiro, 1990). This suggests that protogyny in C. nicholsii was maintained after the species invaded the eastern Pacific coast into the temperate zone.

The population structure of C. nicholsii shows the characteristics generally associated with protogyny. The sex ratio is female biased, with approximately two females to every male (Wiley, 1973; Cole, 1983; Breitburg, 1987). The size-frequency distribution is bimodal, with males attaining a greater length (max. 9.0 cm standard length (SL), measured from the tip of closed snout to the base of the caudal fin) than females (max. 7.6 cm SL) (Wiley, 1973; Cole, 1983). Scale analysis of a C. nicholsii population in Laguna Beach, California, showed that individuals may live for up to five years (Wiley, 1973). Apart from a difference in size, sexual dimorphism in C. nicholsii is limited to differences in the shape of the genital papilla, being long and truncate in males, and short and blunt in females (Wiley, 1973; Cole, 1983), and in coloration of the pelvic fins, which are grey in females throughout the year, but turn black in males during the breeding season (Wiley, 1973).

The pattern of sexual ontogeny in C. nicholsii is not clear. All males may be derived from females (referred to as secondary males), since testicular tissue of all males is identical (Cole, 1983). Hence, C. nicholsii could exhibit a condition known as monandry. On the other
hand, males much smaller (3.1 cm) than the smallest mature female (5.0 cm) were detected at Bamfield (Cole, 1983). This suggests that *C. nicholsii* may be diandric, with both secondary males, and individuals starting out as males (referred to as primary males). However, since testes of complete sex changers do not reveal any structures indicating their former ovarian function (Cole, 1983), primary (if existing) and secondary males cannot be distinguished on the basis of their gonadal structure.

*Coryphopterus nicholsii* has a restricted breeding season, which varies from five months at Bamfield (49°49' N, 125°10' W), British Columbia, Canada (Cole, 1983), to seven months at Naples Reef (34°25' N, 119°57' W) and Laguna Beach (33°50' N, 117°90' W), California, U.S.A. (Wiley, 1973; Breitburg, 1984). Spawning takes place at the male's nest site, and eggs are placed in a single layer on the underside of a rock (Ebert and Turner, 1962; Wiley, 1973). Males care for their clutches until hatching, after 10 to 33 days (Cole, 1982a; Breitburg, 1984). Fry then enter a pelagic larval phase, the duration of which is unknown (Wiley, 1973).

**B) Study Area**

The study was carried out at Bamfield, on the west coast of Vancouver Island, British Columbia, Canada (Figure 2.1). Sea and weather conditions limited the study areas to the more protected sites throughout Barkley Sound. In 1994 and 1995, temperatures of the surface water ranged from a minimum of 5.0°C in December to a maximum of 16.0°C in August (data from Cape Beal Lightstation). The study areas consisted of subtidal, rocky reefs, which run continuously along and parallel with the shoreline. The reefs were comprised of rocks 10 to >50 cm in diameter on a sand-shell substrate. The inshore regions of the reefs (0 - 5 m depth) were overgrown with the kelp *Nereocystis luetkeana*, and the deeper, offshore areas (10 - 20+ m depth) were bounded by a sloping silt plain. In general, the size of rocks decreased with depth, with large boulders (>50 cm diameter) at the inshore region, and smaller rocks and sometimes
Figure 2.1  Location of study area around Bamfield Inlet, Vancouver Island, British Columbia, Canada (49°49' N, 125°10' W). T refers to locations where transects were surveyed (Chapter 3); collections were carried out on rocky reefs at Dixon and at Seaciff (Chapter 3); the social organization was described at 'Gobytown' (Chapter 4); the competition experiments were carried out close to and at 'Gobytown' (Chapter 4); and male clutch sizes were estimated at Dixon, Helby, O'Hlat, and Seacliff (Chapter 5).
pebbles (<10 cm diameter) at the deeper, offshore end. All field work was carried out using SCUBA.

C) Measuring, Weighing, and Tagging

Following capture, fish were anaesthetized with tricaine methane sulfonate (MS 222, Syndel Laboratories, Vancouver, Canada), measured (SL to 0.1 cm), weighed (W to 0.1 g), and sexed according to the shape of their genital papilla. Subsequently, fish were either used in field and laboratory studies, or preserved for histology and steroid extraction.

To enable recognition of individuals, I tagged fish with coloured beads attached by a loop of monofilament line (6 lbs) through the dorsal musculature. Tags consisted of a piece of line with one or two beads tied onto one end. A small, open knot was made just below the beads. With a fine needle, the line was threaded through the dorsal musculature between the two dorsal fins. Care was taken to thread in between the scales, to avoid pushing scales into the flesh. The line was pulled through and threaded through the open knot. Subsequently, the open knot was pulled tight and pushed close to the body. The line was cut off as close to the body as possible. In both field and laboratory studies, I assigned bead colours randomly, to avoid possible bias that might arise from the effect of tag colour on social interactions (Burley, 1986).

D) Laboratory Holding Conditions

During all laboratory experiments, I kept fish in tanks with flow-through sea water. Tanks had a 5 cm layer of coarse beach sand on the bottom. Length, width, and height of tanks varied, and are presented separately for each experiment. Refuges and nest sites of similar dimensions to natural shelters were provided as described for each experiment. Fish were kept in natural light or artificial illumination at natural daylight hours, and were fed frozen brine shrimp (ad lib.) daily.
E) Histology

Whole gonads were dissected from specimens, cut into three equal parts transversely and preserved in Dietrich's fixative (1994 samples), or in formalin fixative (10% buffered solution of 37-40% formaldehyde) (1995 samples). In several instances, however, gonads were too small for dissection, and whole abdomens (from gills to the genital papilla) of fish were preserved.

To facilitate sectioning of abdomens, I decalcified vertebrae using a 10% EDTA (Ethylenediaminetetraacetic acid; Sigma Chemical Co., St. Louis) solution (Lynch, 1983). To allow the EDTA to penetrate the abdomen completely, the abdomen was cut into three equal parts transversely and submersed in the EDTA solution for six days. The EDTA solution was slowly stirred, and was renewed every 24 hours. After six days the abdomens were washed in distilled water for six to eight hours. The distilled water was slowly stirred, and was renewed every two to three hours. The abdomens were subsequently transferred into 70% ethanol until further processing.

Preparation for histological analysis consisted of dehydrating the middle part of each gonad or abdomen in increasing concentrations of ethanol. The samples were transferred into toluene to wash out excess alcohol, and embedded in wax so that transverse sections along the anterior-posterior axis could be obtained. Samples of each gonad were sectioned serially at 5 μm, mounted onto microscope slides, and stained with Harris' Haematoxylin and Eosin. Slides were mounted with cover slips, sealed with Permount (Fisher), and examined under a light microscope.
CHAPTER THREE

Sex Change and Seasonal Changes in Population Structure in Coryphopterus nicholsii

A. Introduction

In a number of tropical, protogynous teleosts, changes in local social conditions initiate sex change (see reviews Shapiro, 1987; Ross, 1990). The exact social factors instigating sex change depend on the species' social organization and mating system (Ross, 1990). For example, the wrasse Labroides dimidiatus, the seabass Anthias squamipinnis (= Subanthias squamipinnis), and the hogfish Bodianus diplotaenia live in small harems with rigid dominance hierarchies. In all three species, the removal of the male resulted in sex change by the largest, dominant female in the harem (Fishelson, 1970; Robertson, 1972; Hoffman, 1983). In species with large social groups lacking rigid dominance hierarchies, it is thought that sex change is induced by specific changes in the social group (Ross, 1990), such as changes in sex ratio (Shapiro, 1979; Shapiro and Lubbock, 1980) or size distribution (Ross et al., 1983; Ross, 1990). No experimental data are available to support the sex ratio model. On the other hand, in the protogynous wrasse Thalassoma duperrey, an increase in the relative numbers of smaller conspecifics in a social unit can induce sex change (Ross et al., 1983).

In protogynous teleosts living on coral reefs, one might expect a relationship between factors instigating sex change and social and mating systems. The relative stability of ecological and physical conditions in these environments generally allows for year-round spawning (Breder and Rosen, 1966; Johannes, 1978), and results in social organizations that are relatively stable over time (Ebeling and Hixon, 1991). Probably this year-round stability allows individuals to accurately assess their status and relative condition within the local population continuously throughout the year. Hence, females can respond to changes in their local social organization and mating hierarchy year-round, and thus to opportunities for sex change. Indeed, in many
tropical protogynous teleosts sex change occurs year-round (see references in Sadovy and Shapiro, 1987).

In contrast, temperate protogynous teleosts occur in a seasonally changing environment that restricts conditions favourable for breeding (e.g. temperature, food availability) to a limited season (Breder and Rosen, 1966; Wootton, 1990b). A restricted breeding season most likely affects a species' social and mating system. The few studies carried out in temperate waters show that males of the protogynous wrasses *Notolabrus celidotus*, *N. tetricus*, *Pictilabrus laticlavius*, *Pseudolabrus psittacus*, *Labrus bergylta*, and *L. ossifagus* defend spawning sites during the breeding season (Jones, 1980, 1981; Potts, 1984; Barrett, 1995). Whether they are territorial year-round is unknown. The home ranges of female *N. celidotus*, *P. laticlavius*, and *P. psittacus* overlap male territories, at least during the breeding season (Jones, 1980; Barrett, 1995). The social organization of the one other temperate, sex-changing teleost to have been studied, the goby *C. nicholsii*, appears to be one of year-round territoriality (Cole, 1984).

In addition, one may assume that sex change during the breeding season is selected against, because an individual undergoing sex change is unable to breed (Reinboth, 1988). Thus, we expect to find that sex change in temperate teleosts is seasonal. Year-round gonadal inspections of *S. pulcher* (Warner, 1975b), *L. bergylta* (Dipper et al., 1977), *L. ossifagus* (Dipper and Pullin, 1979), and *N. celidotus* (Jones, 1980) support this prediction, with sex change being restricted to the non-breeding season. In contrast, the few transitional *C. nicholsii* detected in the field were captured in both breeding and non-breeding seasons (Cole, 1983).

This seasonal occurrence of sex change, and the indication of seasonal territoriality of most of the temperate protogynous teleosts, poses the question how individuals assess opportunities for sex change. Shapiro (1984) proposed that, in species with social mediation of sex change, seasonal sex change may result from seasonal alterations in the social environment. He hypothesized that a post-spawning increase in juvenile recruitment and adult mortality may result in such changes, and thus may mediate sex change. In temperate, gonochoristic teleosts, both seasonal recruitment (Thompson, 1981; Ayling and Cox, 1982) and a post-spawning
increase in adult mortality (Gibson and Ezzi, 1981) have been documented. No data are currently available on seasonal mortality rates in temperate, protogynous teleosts. On the other hand, in two temperate, protogynous species, *N. celidotus* and *S. pulcher*, the seasonal appearance of transitionals indeed coincides with a post-spawning increase in recruitment (Warner, 1975b; Jones, 1980; Cowen, 1985). However, whether this correlation is causal, or whether both events are the result of a common causal factor, such as seasonal changes in day length and temperature, or in the abundance or quality of food, is unknown.

In an attempt to identify the conditions that promote sex change in *C. nicholsii*, I examined the abundance, size distribution and sex ratio of this species in breeding and non-breeding seasons. First, the distribution and abundance of *C. nicholsii* in relation to available substrate were determined along transects at several locations. This was followed by extensive collections of fish every four to five weeks. I examined gonadal material and gonadosomatic indices to determine the breeding season, to detect individuals in the process of sex change, and to describe seasonal patterns in fish density, size distribution and sex ratio. Finally, the effect of two socio-ecological conditions (i.e. sex ratio and availability of shelter rocks) on sex change were examined in laboratory groups.

B. Material and Methods

Distribution and Abundance of Gobies along Transects

From June 7 to June 21, 1994, I surveyed seven transects at different locations in Barkley Sound (Figure 2.1). A measuring tape was laid out on the substrate perpendicular to the shoreline, from the shore to the beginning of the silt plain. One meter square quadrats were marked onto the substrate using PVC tubing alongside the tape. I estimated the number of gobies and rocks every square meter, changing sides every square meter. The number of gobies in three size classes (small, <5 cm; medium, 5 to 7 cm; large, >7 cm) was determined by visual counts. The reliability of visual estimates of size was checked against a large sample
(n>200) for which size was measured in the laboratory. I could not determine the sex of the gobies, since the sexes overlap in size and have almost identical colouration (Wiley, 1973). In addition, the number of rocks in each of three size classes (10-25 cm, 25-50 cm, and >50 cm along maximum diameter) was estimated for each quadrat, and the substrate type (sand, shell, or sand-shell mixture) noted.

Collection of Gobies in Breeding and Non-Breeding Season

I collected fish on rocky reefs at Dixon and Seacliff (Figure 2.1) in the breeding season (May and June 1995; June and July, 1994) and in the non-breeding season (August, September, and October, 1994) at four to five week intervals. Due to logistical problems, I was unable to collect gobies from November until April. A one meter square quadrat was marked onto the substrate using PVC tubing. The quadrat was then surrounded with a net (5 by 0.5 m), which was held to the bottom with anchor chain and floated with buoys. The weighted side of the net was embedded into the substrate to prevent fish from escaping. All gobies within the quadrat were caught using hand nets. The number of rocks was estimated for each quadrat as described earlier. On average, I surveyed six quadrats at Dixon and at Seacliff, resulting in twelve samples for each monthly collection. I took subsequent collections at different places on the same reefs, to avoid effects of depletion on population structure.

I measured, weighed, sexed, and prepared fish for histological analyses as described earlier. Fish bodies were frozen at -70°C until hormone extraction (Chapter 6). In the collections from May until August, gonads of fish ≥4.8 cm were dissected out, blotted dry, and weighed (to 0.1 g) to determine Gonadosomatic Index (GSI = (gonad weight / body weight) x 100). In the September and October collections, whole abdomens of fish ≥4.8 cm were preserved since gonads were too small for dissection. Consequently, GSI's were not calculated for these two months. For all collections, the gonads of fish ≥4.8 cm without clearly distinguishable female or male genital papilla were analyzed histologically to determine sex. The sex of fish smaller than
4.8 cm could not be determined reliably using shape of genital papilla. Since gonads of these fish were too small for dissection, whole abdomens were preserved to determine sex histologically. The breeding season was determined directly through histological examination of gonadal material and inferred from the GSI.

Size-frequency distributions were established for females and males separately, for each monthly collection. Subsequently, female and male size-frequency distributions within one month were compared by Kolmogorov-Smirnov two-sample tests (Zar, 1984).

Density was determined for each quadrat. I divided fish into four categories, based on my observations that (i) the smallest mature females were 4.8 cm (see results), and, (ii) males smaller than 7.0 cm were never observed to guard clutches in the field (Chapter 5). The four categories consisted of: (a) immature females (<4.8 cm), (b) mature females (≥4.8 cm), (c) small, non-nesting males (<7.0 cm), and, (d) nesting-sized males (≥7.0 cm).

Sex ratio was determined for each quadrat and for each monthly collection. A problem with estimating sex ratio in C. nicholsii is that the division between breeding and non-breeding males is not clear. Males as small as 4.1 cm have mature testes in the breeding season (see results), suggesting they are reproductively active. However, only males ≥7.0 cm guarded clutches in the field, and alternative mating tactics by smaller males were not observed in the field. Hence, two estimates for sex ratio were obtained, one of which uses the same minimum size for breeding males as for females:

i) the number of males ≥4.8 cm : the number of females ≥4.8 cm, and

ii) the number of males ≥7.0 cm : the number of females ≥4.8 cm.

Temporal changes in both ratios were tested by G-tests (Zar, 1984).

Two-tailed tests were used for all analyses unless stated otherwise, and non-parametric tests were used when assumptions of normality and homoscedasticity were not met (Zar, 1984); α was set at 0.05.
**Induction of Sex Change in the Laboratory**

In one experiment, I placed gobies in groups with different sex ratios in the non-breeding season. Five tanks contained no males (6 or 8 females per tank). The other groups contained males and females in the ratio 1:4 (5 fish), 1:3 (8 fish), and 1:1.5 (5 fish), with each ratio group being replicated three times (Table 3.1). In a second experiment, I placed females in groups of six in tanks containing six or sixteen rocks of same sizes, during both breeding (3 replicates at each shelter rock density) and non-breeding seasons (3 replicates at each shelter rock density). Within all experimental groups, fish were matched for size as closely as possible between tanks. All experimental groups were kept in outside tanks (150 x 150 x 50 cm or 160 x 60 x 20 cm), and were visually, chemically, and physically isolated from one another. After six weeks (sex change of mature female *C. nicholsii*)

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**Table 3.1** Design and results of laboratory experiments, in which the effect of (A) sex-ratio and (B) number of refuges and nest sites on initiating sex change were tested. Abbreviations for both tables: M = number of males, F = number of females, Sh = number of shelters, n = number of replicates, SL ± SE = mean Standard Length of females in cm ± one Standard Error of mean, SL range = minimum - maximum SL of females in cm. Sex change refers to the number of females that fully transformed into a male.

### A) SEX-RATIO

<table>
<thead>
<tr>
<th>M</th>
<th>F</th>
<th>fish/m²</th>
<th>Sh</th>
<th>n</th>
<th>SL ± SE</th>
<th>SL range</th>
<th>Dates</th>
<th>sex change</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6</td>
<td>5.0</td>
<td>8</td>
<td>2</td>
<td>5.3 ± 0.07</td>
<td>5.0 - 5.6</td>
<td>Nov. - Dec.</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>6.7</td>
<td>12</td>
<td>1</td>
<td>5.8 ± 0.23</td>
<td>4.9 - 6.5</td>
<td>Sept. - Oct.</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
<td>7.7</td>
<td>12</td>
<td>2</td>
<td>5.6 ± 0.16</td>
<td>5.0 - 6.5</td>
<td>Oct. - Dec.</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4.8</td>
<td>8</td>
<td>3</td>
<td>6.9 ± 0.07</td>
<td>6.6 - 7.1</td>
<td>Sept. - Nov.</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>7.7</td>
<td>12</td>
<td>3</td>
<td>5.9 ± 0.11</td>
<td>4.9 - 6.5</td>
<td>Oct. - Dec.</td>
<td>2 (in 1 tank)</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>4.8</td>
<td>8</td>
<td>3</td>
<td>7.0 ± 0.08</td>
<td>6.6 - 7.0</td>
<td>Sept. - Nov.</td>
<td>0</td>
</tr>
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### B) NUMBER OF SHELTER ROCKS

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<th>M</th>
<th>F</th>
<th>fish/m²</th>
<th>Sh</th>
<th>n</th>
<th>SL ± SE</th>
<th>SL range</th>
<th>Dates</th>
<th>sex change</th>
</tr>
</thead>
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<tr>
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<td>6</td>
<td>5.0</td>
<td>6</td>
<td>3</td>
<td>5.7 ± 0.11</td>
<td>5.1 - 6.5</td>
<td>June - July</td>
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<tr>
<td>0</td>
<td>6</td>
<td>5.0</td>
<td>16</td>
<td>3</td>
<td>5.8 ± 0.11</td>
<td>5.1 - 6.7</td>
<td>June - July</td>
<td>2 (1 in 2 tanks)</td>
</tr>
<tr>
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<td>6</td>
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<td>July - Sept.</td>
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<td>5.0</td>
<td>16</td>
<td>3</td>
<td>6.3 ± 0.09</td>
<td>5.5 - 6.8</td>
<td>July - Sept.</td>
<td>0</td>
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takes a minimum of four weeks in the laboratory; Cole, 1983), fish were measured, weighed, and sexed as described earlier. Gonads of all fish were dissected out and preserved for histological analysis to confirm assigned sex.

**Gonadal Histology**

I classified fish ≥4.8 cm from field collections and laboratory experiments as female and male according to the shape of the genital papilla. In addition, fish ≥4.8 cm without clearly distinguishable female or male genital papilla were classified as transitionals. Subsequently, I analyzed gonads of these fish in terms of cell types present (Table 3.2), following the descriptions of Khoo (1974), Grier (1981), Wallace and Selman (1981), and Nagahama (1983). Gonads were considered to be in breeding condition, when ovaries contained both oocytes in yolk vesicle stage and in yolk platelet stage, and testes contained both spermatids and spermatozoa. A total of 249 gonads were examined under the light microscope (217 females, 3 transitionals, 20 males, and 9 complete sex changers).

**C. Results**

**Distribution and Abundance of Gobies along Transects**

A total of 165 quadrats were surveyed, of which 125 were used for analyses. The remaining quadrats were sampled in kelp beds, but were not included due to difficulties estimating gobies in this environment. The average density of gobies in quadrats surveyed along transects was 6.5 fish/m², and ranged from 1 to 17 gobies.

I detected no systematic changes in distribution or abundance of gobies along four of the seven transects (Bamfield Inlet, Dixon, Seacliff, and O'Hiat). On two of the remaining transects, the density of large gobies decreased with increasing distance from the shoreline.
Table 3.2  Different cell types examined in gonads of female, transitional, and male C. nicholsii. Abbreviations: oog = oogonia, pvo = pre-vitellogenic oocyte, yvs = oocyte in yolk vesicle stage, yps = oocyte in yolk platelet stage, ato = atretic oocyte, l = lumen, scy = seminiferous tubules, sg = spermatogonia, sc = spermatocytes, st = spermatids, sz = spermatozoa; ++ = common, + = infrequent, +/- = rare, - = absent; (n) = sample size.

<table>
<thead>
<tr>
<th>Category</th>
<th>oog</th>
<th>pvo</th>
<th>yvs</th>
<th>yps</th>
<th>ato</th>
<th>l</th>
<th>scy</th>
<th>sg</th>
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(Pearson correlation: Ellis Island, $r=-0.64$, n=16, p=0.008; San Jose Islands, $r=-0.93$, n=12, p<0.005). At the deep end of the last transect (Grappler Inlet), a large number of gobies (7 to 17 fish/m$^2$) were found on sand substrate. This resulted in a significant, positive correlation between small and medium sized gobies, respectively, and distance from the shoreline (small: $r=0.75$, n=12, p=0.007; medium: $r=0.61$, n=12, p=0.04).

A comparison of goby distribution and abundance along the different transects revealed no striking differences. However, goby distribution was positively correlated with rock distribution: the total number of gobies in a quadrat increased significantly with the total number of rocks (Table 3.3). Of the three size classes, the number of large gobies was most strongly,
and significantly, correlated with the number of rocks in all size classes (Table 3.3). Goby density was highest on a mixture of sand-shell substrate (One-Way ANOVA: p=0.04; Student-Newman-Keuls test: p<0.05).

Table 3.3 Correlations between density of *C. nicholsii* and number of rocks, in 7 transects surveyed in Barkley Sound from June 7 to June 21, 1994. For each correlation, the Spearman rank correlation coefficient (r_s) is given; sample size is 123 for all correlations. * indicates a statistically significant correlation (p<0.05) between goby density and rock density.

<table>
<thead>
<tr>
<th>GOBY SIZE CLASS</th>
<th>10-25 cm</th>
<th>25-50 cm</th>
<th>&gt;50 cm</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (&lt;5 cm)</td>
<td>r_s=0.18 *</td>
<td>r_s=0.01</td>
<td>r_s=0.01</td>
<td>r_s=0.15</td>
</tr>
<tr>
<td>Medium (5-7 cm)</td>
<td>r_s=0.24 *</td>
<td>r_s=0.18 *</td>
<td>r_s=0.01</td>
<td>r_s=0.23 *</td>
</tr>
<tr>
<td>Large (&gt;7 cm)</td>
<td>r_s=0.56 *</td>
<td>r_s=0.44 *</td>
<td>r_s=0.32 *</td>
<td>r_s=0.61 *</td>
</tr>
<tr>
<td>Total</td>
<td>r_s=0.38 *</td>
<td>r_s=0.26 *</td>
<td>r_s=0.14</td>
<td>r_s=0.38 *</td>
</tr>
</tbody>
</table>

Collection of Gobies in Breeding and Non-Breeding Seasons

Determination of Breeding Season

Cole (1982) determined that, at Bamfield, *C. nicholsii*'s breeding season starts early March, two months before I started the collections. The detection of both ovaries and testes in breeding condition in the May, June, and July collections suggests that the breeding season lasts until early August. In addition, testes of a small number of males in the September and October collections contained both spermatids and spermatozoa. The GSI data further support the suggestion that the breeding seasons ends early August. After May, GSI of both sexes
decreased steadily to its lowest point in the first week in August (Figure 3.1). Thus, the reproductive season of *C. nicholsii* at Bamfield lasts approximately 5 months.

During the breeding season, gonad weight made up a higher proportion of body weight in females (mean=5.8%, n=103) compared to males (mean=1.6%, n=32). Gonadosomatic index was highest in May for both females (8.8%) and males (2.9%) (Figure 3.1). The mean GSI of small, non-nesting males (mean=1.2%, n=8) was smaller than that of nesting-sized males (mean=2.0%, n=18).

**Size-Frequency Distributions**

Male and female size-frequency distributions differed significantly (Kolmogorov-Smirnov two-sample test, p<0.05) in four out of six collections (June, July, August, and September), with males attaining larger sizes than females (Figure 3.2). In all collections, female and male size distributions overlapped considerably. Fourteen males were collected, which were smaller than the smallest mature females (<4.8 cm). These small males were mainly detected throughout the breeding season, and were almost absent thereafter.

**Densities and Sex Ratios**

The average density of *C. nicholsii* was 8.4 fish/m$^2$. Mean number of gobies almost doubled from 8 fish/m$^2$ in May to 14.8 fish/m$^2$ in June (U=0.13, p=0.03; Figure 3.3a). Between June and July, the density of gobies decreased again to 9.2 fish/m$^2$, and remained around that level after the end of the breeding season.

The mean density of immature females showed a similar pattern, i.e. increased five-fold from 1.0 fish/m$^2$ in May to 4.9 fish/m$^2$ in June (t=-2.61, p=0.03; Figure 3.3b). After June, the number of immature females slowly decreased to around 2.0 fish/m$^2$ in September and October.

The average density of mature females almost doubled from May (4.5 fish/m$^2$) to June (7.5 fish/m$^2$) (Figure 3.3c). From June to July, the number of mature females dropped to 3.0 fish/m$^2$ (t=3.38, p=0.004), and remained around 3.5 fish/m$^2$ after that.
Figure 3.1  Temporal changes in Gonadosomatic Indices of *C. nicholsii* (mean ± SE), for females (●) and males (□) ≥4.8 cm. Data are based upon fish collected from m² quadrats at different locations in each month. Number of fish are given in between parenthesis. The black bar indicates the breeding season.
Figure 3.2  Size-frequency distribution of female and male *C. nicholsii* from May to October. Data are based upon fish collected from m² quadrats at different locations in each month. Median sizes of females and males for each collection are shown. * indicates a statistically significant difference between female and male size.
Figure 3.2 Continued
Figure 3.3 Temporal changes in density of *C. nicholsii* (mean ± SE), for (a) all fish, for (b) immature and (c) mature females, and, for (d) small, non-nesting and (e) nesting-sized males. Data are based upon fish collected from m² quadrats at different locations in each month. Sexual status of females was determined histologically, whereas that of males was based on size. Number of quadrats sampled are given in (a), and are the same for the other graphs. Results of comparison with previous month (t-test) are shown above selected months (* = p<0.05). The black bar indicates the breeding season. Note different scales on y-axes.
In contrast, mean density of both categories of males were fairly constant. The number of small, non-nesting males was 0.8 fish/m$^2$, and ranged from 0.3 to 1.3 fish/m$^2$ (Figure 3.3d). The highest number of small, non-nesting males were found in the breeding season. The average density of nesting-sized males was 1.0 fish/m$^2$, and almost halved from 1.4 fish/m$^2$ in June to 0.8 fish/m$^2$ in September (Figure 3.3e).

All data combined revealed that densities of the four categories of gobies were weakly but positively, and mostly significantly, correlated with densities of the three size classes of rocks (Table 3.4).

On average, the male-to-female sex ratio of fish ≥4.8 cm was 1:2.7 (Figure 3.4a), and the ratio of nesting-sized males to mature females was 1:4.1 (Figure 3.4b). Both estimates became more female-biased between May and June. The drop in number of mature females between June to July resulted in less female-biased sex ratios in July. After the end of the breeding season, both sex ratios remained fairly constant.

All data combined revealed that both the sex ratio of fish ≥4.8 cm, and the ratio of nesting-sized males to mature females, became significantly less female-biased with an increase in both goby density (Spearman rank correlation: $r_s=0.37$, n=64, p=0.002; $r_s=0.52$, n=64, p<0.005) and in rock density (Table 3.4).

**Induction of Sex Change in the Laboratory**

Nine females changed sex in the laboratory. Neither sex ratio nor number of available shelter rocks affected the frequency of sex change (Table 3.1A, B). In the tanks with low and high shelter densities, the number of sex changers was higher in breeding than in non-breeding seasons (Table 3.1B). The size range of the nine transformed females was 5.8 to 6.6 cm, with a mean of 6.3 cm ± 0.1 SE. Five of these females were the largest females in the tanks, whereas the other four were the second (n=2) and third (n=2) largest females in their group with larger,
Figure 3.4 Temporal changes in sex ratios of *C. nicholsii*: (a) the number of mature females relative to the number of breeding males, and, b) the number of mature females relative to the number of nesting-sized males. Data are based upon fish collected from m² quadrats at different locations in each month. Number of quadrats sampled are given in between parenthesis. Results of comparison with previous month (G-test) are shown above selected months (* = p<0.05). The black bar indicates the breeding season.
non sex-changing females present. The smallest females never changed sex. Sex change was complete in all nine individuals: female gonads were completely replaced by male gonads.

Table 3.4 Correlations between density and sex ratio of *C. nicholsii*, and number of rocks, in m² quadrats sampled at Seacliff and Dixon in 1994 and 1995. For each correlation, the Spearman rank correlation coefficient ($r_s$) is given; sample size is 51 for fish density correlations, and 48 for sex ratio correlations. * indicates a statistically significant correlation ($p<0.05$) between goby density and rock density.

<table>
<thead>
<tr>
<th>ROCK SIZE CLASS</th>
<th>10 - 25 cm</th>
<th>25 - 50 cm</th>
<th>&gt;50 cm</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DENSITY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>immature (&lt;4.8 cm)</td>
<td>$r_s=0.38^*$</td>
<td>$r_s=0.04$</td>
<td>$r_s=-0.02$</td>
<td>$r_s=0.33^*$</td>
</tr>
<tr>
<td>mature (&gt;4.8 cm)</td>
<td>$r_s=0.22$</td>
<td>$r_s=0.37^*$</td>
<td>$r_s=0.28^*$</td>
<td>$r_s=0.30^*$</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>small, non-nesting (&lt;7.0 cm)</td>
<td>$r_s=0.17$</td>
<td>$r_s=0.27$</td>
<td>$r_s=0.14$</td>
<td>$r_s=0.24$</td>
</tr>
<tr>
<td>nesting-sized (&gt;7.0 cm)</td>
<td>$r_s=0.42^*$</td>
<td>$r_s=0.40^*$</td>
<td>$r_s=0.43^*$</td>
<td>$r_s=0.48^*$</td>
</tr>
<tr>
<td>Total</td>
<td>$r_s=0.46^*$</td>
<td>$r_s=0.33^*$</td>
<td>$r_s=0.25$</td>
<td>$r_s=0.49^*$</td>
</tr>
<tr>
<td><strong>SEX RATIO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males ≥4.8 cm : females ≥4.8 cm</td>
<td>$r_s=0.45^*$</td>
<td>$r_s=0.39^*$</td>
<td>$r_s=0.32^*$</td>
<td>$r_s=0.52^*$</td>
</tr>
<tr>
<td>Males ≥7.0 cm : females ≥4.8 cm</td>
<td>$r_s=0.34^*$</td>
<td>$r_s=0.37^*$</td>
<td>$r_s=0.46^*$</td>
<td>$r_s=0.39^*$</td>
</tr>
</tbody>
</table>
Gonadal Histology

Only ten out of 359 fish ≥4.8 cm were classified as transitionals on the basis of the shape of their genital papilla. All ten fish were collected at the end of the breeding season. Histological examination of the gonads of these fish, however, showed that none of them was undergoing sex change: all had either completely ovarian (n=7) or testicular (n=3) gonads. Of the 204 fish smaller than 4.8 cm (size range 3.0 - 4.7 cm), 14 were males, 179 females, and three fish (3.6, 3.7 and 4.5 cm) had both ovarian and testicular tissue in their gonads. These latter three fish could not be identified by external features as transitional. The sex of 10 fish out of 206 smaller than 5.0 cm could not be determined, due to loss of samples.

Histology of the Ovary

Ovaries of females in breeding condition were observed in females ≥4.8 cm during the breeding season. These ovaries formed a compact mass with a central lumen and consisted mainly of large oocytes in yolk vesicle stage and yolk platelet stage (Table 3.2; Figure 3.5a). Ovulated oocytes were seen in some ovaries, whereas atretic cells were only noticed in a few ovaries. In addition, a thick cortex surrounded the ovary. The presence of all stages of oocytes, from oogonia to ovulated oocytes, in the same ovary indicates that several batches of oocytes may ripen during one breeding season.

Ovaries of females in non-breeding condition were observed in females ≥4.8 cm during the non-breeding season. These ovaries were less compact, but still had a central lumen and were surrounded by a thick cortex (Figure 3.5b). Only very few yolk vesicle stage oocytes were present, and yolk platelet stage oocytes were completely absent. The majority of these ovaries contained atretic oocytes, from early to advanced stages. In addition, some of these ovaries contained large pink bodies, which had the size of oocytes and were filled with phagocytes. The structure of ovaries of females smaller than 4.8 cm were similar to those of mature females in non-breeding condition, except that atretic oocytes and pink bodies were rarely present in the
Figure 3.5 Transverse sections through ovaries of *C. nicholsii*; (a) breeding condition, and, (b) non-breeding condition. Abbreviations: oog=ooogonia, pvo=pre-vitellogenic oocyte, ato=atretic oocyte, ypo=yolk platellet oocyte, L=lumen. Magnifications: (a) 25X, (b) 100X.
former. No differences were detected in immature ovaries between the breeding and non-breeding seasons.

**Histology of the Testis**

Testes of males in breeding condition were observed in males as small as 4.1 cm during the breeding season, and in three sex changers in the laboratory during the breeding season. In addition, such testes were sometimes seen in males as long as two months after the end of the breeding season. The seminiferous tubules of these testes were packed with spermatids and spermatozoa, and the testicular wall was thin (Figure 3.6a).

Testes of males in non-breeding condition were observed in males during the non-breeding season, and in six sex changers in the laboratory during both breeding and non-breeding seasons. Seminiferous tubules were fully developed, and were lined with spermatogonia and spermatocytes; however, spermatids were only seen in some sections (Figure 3.6b). Most of these testes were surrounded by a thick layer of connective tissue.

**Histology of the Transitional Gonad**

Transitional gonads were detected in only three fish, all smaller than 4.8 cm. Date of capture and size of these fish was: July 1, 3.6 cm; July 4, 3.7 cm; August 2, 4.5 cm. The gonad of one transitional (3.7 cm) formed a loose mass, with a disintegrating lumen. This ovotestis consisted mainly of pre-vitellogenic oocytes, with a small number of oogonia and oocytes in the yolk vesicle stage, and was surrounded by a thick cortex. Atretic cells were abundant in most sections, from early to advanced stages, together with few to numerous pink bodies. A small number of crypts of spermatocytes lined the ovarian lumen, but seminiferous tubules were absent. The gonad of the second transitional (3.6 cm) was made up of approximately equal proportions of ovarian and testicular tissue (Figure 3.7a). The ovarian tissue was spread out throughout the gonad, and contained predominantly pre-vitellogenic oocytes. The testicular
Figure 3.6  Transverse sections through testes of *C. nicholsii*; (a) breeding condition, and, (b) non-breeding condition. Abbreviations: sc=spermatocytes, st=spermatids. Magnifications: (a) 100X, (b) 100X.
Figure 3.7 Transverse sections through transitional gonads of *C. nicholsii;* (a) 3.6 cm individual, and, (b) 4.5 cm individual. Abbreviations: oo=oocyte, pvo=pre-vitellogenic oocyte, L=lumen, sc=spermatocytes, st=spermatids. Magnifications: (a) 100X, (b) 50X.
tissue was formed by many, small and larger crypts of spermatocytes, which were lining the remnants of the ovarian lumen. In addition, seminiferous tubules and a few spermatids appeared in some sections, and not in others. In the gonad of the third transitional (4.5 cm), ovarian remnants consisted of a few early-stage oocytes (Figure 3.7b). Seminiferous tubules were fully developed, and were lined with spermatogonia and spermatocytes; spermatids were seen in some sections.

D. Discussion

Distribution, Abundance, and Population Structure

The distribution of *C. nicholsii* in Barkley Sound was non-random; goby density was positively correlated with density of shelter rocks. *Coryphopterus nicholsii* excavates refuges underneath these shelter rocks. A close association with shelters is common in teleosts (Gibson, 1969; Potts, 1984; Hixon and Beets, 1989, 1993), in particular in gobies (Grossman, 1980; Cole, 1983; Wilkins and Myers, 1993), blennies (Stephens *et al*., 1970; Buchheim and Hixon, 1992), and damselfish (Moran and Sale, 1977; Schulman, 1985). Sheltering behaviour may have various functions, such as reduction of predation, reproduction, and avoidance of adverse environmental conditions. *Coryphopterus nicholsii* retreated into their refuges in response to close-by presence of large piscivores, such as the kelp greenling, *Hexagrammos decagrammus*, and painted greenling, *Oxylebius pictus*. In addition, the defence of a shelter rock is a prerequisite for male *C. nicholsii* to attract breeding females and obtain clutches (Chapter 5), as in many goby species (Breder and Rosen, 1966; Miller, 1984; Thresher, 1984). Finally, shelters may provide protection against turbulence, in particular during winter storms.

Individual *C. nicholsii* used shelters non-randomly, as indicated by the correlations between different goby size classes and the three rock size classes (Table 3.3, 3.4). A size-differentiated shelter rock use has been observed in the gobies *Thorogobius ephippatus* and *Gobius cruentatus* by Wilkins and Myers (1993), who suggest that this is a mechanism of
reducing intraspecific competition. However, previous laboratory work on *C. nicholsii* showed that fish of all sizes and both sexes preferred large shelter rocks over small ones (De Graaf, 1995). A larger shelter rock may provide a better refuge for both sexes, and a larger spawning substrate for males. The non-random use of shelter rocks in the field thus suggests that larger fish competitively dominate smaller fish for access to large shelters. Hence, in *C. nicholsii* size-differentiated utilization of shelter rocks is more likely to result from intraspecific competition for shelter rocks, rather than from a mechanism to reduce intraspecific competition.

In addition to intraspecific competition, the number of shelter rocks available for *C. nicholsii* may be further limited by a preference for substrate, and by interspecific competition. Both at Bamfield (present study) and at Naples Reef, California (Breitburg, 1987), *C. nicholsii* showed a preference for sandy substrates, most likely because such substrates permit excavation under shelter rocks. Competition for shelter rocks between *C. nicholsii* and the plainfin midshipman fish, *Porichthys notatus*, was documented at Naples Reef (Breitburg, 1984). However, I never saw the plainfin midshipman fish at my study sites. A limited availability of large shelter rocks may have important implications for the rate and timing of sex change in *C. nicholsii*, since females may change sex as a function of competition for nest sites. I will address this further in Chapter 7.

The population structure of *C. nicholsii* showed features typically associated with protogyny. Sex ratios were female biased, with on average one male for every four females. Sex ratios of *C. nicholsii* documented in other studies were closer to unity, varying from 1:1.7 at Bamfield (Cole, 1982b) to 1:1.2 and 1:1.7 in California (Wiley, 1973; Breitburg, 1984). Whether these differences in sex ratios reflect geographic differences in population structure, differences in habitat structure, or differences in collection procedures is not clear. In addition, the size-frequency distributions were bimodal, with median size of males larger than that of females. The overlap in size distributions of females and males were similar at Bamfield (Figure 3.2; Cole, 1983) and Laguna Beach (Wiley, 1973). This indicates that the size range in which individuals change sex is similar for these populations.
The Timing of Sex Change in the Annual Breeding Cycle

Ten fish ≥4.8 cm, collected at the end of the breeding season, were classified as transitionals on the basis of the shape of their genital papilla. However, histological examination of the gonads of these fish showed that all gonads were either ovarian or testicular. Hence, the shape of the genital papilla is not a reliable external characteristic to identify transitional *C. nicholsii*. On the other hand, histological examination by Cole (1983) revealed that female and male *C. nicholsii* can be identified reliably from the shape of the genital papilla.

The detection of only three transitionals suggests that sex change in *C. nicholsii* is infrequent from early May to early October. The absence of individuals without clearly distinguishable female or male genital papilla in field collections from early April to late October in 1994 and 1995 corroborates this suggestion. Also, it extends the period during which sex change occurs infrequently another two months. In contrast, the four transitionals detected by Cole (1983) were found in both breeding and non-breeding seasons. This leaves the question whether sex change in this species occurs at a steady rate throughout the year, or whether there are periods when a significant proportion of females changes sex.

Year-round gonadal inspections of four temperate protogynous wrasses, *S. pulcher* (Warner, 1975b), *L. bergylta* (Dipper et al., 1977), *L. ossifagus* (Dipper and Pullin, 1979), and *N. celidotus* (Jones, 1980), revealed that sex change occurred most frequently immediately after the end of the breeding season. Breitburg (1984) speculated that sex change in *C. nicholsii* may be maladaptive early in the non-breeding season. The future social environment will be unpredictable due to disturbances caused by winter storms and year-to-year fluctuations in intra- and interspecific competition for nest sites (Breitburg, 1984). This will affect males in particular: whether a male will attract breeding females and obtain clutches depends largely upon the availability of nest sites (Chapter 4, 5). Hence, sex change in *C. nicholsii* may occur primarily just before the onset, or early in the reproductive season.
Shapiro (1984) hypothesized that, in teleost species with social control of sex change, seasonal sex change is related to seasonal changes in the social environment. Such changes may involve post-spawning adult mortality and juvenile recruitment (Shapiro, 1984). For *C. nicholsii*, suggestions of a relationship between the timing of sex change and seasonal changes in its population structure have to be treated with caution, until the exact timing of sex change has been determined. The decrease in densities of both immature and mature females between May and June, without a concomitant increase in the two male categories, suggests a high mortality for female gobies. However, this decrease may also be the result of emigration of females: towards the end of the breeding season, mature females moved rapidly into vacant areas to occupy newly available shelter rocks (Chapter 4). With the available data, I cannot distinguish between the effects of mortality and emigration on female density. The timing of juvenile recruitment in *C. nicholsii* could not be detected from the size distributions (Figure 3.2). At Laguna Beach, California, however, newly settled gobies were first observed just before the onset of the breeding season, in February (Wiley, 1973). Thus, seasonal changes in the population structure of *C. nicholsii* appear to occur.

**The Timing of Sex Change: Pre- or Post-Maturational?**

In some protogynous wrasses and parrotfish, two different types of males exist: those that start out as males (primary males), and those that result from sex change of females (secondary males) (Robertson and Warner, 1978; Warner and Robertson, 1978). In addition, in some protogynous species males are also derived from immature females (so called pre-maturational sex change or secondary gonochorism (Sadovy and Shapiro, 1987).

My results show that *C. nicholsii* is a protogynous species. Hence, it is reasonable to expect that at least some males are secondary males. However, males smaller than the smallest mature females were detected in this and other studies (Wiley, 1973; Cole, 1983; Breitburg, 1987). These males may be primary males, or they may be the result of pre-maturational sex change. The presence of immature individuals with both ovarian and testicular structures in their
gonads, together with the absence of males among the smallest size classes (see also Cole, 1983) suggests that these small males are derived from females. Nevertheless, the presence of males in recently hatched larvae would confirm the existence of primary males. Unfortunately, experiments established to obtain newly hatched larvae were unsuccessful. Hence, the pattern of sexual ontogeny in *C. nicholsii*, as derived from the data collected, consist of two different life-history pathways: secondary males resulting from both pre- and post-maturational sex change.

In protogynous teleosts, the presence of small, non-territorial, but sexually mature males in a population generally reflects opportunities for sneaking. Sneaking is an alternative mating tactic, during which males release sperm in the vicinity of a demersal spawning pair (Gross, 1984). The proportion of such small males is often correlated with population density (Warner and Robertson, 1978; Warner and Hoffman, 1980a, b; Van Rooij et al., 1996b) and habitat characteristics, such as number of hiding places (Robertson and Warner, 1978; Robertson et al., 1982). In *C. nicholsii*, small, non-nesting males ≥4.8 cm made up a large proportion (24%) of the male population in the breeding season. These males are most likely reproductively active, since males as small as 4.1 cm had testes in breeding condition. In addition, *C. nicholsii*'s habitat is suitable for sneaking, with numerous hiding places for smaller males, allowing close access to spawnings. The positive, significant correlation between the proportion of small, non-nesting males ≥4.8 cm and the total number of rocks (Spearman rank correlation: $r_s=0.32$, $n=48$, $p=0.03$) indeed suggests that rocks may provide hiding places for sneaking males. Finally, males smaller than 4.8 cm were only detected in the breeding season, suggesting their presence is related to sneak spawning. Whether these small males migrated or died after the end of the breeding season is unknown. In contrast, the smaller GSI's of small, non-nesting males compared to those of nesting-sized males is not typical of a sneaking tactic (however, see Cole, 1982, who detected a significant higher GSI in small males than in large males). Also, although I observed two spawning acts involving more than two fish in the field, I was unable to confirm sneaking. Thus, it is not clear whether sneaking occurs in *C. nicholsii*. 
Summary

My histological evidence of complete sex reversal in mature females in the laboratory, corroborates the findings of Cole (1983) that *C. nicholsii* is a protogynous hermaphrodite. In addition, the population structure of *C. nicholsii*, both at Bamfield (see also Cole, 1983) and in California (Wiley, 1973; Breitburg, 1984), shows the characteristics generally associated with protogyny: a female biased sex ratio, and a bimodal size-frequency distribution, with males attaining a greater length than females. Further, these data strongly suggest that the pattern of sexual ontogeny in *C. nicholsii* consists of two different life-history pathways, with all males resulting from either pre- or post-maturational sex change. Finally, the sex ratios and the size distribution of males indicate that sex change must occur quite frequently. However, the exact timing of sex change in *C. nicholsii's* annual breeding cycle remains unclear.
CHAPTER FOUR

Social Organization and Competition for Refuges and Nest Sites in Coryphopterus nicholsii

A. Introduction

The role of social organization and mating systems in the evolution of protogyny mechanisms in fish has been stressed by Ross (1990). Social and mating systems are influenced by the distribution of resources in space and time (Emlen and Oring, 1977; Davies, 1991). Thus the ability of males to control resources and monopolize spawnings affects whether protogyny is likely to occur in any given species (Ross, 1990). The social organization and mating system of several protogynous teleosts, mainly coral reef species, have been described in detail. In these species, the defence of females (Baird and Liley, 1989), or resources important to females, such as food (Robertson, 1972), spawning sites (Hoffman et al., 1985; Warner and Lejeune, 1985; Lejeune, 1987), nest sites (Cole and Shapiro, 1990; Schwarz and Lavett-Smith, 1990), or a combination of these (Hoffman et al., 1985; Cardwell 1989; Van Rooij et al., 1996c), enables males to monopolize more than one mate.

The social organization of the temperate sex-changing goby Coryphopterus nicholsii was defined as being one of year-round territoriality (Cole, 1984). However, Cole (1984) made her observations on untagged individuals, which made it impossible for her to follow individuals of known sex and size for a certain amount of time. As a result, it is unclear whether individual C. nicholsii are territorial year-round, whether the sexes differ in territorial behaviour, and whether territoriality is size-specific. The collection of such detailed information on C. nicholsii's social organization is of interest, for it will clarify the social conditions promoting protogynous sex change in this species.

A number of lines of evidence suggest that rocks suitable as refuges and nest sites are a limited resource for C. nicholsii. First, densities of gobies are strongly and positively correlated
with shelter rock densities (Chapter 3). Second, only males $\geq 7.0$ cm are nesting in the field (Chapter 5). Up to 25% of sexually mature males, in particular males smaller than 7.0 cm, do not obtain clutches. Nevertheless, in the laboratory these smaller males are capable of spawning and guarding a clutch (Chapter 5). Finally, territories often contain more than a single shelter rock (Cole, 1984). This means that intraspecific competition may prevent other fish from using the additional shelter rocks available in a territory, since only one shelter rock can be used by the occupant at a time.

In this chapter, I first describe the breeding and non-breeding social organization, including use of space and intra-specific agonistic behaviour, of tagged *C. Nicholsii* at Bamfield. I present evidence that both sexes defend territories encompassing one or more shelter rocks, which are both used as a refuge and a nest site. An additional objective was to determine whether the density of shelter rocks affects social organization. As mentioned earlier, densities of gobies increase with shelter rock densities (Chapter 3). In a number of protogynous teleosts, variation in density influences the rate and timing of sex change (Warner, 1975a; Warner and Robertson, 1978; Warner and Hoffman, 1980a; Jones, 1980; Lejeune, 1987), presumably by affecting the ability of males to control resources (Warner and Hoffman, 1980b).

My second objective in this chapter was to examine the influence of intraspecific competition on territory establishment by smaller individuals. I did this by testing the following hypotheses: i) that shelter rock availability limits territory establishment by small individuals, and, ii) that aggression by large fish limits territory establishment by smaller individuals. In the first experiment, I removed territorial, non-nesting males from their shelter rocks. In the second experiment, I established artificial reefs in a previously uninhabited area. The prediction for both these experiments was that small, non-territorial fish in the surrounding populations would quickly occupy the available shelter rocks. In the third experiment, I removed males guarding clutches from their nest sites. I also established artificial nest sites in a populated area. The prediction for these experiments was that if males in breeding condition, but smaller than 7.0 cm, are unable to
establish territories due to aggression by already established males, they should occupy the newly available nest sites.

Finally, I examine the role of sex in determining the ability of an individual to gain access to a shelter rock. Males were expected to be more successful in competition for shelter rocks than females, in particular during the breeding season, because male reproductive output is tied to defending a nest site (Chapter 5).

Overall, my aims were to (1) describe in detail the social organization in the field, (2) determine whether suitable shelter rocks are a limited resource for *C. nicholsii*, (3) determine which individuals in the population are prevented from occupying a shelter rock, and (4) examine the effect of fish size and sex on competition for a limited, critical resource. The results were expected to reveal the resource(s) controlled by males, and to clarify the role of intraspecific competition in governing population density and social organization of *C. nicholsii*.

B. Material and Methods

Field Studies

Establishment of Study Population

I collected and tagged fish in August and October 1994 (non-breeding season) and in April and May 1995 (breeding season) at the southern part of 'Gobytown' (Figure 2.1). A detailed description of the collection procedure is given in Chapter 3. After collection, fish were transported to the boat (non-breeding season), or to the Bamfield Marine Station (breeding season). Fish were measured, weighed (only during the breeding season), sexed and tagged as described earlier. The nets were left around the quadrats to prevent other gobies from immigrating into the area. The fish were returned and released into their quadrats within four hours, and the nets were removed after the fish had settled down. Within each season, I tagged fish in a total of sixteen separate quadrats, 0.5 to 2 m apart.
During the non-breeding season, I studied populations in two different, adjacent habitats. The first habitat Rocky, was in the centre of the rocky reef, and had a high density of suitable shelters. Depth of this habitat varied from 6-10 m. The second habitat Sandy, was on the edge of the reef and the silt plain, at a depth of 10-13 m. This habitat had a low density of suitable shelters, and the shelters present were smaller on average than those in the Rocky habitat. Both habitats had a sand-shell substrate. Within each habitat, I tagged gobies in eight quadrats.

During the non-breeding season of 1994, I tagged a total of 138 fish, of which 76 were eventually used for observations: 47 in the Rocky habitat and 29 in the Sandy habitat. None of these tagged fish were present during the breeding season of 1995. This was most likely due to loss of tags; after tagging, fish with scarred dorsal musculature were regularly detected in the study area. Therefore, in May 1995, I tagged 143 new fish, of which 75 were eventually used for observations. Approximately two months after tagging, all tagged fish were recaptured, sexed, measured, and returned, in both the non-breeding season (recapture date: Oct. 29, 1994; Rocky habitat only) and the breeding season (recapture date: July 5, 1995). Fish recaptured during the breeding season were also weighed. Growth data of these fish are analyzed and discussed in Chapter 5.

Observation Procedures

I observed use of space and agonistic behaviour of tagged individuals during the non-breeding season (Sept. 21 - Oct. 24, 1994) and the breeding season (May 22 - June 12, 1995). Each fish was observed three times for 40 minutes on different days, at different times of the day. Due to low levels of activity, more than one fish per quadrat could be observed at a time. The observations consisted of recording movements and intraspecific, agonistic interactions of individual fish onto detailed maps of the quadrats.

The three observations for each fish were combined to determine size of home range and territory, and frequency of agonistic interactions. Home range was defined as the total area
a fish moved around in, and territory was defined as the exclusive area defended against conspecifics by the occupant (Brown and Orians, 1970). Agonistic behaviour was defined as an interaction between two conspecifics, during which one fish rapidly approached the other, generally followed by avoidance behaviour of the approached fish. The location and outcome of these interactions (whether a focal animal displaced: 'interaction won', or was displaced: 'interaction lost') were scored for each focal animal. Infrequently, the approached conspecific did not move away, in particular when the two interacting fish were of equal size. This usually resulted in both fish performing display behaviour. This behaviour consisted of extending all fins and moving the body in lateral undulations, often accompanied by mouth gaping (Cole, 1984). Eventually both individuals would return to their respective home ranges or territories, without displacement having taken place. In these cases, fish were said to be equally matched ('equal interaction').

**Data Analyses**

Density was estimated for each square meter. Fish were divided into four categories, based on the observations that: (i) the sex of fish smaller than 4.8 cm could not be reliably determined using genital papilla shape (Chapter 3); (ii) the smallest mature females were 4.8 cm (Chapter 3), and, (iii) males smaller than 7.0 cm were never observed to guard clutches in the field (Chapter 5). The female category consisted of mature females (≥4.8 cm); the two male categories breeding males (≥4.8 cm) and nesting-sized males (≥7.0 cm); fish smaller than 4.8 cm were classed as "small fish". Each category of density was compared between habitats and between seasons by t-tests (Zar, 1984).

Two estimates of sex ratio were obtained (see Chapter 3 for discussion of sex ratios in *C. nicholsii*): i) ratio of breeding males to mature females, and, ii) ratio of nesting-sized males to mature females. Each estimate of sex ratio was compared between habitats and between seasons by G-tests (Zar, 1984).
Female *C. nicholsii* are generally smaller than males (present study, Chapter 3; Wiley, 1973; Cole, 1983). Therefore, analyses of home range size, territory size, and agonistic behaviour were initially performed for each sex separately in order to reduce confounding effects of size on social behaviour. Males guarding clutches spent most of their time in their nest sites, and were therefore not included in the main analyses.

Home range size and territory size were determined using the outer convex polygon method (Southwood, 1966). Only data from fish observed for longer than 100 minutes were used in this analysis. Home range size, territory size, and fish size were log transformed (Zar, 1984). Preliminary analyses revealed that the effect of fish size on home range was negligible. Therefore, size was removed as a covariate from the analysis, and home range sizes were compared between habitats and sexes, and between seasons and sexes, by Two-Way Anova. On the other hand, fish size did affect territory size, and was therefore included in the analyses as a covariate. The effect of habitat on territory size during the non-breeding season was determined by analysis of covariance (ANCOVA). A habitat effect proved to be negligible, and data from the two habitats were combined. Subsequently, the effect of season on territory size was tested by ANCOVA. Finally, female and male territory size were compared using individuals with overlapping sizes, for each season separately, by ANCOVA.

The proportion of interactions won was calculated for all fish observed. The analyses of proportion of interactions won were the same as the analyses of territory size, except data were not transformed unless stated otherwise. In the analyses of variance models, all interaction terms were included initially, but were removed when not significant.

**Removal of Territorial, Non-Nesting Males**

On June 20, 1995, I mapped use of space and agonistic behaviour of tagged fish in two quadrats. Subsequently, two (7.0 and 7.3 cm) and three (6.1, 7.5 and 7.8 cm) territorial males were removed from each quadrat, and kept overnight at the Bamfield Marine Station. Neither male was guarding a clutch at the time of removal. The next day I monitored movements and
agonistic interactions of both tagged gobies left and newly present gobies in the two quadrats for 45 min, and released the removed males into their old territories. The following day I repeated the behavioural observations on all gobies in the quadrats for 45 min.

**Artificial Reefs**

In July 1995, at the end of the breeding season, I established three artificial reefs at the southern part of Gobytown (Figure 2.1). Artificial reefs were 1 by 0.5 m, and were made up of concrete tiles (25 x 5 x 25 cm and 12.5 x 5 x 12.5 cm). The tiles were placed on a sloping silt plain at a depth of 13 m, four to five meters away from the nearest rocky reef. One and five days after the artificial reefs were established, all gobies on the reefs were caught, measured, weighed, and sexed as described earlier, and released 2 km away. Four months later, all gobies on the artificial reefs were captured and handled as described above. I compared the population structure on the artificial reefs in July with that on natural reefs at the same time of the year (Chapter 3), and with that on the artificial reefs in November. Two estimates for sex ratio were obtained: i) ratio of breeding males to mature females, and ii) ratio of nesting-sized males to mature females. Sizes of females and males were compared by t-tests, and sex ratios by G-tests (Zar, 1984).

**Removal of Males Guarding Clutches**

On June 13 and 14, 1995, I removed twenty-two nesting males from their nest sites at the centre of Gobytown (Figure 2.1), in an area approximately 20 x 50 m between 9 and 12 m depth. Nest sites were checked weekly for a period of four weeks (June 20 - July 16) for occupancy and new clutches.

**Artificial Nest Sites**

On June 15, 1995, I established forty-one artificial nest sites at the northern part of Gobytown (Figure 2.1) in an area approximately 20 x 10 m, at the edge of a rocky reef at a depth
of 6 to 9 m. Twenty-one large (25 x 5 x 25 cm) and 20 small (12.5 x 5 x 12.5 cm) concrete tiles were added to the area, separated from each other by at least 0.5 m. Censuses to check for occupancy and clutches were carried out 5, 6, 15, and 17 days after the nest sites were established. Male size and clutch size were estimated as described in Chapter 5.

Laboratory Study

Effect of Sex on Shelter Rock Competition

Fish were captured in the field using SCUBA and hand nets, and subsequently measured, weighed, sexed, and tagged as described earlier. Some fish were kept individually in plastic containers (15 - 20 cm in diameter) for up to three days. At the end of a trial, tags were removed and fish were released into the field.

I established pairs of females and males of the same size (± 0.1 cm) and weight (± 0.1 g) in inside tanks (160 x 60 x 20 cm) with only one shelter rock (20 x 20 cm, with a slight concave underside). A PVC tube (10 x 2.5 x 5 cm) was also provided as a hiding spot for the subordinate fish. The rock and PVC tube were in the middle of the tank along its long axis, with 40 cm between one end of the tank and the first structure, 80 cm between the two structures, and 40 cm between the second structure and the other end of the tank. To prevent any position bias, I switched the rock and PVC tube between trials.

One day after the fish were established, I recorded the positions of both fish 20 times, once every 15 minutes. A fish was assigned to the shelter rock if it was underneath it. At the end of the first series of observations, I removed the fish occupying the rock most often. On the second day, I observed the position of the remaining fish ten more times, at 15 minutes interval. This experiment was replicated 20 times with new fish during both breeding (1995) and non-breeding seasons (1994).

For each tank, I calculated the difference in number of times the rock was occupied by the female and male. To test if females and males were occupying the shelter rock at similar frequencies, the 20 numbers within each season were tested in a one-sample t-test (Zar, 1984).
The number of times the subordinate fish was observed underneath the shelter rock was compared between day 2 and day 1, using a paired t-test (Zar, 1984).

C. Results

Field Studies

Densities and Sex Ratios

The average density of blackeye gobies in all four categories was two to three times higher in the Rocky habitat than in the Sandy habitat (Table 4.1). This difference in density was significant for both females (t-test: t=3.47, p<0.001) and small fish (t=2.32, p=0.04). In addition, both the ratio of breeding males to mature females (Rocky, 1:3.1, n=73; Sandy, 1:4.0, n=35), and the ratio of nesting-sized males to mature females (Rocky, 1:3.9, n=69; Sandy, 1:5.6, n=33) were slightly more female-biased in the Sandy habitat than in the Rocky habitat, but not significantly so (G=0.29, 0.50<p<0.75 and G=1.88, 0.10<p<0.25) (Table 4.1).

The mean densities of total number of gobies were similar in breeding and non-breeding seasons (Table 4.1), and did not differ significantly (t=1.61, p=0.12). However, both density of breeding males and density of nesting-sized males were approximately 1.5 times higher in the breeding season than in the non-breeding season, whereas density of mature females decreased approximately a third. Consequently, both the ratio of breeding males to mature females (breeding season: 1:1.3, n=97; non-breeding season: 1:3.3, n=108) and the ratio of nesting-sized males to mature females (breeding season: 1:1.9, n=82; non-breeding season: 1:4.4, n=102) were significantly less female-biased in the breeding season than in the non-breeding season (G=10.42, 0.005<p<0.001; G=5.74, 0.01<p<0.025) (Table 4.1).
Table 4.1  Density and sex ratio of *C. nicholsii* per square meter (mean ± SE), in Rocky and Sandy habitat during non-breeding season, and in non-breeding (Rocky and Sandy habitat combined) and breeding seasons. Data are based upon fish collected in 16 square meter quadrats during each season (8 within each habitat during the non-breeding season). * indicates a statistically significant difference (p<0.05) between Rocky and Sandy habitats, or non-breeding and breeding seasons; n.s. = non-significant.

<table>
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<th>NON-BREEDING SEASON</th>
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<td>ROCKY</td>
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<td>DENSITY</td>
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<td>Small fish/m²</td>
<td>2.4 ± 0.7</td>
<td>0.8 ± 0.3</td>
<td>1.6 ± 0.4</td>
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<td>Females/m²</td>
<td>6.9 ± 0.8</td>
<td>3.5 ± 0.5</td>
<td>5.3 ± 0.7</td>
<td>3.5 ± 0.5</td>
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<tr>
<td>Males ≥4.8 cm/m²</td>
<td>2.3 ± 0.5</td>
<td>0.9 ± 0.4</td>
<td>1.5 ± 0.4</td>
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<td>n.s.</td>
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<td>Males ≥7.0 cm/m²</td>
<td>1.8 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.8 ± 0.2</td>
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<td>n.s.</td>
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<tr>
<td>Total/m²</td>
<td>11.9 ± 1.0</td>
<td>5.3 ± 0.5</td>
<td>8.7 ± 1.1</td>
<td>6.7 ± 0.7</td>
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<td>SEX RATIO</td>
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<td>Males ≥4.8 cm :</td>
<td>1 : 3.1</td>
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<td>females ≥4.8 cm</td>
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<td>Males ≥7.0 cm :</td>
<td>1 : 3.9</td>
<td>1 : 5.6</td>
<td>1 : 4.4</td>
<td>1 : 1.9</td>
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<tr>
<td>females ≥4.8 cm</td>
<td>n.s.</td>
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Home Range Size

The average home range size was 0.32 m² (n=104), and ranged from 0.01 m² to a maximum of 1.18 m². Feeding occurred throughout the home range, both in the centre and on
the periphery. In contrast, intraspecific, agonistic interactions took place primarily, but not exclusively, on the periphery of the home range.

Fish size had a negligible effect on home range size ($R^2=0.02$, $F_{1,114}=2.09$, $p=0.15$), and was therefore not included as a covariate in these analyses. In the non-breeding season, home ranges of both sexes were approximately 30% larger in the Sandy habitat than in the Rocky Habitat. However, no significant difference was detected (Two-way ANOVA: $F_{1,49}=0.97$, $p=0.33$; Figure 4.1). Within each habitat, home range sizes of females and males were very similar: sex had no effect on home range size ($F_{1,49}=0.03$, $p=0.87$; Figure 4.1).

Home ranges of both sexes were approximately 35%, and significantly, smaller in the breeding season than in the non-breeding season ($F_{1,101}=4.76$, $p=0.03$; Student-Newman-Keuls test: $p<0.05$; Figure 4.2). Within each season, female and male home range sizes were very similar: sex had no effect on home range size ($F_{1,101}=0.73$, $p=0.39$; Figure 4.2).

**Territory Size**

The average territory size was 0.14 m$^2$ ($n=56$), and ranged from 0.01 m$^2$ to a maximum of 0.50 m$^2$. Female and male *C. nicholsii* defended territories in both breeding and non-breeding seasons. A territory always included one or more shelter rocks, used as a refuge by both sexes year-round, and also as a nest site by males in the breeding season. Both sexes were similarly likely to be territorial: the proportion of territorial fish did not differ among similarly-sized females and males, in either breeding ($G=2.90$, 0.05<$p$<0.10) or non-breeding seasons ($G=3.54$, 0.05<$p$<0.10). However, fish were more likely to be territorial when large: the proportion of territorial fish per 0.5 cm size class increased strongly, and significantly, with size (Pearson correlation: $r=0.95$, $n=9$, $p<0.001$).

Large gobies defended larger territories than small fish ($R^2=0.20$, $F_{1,55}=13.92$, $p<0.001$). Fish size was therefore included in the analyses as a covariate. In the non-breeding season, territory size did not differ between Rocky and Sandy habitat, for either females (ANCOVA: $F_{1,11}=0.61$, $p=0.45$; Figure 4.3a) or males ($F_{1,12}=0.18$, $p=0.67$; Figure 4.3b). In addition, territory size
Figure 4.1  Female and male home range size of *C. nicholsii* (mean ± SE) in the Rocky and the Sandy habitat during the non-breeding season. Different letters indicate a statistically significant difference (p<0.05) in home range size for natural logarithm transformed data (see text); (n) = sample size.
Figure 4.2  Female and male home range size of *C. nicholsii* (mean ± SE) during the breeding and the non-breeding season. Different letters indicate a statistically significant difference (p<0.05) in home range size for natural logarithm transformed data (see text); (n) = sample size.
Figure 4.3  Relationship between territory size and fish size in *C. nicholsii* during the non-breeding season in the two habitats, for (a) females, and, (b) males. Open symbols and broken lines are for Rocky habitat, closed symbols and solid lines for Sandy habitat. The regression lines are given for natural logarithm of territory size on natural logarithm of size within each habitat.
did not differ between the two seasons, for either females (F,131=0.04, p=0.84; Figure 4.4a) or males (F,135=1.49, p=0.23; Figure 4.4b). Finally, sex did not affect territory size: female and male territory size did not differ in either the breeding season (F,133=0.004, p=0.95) or the non-breeding season (F,135=2.71, p=0.12).

During a night dive in the non-breeding season, all observed gobies were either hidden underneath a rock, or lying on the sand against a rock. All tagged fish located during this night dive were within the boundaries of their daytime home range (n=17), or in their neighbour’s home range (n=2). Fish defending a territory during the day were found deep in their excavated refuge, whereas smaller, non-territorial fish were lying close against a rock or in a crevice.

**Agonistic Behaviour**

Gobies interacted with conspecifics throughout the day, with on average 5 agonistic interactions per hour (n=142), ranging from 0 to a maximum of 25. In general, gobies would approach and chase conspecifics from a spot central to the home range or territory. A chase generally resulted in avoidance by the intruder.

Large gobies won a higher proportion of intraspecific, agonistic interactions than small fish ($R^2=0.57, F_{1,124}=168.14, p<0.001$): large males almost always won interactions, whereas small fish lost most. Fish size was therefore included in the analyses as a covariate. In the non-breeding season, the proportion of interactions won by individual fish did not differ between Rocky and Sandy habitats, for either females (ANCOVA: $F_{1,36}=0.82, p=0.37$; Figure 4.5a) or males (arcsin-transformed data; $F_{1,17}=2.87, p=0.11$; Figure 4.5b). In addition, the proportion of interactions won by individual females did not differ between the two seasons ($F_{1,72}=0.71, p=0.40$; Figure 4.6a, b). Due to extreme non-normality and non-homogeneity of variance of the data (resulting from the high number of large males that won most, or all of their interactions), even for transformed data, the effects of season and male size on the proportion of interactions won could not be determined using ANCOVA's. The effect of season was therefore tested separately. The proportion of interactions won by individual males in the breeding season (median=100%, n=23)
Figure 4.4  Relationship between territory size and fish size in *C. nicholsii*, (a) during the breeding, and, (b) the non-breeding season. Symbols for each graph are: females (●), transitional (◆), and males (□). The regression lines are given for natural logarithm of territory size on natural logarithm of size for females (solid line) and males (broken line).
Figure 4.5  Relationship between proportion of interactions won and fish size in *C. nicholsii*, during the non-breeding season in the two habitats, for (a) females and (b) males. Open symbols and broken lines are for Rocky habitat, closed symbols and solid lines for Sandy habitat. The regression lines are given within each habitat for females.
Figure 4.6  Relationship between proportion of interactions won and fish size in *C. nicholsii*, (a) during the breeding, and (b) the non-breeding season. Symbols for each graph are: small fish (✧), females (●), transitional (●), and males (□). The regression lines are given for females (solid line) and males (broken line).
and the non-breeding season (median=99%, n=20) were almost the same, and did not differ significantly (U=422, p=0.64; Figure 4.6a, b). Finally, sex did not affect the proportion of interactions won: the proportions did not differ for individual females and males with overlapping sizes, in either breeding (F_{1,37}=1.11, p=0.30; Figure 4.6a) or non-breeding seasons (F_{1,32}=0.13, p=0.72; Figure 4.6b).

**Recapture Data**

In the non-breeding season thirty-four (36%) of the fish tagged in Rocky habitat were recaptured. Of those, only one female (7.2 cm) had changed into male. At the end of the breeding season fifty-nine (41%) of the fish tagged were recaptured. One fish had changed from female to male (7.0 cm).

**Removal of Territorial, Non-Nesting Males**

One day after removal of five territorial males, three territories were occupied by large (>7.0 cm), untagged fish. These fish were most likely males, since only 20% of such large fish are females (Chapter 3). Two of these fish used the shelter rock of the previous owner, whereas the third fish used another one approximately 30 cm. away. The two remaining territories were not taken over by new fish.

One day after returning the previous owners, three of the five removed males regained their original shelter rocks. Two of these removed males (size: 7.0, 7.3 cm) displaced the new fish completely or partially from their territory. The shelter rock of the third removed male (7.8 cm) had not been taken over by a new fish. A fourth returning male (7.5 cm) was not able to displace the new resident from his old shelter rock. The former defended a new territory next to his old one. Whether this was done by evicting another fish could not be determined. The new male guarded a clutch 13 days after removal of the original male. The fifth removed male (6.1 cm) disappeared. The smaller fish did not change their use of space noticeably after removal and return of the original males.
Artificial Reefs

In July, seventeen fish were caught on the artificial reefs, 10 at day one and 7 at day five (Table 4.2). Fourteen were female, two male, and the sex of a small fish was unknown. The average size of females was 6.7 cm ± 0.3 SE (range 5.7 - 8.2 cm); the two males measured 6.2 and 6.9 cm, and the small fish 4.4 cm. The ratio of breeding males to mature females was more female biased on the artificial reefs (1:7.0, n=16) than in the natural population (1:2.0, n=45). However, probably due to the low sample size on the artificial reefs, no significant difference was detected (G-test: G=2.93, 0.05<p<0.10). In contrast to the natural population, no nesting-sized males were found on the artificial reefs. Females on the reefs were significantly larger than those in the natural population (t-test: t=3.52, p=0.001), but size of males did not differ (t=-0.43, p=0.68).

Four months after the artificial reefs were established, a total of 13 fish were caught (Table 4.2). Seven were females and 6 were males. The average size of females was 6.1 cm ± 0.3 SE (range 5.2 - 7.4 cm), and of males 7.7 cm ± 0.1 SE (range 7.3 - 8.0 cm). The relatively large number of males in November resulted in a less female-biased ratio of breeding males to mature females (1:1.2, n=13) than in July (1:7.0, n=16) (G=4.17, 0.025<p<0.050). Also, in contrast to the population on the artificial reefs in July, all males on the reefs in November were ≥7.0 cm. As a result, males on the artificial reefs were significantly larger in November than in July (t=-4.84, p=0.003). In contrast, size of females did not differ (Mann-Whitney test: U=56, p=0.13).

Removal of Males Guarding Clutches

Of the 22 nest sites from which I removed the nesting males, only two nest sites (9.1%) were taken over by nesting males, one after six days (8.3 cm) and one after 18 days (7.9 cm).
Table 4.2  Size (mean ± SE) of female and male ≥4.8 cm, and sex ratios on the artificial reefs in July and November (1995), and in the natural population in July (1994 and 1995). * indicates a statistically significant difference (p<0.05) between the natural and artificial reef populations in July, or the artificial reef populations in July and November; n.s. = non-significant; (n.t.) = not tested. Range and sample size are given in between parenthesis.

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<td></td>
<td>July</td>
<td>July</td>
<td>November</td>
</tr>
<tr>
<td><strong>SIZE</strong></td>
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<tr>
<td>Females</td>
<td>6.0 ± 0.1 (4.8-6.9; 30)</td>
<td>6.7 ± 0.2 (5.7-8.2; 14)</td>
<td>6.1 ± 0.3 (5.2-7.4: 7)</td>
</tr>
<tr>
<td></td>
<td>* n.s.</td>
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<tr>
<td>Males</td>
<td>7.0 ± 0.3 (4.8-8.9; 15)</td>
<td>6.6 ± 0.4 (6.2 - 6.9; 2)</td>
<td>7.7 ± 0.1 (7.3-8.0; 6)</td>
</tr>
<tr>
<td></td>
<td>* n.s.</td>
<td></td>
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<tr>
<td><strong>SEX RATIO</strong></td>
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<tr>
<td>Males ≥4.8 cm :</td>
<td>1:2.0 <strong>1:7.0</strong>________</td>
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<tr>
<td>females ≥4.8 cm</td>
<td>n.s.</td>
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</tr>
<tr>
<td>Males ≥7.0 cm :</td>
<td><strong>1:3.8 <strong>0:14.0</strong></strong>______</td>
<td>1:1.2</td>
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<td>females ≥4.8 cm</td>
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**Artificial Nest Sites**

Seventy-eight percent of all the tiles (17 large and 15 small tiles) was used as nest sites within 17 days. All males nesting under the tiles were larger than 7.0 cm, the minimum size of males nesting on natural nest site. The mean size of males nesting on artificial nest sites and of males nesting on natural nest sites did not differ significantly (Table 4.3). However, size of males was larger on the large tiles than on the small ones (t-test: t=2.86, p=0.008; Figure 4.7).

The available spawning substrate affected clutch size: clutches on the tiles was larger than those on natural nest sites (Mann-Whitney test: U=329, p=0.003; Table 4.3), and clutches were also larger on the large tiles than on the small ones (t=2.67, p=0.012; Figure 4.7).
Figure 4.7  Size of nesting male *C. nicholsii* and size of clutch guarded on small and large tiles (mean ± SE). * indicates a statistically significant difference (p<0.05) between small and large tiles; (n) = sample size.
However, in contrast to natural nest sites, clutch size and male size were not significantly correlated on the tiles (Pearson correlation: large tiles, $r=-0.26$, $n=15$, $p=0.36$; small tiles, $r=-0.31$, $n=14$, $p=0.28$).

Table 4.3 Male size (cm) and clutch size (number of eggs) (mean ± SE) on natural and artificial nest sites. * indicates a statistically significant difference ($p<0.05$) between natural and artificial nest sites; n.s. = non-significant. Range and sample size are given in between parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Natural nest sites</th>
<th>Artificial nest sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male size</td>
<td>7.9 ± 0.0 (7.0 - 8.9; 122)</td>
<td>7.7 ± 0.1 (7.1 - 8.3; 30)</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Clutch size</td>
<td>8,730 ± 322 (1,623 - 27,202; 154)</td>
<td>14,410 ± 957 (3,716 - 29,185; 32)</td>
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Laboratory Study

Effect of Sex on Shelter Rock Competition

Males occupied the shelter rock significantly more than females, in both breeding (Mann-Whitney test: $U=300$, $p<0.001$) and non-breeding season ($U=300$, $p<0.001$; Figure 4.8). Both females and males occupied the rock slightly more often in the breeding season. One day after removal of the dominant fish (mostly the male), the subordinate fish occupied the rock significantly more, in both breeding (Wilcoxon signed ranks test: $Z=3.32$, $p=0.001$) and non-breeding season ($Z=2.35$, $p=0.02$).

D. Discussion

Territorial Defence of Shelter Rocks

*Coryphopterus nicholsii* has a stable social organization, based on the year-round defence of a territory by females and males. Territories always include one or more shelter
Figure 4.8  Shelter rock occupancy (mean ± SE) for female and male C. nicholsii, in breeding and non-breeding seasons. * indicates a statistically significant difference (p<0.05) between female and male shelter occupancy.
rocks. These were used as refuges by both sexes year-round, and also as nest sites by males during the breeding season. A principal role for shelter rocks for *C. nicholsii*’s survival is further inferred from the positive correlation between goby density and shelter rock density (Table 4.1; Chapter 3). A close association with shelter rocks has been documented in a number of other temperate teleosts, such as the rockfish *Sebastes chrysomelas* and *S. carnatus* (Larson, 1980b), and the gobies *Gobius cruentatus* and *Thorogobius ephippiatus* (Wilkins and Myers, 1993). The defence of a shelter rock is most likely important in reproduction, in the reduction of predation, and the avoidance of adverse environmental conditions.

The defence of a nest site is a prerequisite for males to obtain a clutch, as seen in most other goby species (Breder and Rosen, 1966; Miller, 1984; Thresher, 1984). At Bamfield, the failure of smaller, non-territorial males to obtain a territory with a shelter rock, means that these males are unable to attract mates. Since competition for shelter rocks is strongly size-specific (Figure 4.7), large males monopolize spawnings, and receive a disproportionate share of the matings at the expense of smaller males (Chapter 5). Thus, *C. nicholsii*’s mating system is one of resource-defence polygyny. The presence of these social conditions is consistent with the hypothesis that the ability of males to control resources and monopolize spawnings affects whether protogyny is likely to occur in any given species (Warner, 1984b; Ross, 1990).

While reproduction is an advantage of territoriality during the breeding season, predator avoidance is most likely an important benefit of the year-round defence of a shelter rock. Large piscivores, such as the kelp greenling, *Hexagrammos decagrammus*, and painted greenling, *Oxyle tus pictus* are present all year at Bamfield (see also Gascon and Miller, 1981) and were observed attacking *C. nicholsii*. Gobies retreat into their refuges when these predators are nearby. In addition, fish defending a territory during the day were found deep in their excavated refuge at night. Predator exclosure experiments at Santa Catalina Island, California, indicated that predation on *C. nicholsii* by the kelp bass *Paralabrax clathratus* and barred sand bass *P. nebulifer* reduced survivorship by as much as 75% (Steele, 1996). In the spinyhead blenny, *Acanthemblemaria spinosa*, survival is dependent on the possession of a shelter hole (Buchheim
and Hixon, 1992). If predation rates are indeed lower for territorial than for non-territorial *C. nicholsii*, the year-round defence of a refuge is clearly advantageous.

In addition to the anti-predator value of a shelter from which a fish is out of the reach of its predators, territoriality allows an individual to become familiar with its surroundings. Laboratory experiments suggest that, for *C. nicholsii*, familiarity with the landscape increases an individual’s chances of escaping predators (Markell, 1993).

Finally, shelter rocks can also provide protection against turbulent water conditions. Laboratory work on yearling small mouth bass (*Micropterus dolomieui*) showed that fish seek shelter when exposed to strong currents (Haines and Butler, 1969). The temperate gobies *G. cruentatus* and *T. ephippiatus* use larger shelter rocks at sites with strong currents than at sites with lower current speeds (Wilkins and Myers, 1993).

The territorial defence of shelter rocks implies that the distribution and availability of suitable shelter rocks affects *C. nicholsii*'s social organization. It was therefore surprising that in the two habitats with different shelter rock densities, no apparent differences were found either in territory size, or in the outcome of agonistic interactions, despite significant differences in goby densities. Differences were expected for the following reasons. In many teleosts, territory size decreases (e.g. De Boer, 1978; Lejeune, 1987; Barnett and Pankhurst, 1996) and agonistic behaviour increases (e.g. De Boer, 1981; Pankhurst and Barnett, 1993; Barnett and Pankhurst, 1996) with increased density. When densities are extremely high, intruder pressure may make territory defence uneconomical, e.g. the wrasse *Thalassoma bifasciatum* (Warner and Hoffman, 1980b).

There are several potential explanations for the lack of differences in territorial behaviour in *C. nicholsii* under different population densities. First, gobies are only found in locations with shelter rocks and their populations densities are directly proportional to the availability of shelters. Consequently, increased population densities may indicate increased shelter availability rather than increased competition for those shelters available. Second, the
densities of *C. nicholsii* described here may not represent a situation sufficiently extreme to result in modification of social behaviour. Finally, my observations may not have focused on the appropriate territorial behaviours, or on the appropriate times. For example, territorial behaviour of the male damselfish *Chromis dispilus* at low and high population densities differed only during the display and spawning period, not during the nesting phase (Barnett and Pankhurst, 1996).

In contrast, both female and male home ranges in the Sandy habitat were approximately 30% larger than those in the Rocky habitat. I speculate that these differences in home range size are probably a result of differences in food availability. Mean home range size increased approximately 35% from the breeding to the non-breeding season. A seasonal change in food utilization by *C. nicholsii*, from more planktonic items during summer to more benthic organisms in winter (Wiley, 1973; Cole, 1982b) coincided with this seasonal change in home range size (Figure 4.3a, b). These concurrent, seasonal changes in diet and average home range size most likely reflect changes in food availability.

**Intraspecific Competition for Shelter Rocks**

The results of this study are consistent with the hypothesis that intraspecific competition determines which individuals gain access to shelter rocks. The rapid colonization of the artificial reefs and the artificial nest sites indicates that suitable shelter rocks are a limited resource. This is further supported by the immediate take-over of most of the empty territories after removal of the original, non-nesting males. In addition, territory holders defend shelter rocks in excess of their own requirements, as was shown by the use of a previously unused shelter rock by the new occupant.

Competition for shelter rocks is strongly size-specific: in the present study smaller males were forced to occupy less favourable (i.e. smaller) nest sites in the field. In addition, artificial reefs were quickly occupied by females and small males. Thus, it was surprising that small, non-territorial fish did not respond to the removal of their territorial, non-nesting neighbours. They either did not compete for the newly available shelter rocks, or were prevented
from occupying them by the new territory holders. The first explanation is unlikely, since smaller fish occupied shelter rocks when these were available both in the field (Table 4.2) and in the laboratory (De Graaf, 1995). More likely, the strong size-component in dominance hierarchy and in competition for shelter rocks documented in this study, suggests that the rapid occupation of these territories by large fish prevented smaller fish from taking over the available shelter rocks. Further, the occupation of these territories by large individuals implies that, even for these fish, high-quality territories are limited, since most fish of this size class already defended territories.

Under natural conditions, small C. nicholsii may never be able to defend territories. Cole (1984) hypothesized that the home ranges of small gobies may be the centres of future territories, as was also suggested for smaller individuals of the spinyhead blenny Ophioblennius atlanticus (Nursall, 1977). Small, non-territorial C. nicholsii could also be floaters, capable of establishing a territory in the absence of territory holders. Floaters have been documented in both the black and yellow rockfish Sebastes chrysomelas and the gopher rockfish S. carnatus (Larson, 1980b). The population structure on my artificial reefs in July revealed that females and small males move rapidly into vacant areas, and thus could be considered floaters.

The prediction that males smaller than 7.0 cm would occupy the newly available nest sites was not supported by the results of this study: all males occupying artificial sites and empty territories were larger than 7.0 cm. In addition to a possible shortage of high-quality territories, large males may prefer artificial nest sites over natural ones, because of the larger interior egg laying surface. Artificial spawning substrate artificially elevated spawning success in male Cortez damselfish, Stegastes rectifraenum (Hoelzer, 1990), and male bicolor damselfish, S. partitus (Cole and Sadovy, 1995). In C. nicholsii, female choice for available spawning surface has been documented in the laboratory (De Graaf, 1995), and is supported by the larger clutch sizes on the large tiles than on the small ones, independent of male size. Thus, if small males in breeding condition were present in the population, larger males probably prevented them from establishing a nest site and obtaining clutches on the newly available nest sites. Nevertheless, the larger size of males on the large tiles indicates a strong, size-specific competition for shelter rocks.
In addition to a strong size-component, sex also affects the outcome of competition for shelter rocks. In the laboratory, males were much more successful than same-sized females at occupying a shelter rock in both breeding and non-breeding seasons. The occupation of a territory during the winter may enhance the ability of males to obtain a territory during the breeding season. A similar effect has been detected in the great tit, *Parus major*, where male defence of territories in the winter increases the survival rate of their young in the summer (Krebs, 1971; Davies and Houston, 1984). Unfortunately, I could not collect data on duration of territorial behaviour and male reproductive output, since none of the tagged gobies observed in the non-breeding season of 1994 were recovered in the spring of the next year.

Sex-specific competition for shelter rocks was not confirmed by the results of the field observations. The proportion of territorial individuals did not differ for similarly-sized females and males in either season. Also, no qualitative differences in agonistic behaviour were documented between similarly-sized females and males. However, other differences in female and male territorial behaviour may exist. For example, the shelters defended by males may be more suitable as nest sites than those defended by females, e.g. with better water circulation or a larger available spawning surface. Males may also be more aggressive just before or during the start of the breeding season, when competition for nest sites is expected to be fiercest.

Overall, the rapid take-overs of the artificially vacated territories of non-guarding males, and the quick occupation of the artificial nest sites and reefs strongly suggest that suitable shelter rocks are a limited resource. Yet, this is in stark contrast with the small number of nest sites that were taken over after removal of the nesting males, and the lack of large males on the artificial reefs in July. How can these differences in re-occupation rates be explained?

Even though nest sites may be limited in the field, used nest sites may not have been attractive to males in breeding condition, and were left abandoned. Previously occupied nest sites contain the remains of dead and decaying clutches and may provide conditions unsuitable for a new clutch. This is supported by the movement of males to new nest sites after their clutch
had hatched, documented both in the field (Chapter 5) and in the laboratory (see also Cole, 1982a). On the other hand, nest sites with clean spawning surfaces, such as the vacated shelter rocks of the non-nesting males and the tiles, were readily occupied.

Another explanation for the differences in re-occupation rates is that neighbours may have incorporated the vacated nest sites into their territories. Expansion of neighbours' territories in removal experiments has been documented in a variety of species, including the great tit, *P. major*, (Krebs, 1971), the two rockfish species *S. chrysomelas* and *S. carnatus* (Larson, 1980a), and the sand tilefish *Malacanthus plumieri* (Baird and Liley, 1989). In the present study such expansion may have gone unnoticed, because use of space of neighbouring fish was not monitored and only the original nest sites were checked for occupancy. In contrast, the artificial nest sites were placed in an area with low goby density, and were thus less likely to be taken over by neighbouring gobies. Consequently, the tiles provided many new places for males in breeding condition to settle.

Finally, the population structure on the artificial reefs in July suggests that there is little immediate migration over longer distances by large, nesting-sized males. Over longer times, however, large males do migrate considerable distances, as revealed by the high number of large males on the artificial reefs in November. This could also imply that large, nesting-sized males become more migratory after the breeding season has ended. However, this is unlikely, as recapture proportions for large, nesting-sized males did not differ between the breeding (59%) and the non-breeding season (47%) (G test: G=0.20, 0.50<p<0.75).

**Summary**

In summary, *C. nicholsii* has a stable social organization, based on the year-round defence of a territory by both females and males. Larger individuals of both sexes were more likely to defend territories than smaller ones. Territories always included a shelter rock which was used as a refuge by both females and males year-round, and also as a nest site by males in the breeding season. Suitable shelter rocks are limited, as was shown by the immediate take-
over of most of the empty territories after removal of the original, non-nesting males, and by the rapid colonization of the artificial reefs and nest sites. Intraspecific competition, which is strongly size-specific, determines which individuals gain access to shelter rocks. The increase with fish size in both the proportion of fish that are territorial, and in the proportion of interactions won, suggest that both territoriality and social hierarchy in *C. nicholsii* are based on larger fish exercising dominance over smaller fish.
A. Introduction

The most universally accepted model explaining the evolution of protogynous hermaphroditism is Ghiselin's (1969) 'size-advantage' model. Ghiselin proposed that protogyny evolves when larger males can monopolize the spawnings of females. Under these circumstances, females of all sizes have a higher reproductive output than small males, but a lower reproductive output than large males. Consequently, an individual that functions as a female when small, and changes sex when large enough to compete successfully for dominant or territorial male status, obtains a greater lifetime reproductive success than an individual that does not change sex (Ghiselin, 1969, Warner, 1975a; Warner et al., 1975).

The predictions of the size-advantage model have been supported by field observations. Protogyny has been verified in many teleosts with polygynous mating systems, mainly on coral reefs (Thresher, 1984; Warner, 1984b), but also on temperate reefs (Dipper et al., 1977; Jones, 1980; Potts, 1984). In these mating systems, larger males monopolize matings, by defending spawning sites, a harem of females, and/or nest sites. An example of the differentially high reproductive output of large males, as compared with large females is the protogynous wrasses Thalassoma bifasciatum: large males can obtain 50 pair spawnings a day, compared to only one for large females (Warner et al., 1975a; Hoffman et al., 1985). However, the size-specific reproductive output of both sexes has yet to be estimated for a protogynous species (Warner, 1988). This is an important omission, since the size-advantage model predicts that the size-specific difference in female and male reproductive output determines whether protogyny evolves or not, and the size at which sex change occurs.
According to the size-advantage model, females change sex after becoming large enough to attain dominant or territorial male status (Ghiselin, 1969, Warner, 1975a; Warner et al., 1975). However, in several protogynous coral reef fish, some females change sex at sizes much smaller than the smallest territorial male. This results in the production of bachelor males, and has been documented in a number of protogynous teleosts, such as the wrasse *Labroides dimidiatus* (Robertson, 1974), and the parrotfish *Sparisoma aurofrenatum* (Clavijo, 1982) and *S. viride* (Cardwell, 1989; Van Rooij et al., 1996c).

Two hypotheses have been proposed to explain the evolution of this 'early' sex change: i) the 'growth-rate-advantage', and, ii) the 'mortality-advantage' model (Charnov, 1982; Iwasa, 1991). These hypotheses predict that early sex change is adaptive if sexually active females suffer reduced growth rates or higher mortality compared with similarly-sized bachelor males. Reproductively active females may incur a growth cost, and/or may be more conspicuous to predators (due to differences in morphology or behaviour) than similarly-sized bachelor males. Although early sex changers may not reproduce following sex change, they may acquire territorial male status sooner by growing faster, or survive better than females which delay sex change until they have attained the size of territorial male.

An alternative, though not mutually exclusive explanation for the presence of early sex changers is that bachelor males obtain a higher fertilization success than similarly-sized females, by using an alternative mating tactic. In protogynous teleosts, both 'streaking' behaviour (during which males release sperm near a pelagic spawning pair) and group-spawning by bachelor males have been documented (Robertson and Choat, 1974; Warner and Robertson, 1978; Warner and Hoffman, 1980a). On the other hand, sneaking (during which males release sperm near a demersal spawning pair) is often inferred in protogynous teleosts (Chapter 3; Cole, 1983; Warner, 1984b), but has never been documented in the field.

In this chapter, I test the predictions of both the size-advantage and the growth-rate-advantage model in the blackeye goby, *Coryphopterus nicholsii*. First, I determine the relationships between (a) size of clutch laid and female size, and (b) size of clutch guarded and
male size. I present evidence that a male’s egg mass is usually made up of clutches deposited by several females, and will from hereon refer to a clutch guarded by a male as a composite clutch. I then estimate the numbers of clutches females can lay, and the number of composite clutches males can obtain over a single breeding season. These data are then used to estimate the potential reproductive output of both sexes. Large males are expected to fertilize more eggs than laid by large females, since they can monopolize spawnings by defending limiting nest sites (Chapter 4). Variability in the quality of nest sites may further concentrate reproductive output among larger males. Therefore, I also examine the relationships between male size, size of composite clutch, and nest site perimeter.

Finally, I compare size-specific growth rates of similarly-sized females and males. Males much smaller than the smallest nesting male have been documented in C. nicholsii (Chapter 3; Wiley, 1973; Cole, 1983; Breitburg, 1987). This suggests that early sex change occurs in this species.

B. Material and Methods

Potential Reproductive Output of Females

I established pairs of females and males of known size in laboratory tanks (150 x 150 x 50 cm or 160 x 60 x 20 cm), with numerous shelter rocks in April, May and June, 1995. Females with extended, orange-coloured abdomens were collected in the field, and checked for running eggs by applying gentle pressure to the abdomen. Females with running eggs, indicating they were close to ovulation, and thus in spawning condition, were established with a male the same day. All males were ≥7.0 cm, the size of nesting males in the field (see results). After spawning, which generally occurred the same or following day, I measured the size of the clutch layed by the female by tracing clutch dimensions on abraded transparencies, and determined clutch area using a digitizer. I estimated the average number of eggs per square cm from five clutches using
a transparency with square mm division and a light microscope. The relationship between clutch size and female size was tested in a linear regression.

The number of clutches a female could lay per season was estimated by immediately removing females after spawning in the laboratory, and establishing them with a new, nesting-sized male. I checked pairs weekly for clutches for approximately two months, and noted time from first to subsequent spawning.

**Potential Reproductive Output of Males**

I examined the relationship between the size of the composite clutch guarded and male size during the breeding seasons of 1994 and 1995. I caught nesting males by dipnets and measured their size under water using a calliper. Measurements of fish size taken underwater (6.7 cm ± 0.4 SE) were as precise as those taken at the surface (6.6 cm ± 0.4 SE) (paired t-test: t=1.78, n=10, p=0.12). The size of the composite clutch guarded was estimated as described above, and nest site dimensions using a measuring tape. Males were released close to their nest after divers finished their measurements. All males returned to their nests immediately and resumed guarding their composite clutches. I measured size of composite clutches and size of nesting males at four different sites (Dixon, Helby, O'Hiat, and Seacliff) in Barkley Sound in 1994 (Figure 2.1). Male size, size of composite clutch, and nest site dimensions were all similar across sites (One-Way ANOVA's: p>0.34). In 1995, I only took measurements at Seacliff. All data were pooled to test the relationship between size of the composite clutch and male size in a linear regression. Correlations were calculated between nest site dimensions, male size, and size of the composite clutch guarded.

The number of composite clutches males can obtain over a single breeding season was estimated using three procedures. First, I checked the nest sites of 29 tagged males used for behavioural observations in the field at 'Gobytown' (Chapter 4) for composite clutches weekly over a six week period (May 22 - July 3, 1995). Second, I checked 17 nest sites with untagged males at 'Gobytown' weekly for both occupancy and composite clutches for an 8 week period.
(May 29 - July 16, 1995). In both procedures, new composite clutches were mapped on transparencies. Third, after a successful spawning in the laboratory, I placed male and nest site in isolation in aquaria (30 x 30 x 60 cm). Male and composite clutch were checked daily to determine duration of egg guarding, and time from spawning to hatching.

**Female and Male Growth Rates**

During the breeding season of 1995, I tagged and released 143 fish at 'Gobytown' (Chapter 4). After approximately two months I recaptured as many as possible of the tagged fish. The recaptured fish were measured, weighed and sexed, as described earlier. The specific growth rate, $g$, which gives the instantaneous rate of growth per unit weight (Wootton, 1990a), was calculated for each fish as the change in the natural logarithm (ln) of weight ($W_i$) from the day of tagging ($t_1$) to the day of recapture ($t_2$):

$$g = \frac{\ln(W_{t_2}) - \ln(W_{t_1})}{t_2 - t_1}.$$  

The specific growth rate was expressed as % of initial body weight gained per unit time.

Preliminary analyses showed that initial size affected growth rate, and was therefore included in the analyses as a covariate. Female and male growth rates were compared in an ANCOVA, using individuals of overlapping size. Only females and males smaller than 7.0 cm were compared, because males of this size did not tend composite clutches in the field (see results).

**C. Results**

**Potential Reproductive Output of Females**

The mean clutch size laid by a female, estimated in the laboratory was 4,517 eggs ± 456 SE (range: 1,868 - 11,327 eggs, n=20). The size of the clutch spawned by a female was
highly dependent on female size: 75% of the variance in clutch size was explained by body size (Figure 5.1a). Both stripping and histological examination showed that females had no ripe oocytes left after spawning.

Only one female spawned twice in the laboratory, with a six week interval between spawnings. This observation suggests that in a five month breeding season females may spawn up to three to four times.

**Potential Reproductive Output of Males**

On average, a nesting male cared for a composite clutch of 8,731 eggs ± 322 SE (range: 1,623 - 27,202 eggs, n=154) at a time. Thus, on average two females contributed to the average composite clutch guarded by a male in the field (range: 1 - 6 females). However, this may be a conservative estimate, as composite clutches under large boulders (rocks >50 cm diameter) were inaccessible. Males smaller than 7.0 cm were never found guarding a composite clutch (Figure 5.1b): nesting males were significantly larger (median=7.5 cm, n=16) than non-nesting males (median=6.5 cm, n=27) (Mann-Whitney test: U=472, p<0.001). Among nesting males, only 12% of the variance in the size of the composite clutch was explained by male size (Figure 5.1b). In addition, size of the composite clutch increased significantly with nest site dimension (Figure 5.2a), although nest site dimension was not correlated with male size (Figure 5.2b). However, in both cases the correlation was very weak.

I followed 29 tagged males for a period of six weeks. Twenty-one of these obtained one to three composite clutches over this period, and all these males were ≥7.0 cm. Of the 26 males ≥7.0 cm, 5 (19%) were not seen nesting. The three remaining males not seen with a composite clutch were all smaller than 7.0 cm. Of the five males which obtained more than one composite clutch, two switched nest sites between composite clutches. The shortest time between hatching and obtaining a new composite clutch was seven days.
Figure 5.1 Relationship between (a) size of clutch laid and female size, and, (b) size of clutch guarded and male size, in *C. nicholsii*. Clutch sizes for females were measured in the laboratory; for males in the field. Coefficient of determination, sample size and level of significance are given for each graph.
Figure 5.2  Relationship between (a) size of clutch guarded and perimeter of nest site, and, (b) perimeter of nest site and size of nesting male, in *C. nicholsii*, measured in the field. Spearman Rank correlation coefficient, sample size and level of significance are given for each graph.
In addition, I observed 17 nest sites with untagged males for a period of eight weeks. Most nest sites were abandoned after the first composite clutch hatched; only two males obtained a second composite clutch in their original site. In five cases new males, distinguished by size differences, took over a nest site, and obtained a composite clutch.

Finally, I followed the development of six composite clutches guarded by males (one in the field, five in the laboratory) from the day of fertilization until the day of hatching. In the laboratory this period was 18 and 19 days in 1994, and 23, 24, and 25 days in 1995, and was 21 days in the field. During this period males guarded their composite clutches continuously. These observations suggests that males may guard a maximum of six composite clutches over a five month breeding season.

Female and Male Growth Rates

During the breeding season, average growth rate of males smaller than 7.0 cm (mean=0.19% day⁻¹, n=5) was more than twice that of similarly-sized females (mean=0.08% day⁻¹, n=26) (Figure 5.3). However, no significant difference in growth rates of females and males was detected (ANCOVA: F₁,28=1.77, p=0.19).

D. Discussion

Size-Specific Difference in Female and Male Reproductive Output

My results show that reproductive output of both female and male *C. nicholsii* increased significantly with body size. Female clutch size was strongly and positively related to body size. In addition, females shed all their ripe eggs in one spawning, but may spawn several times in a season. Sequential spawning by female *C. nicholsii* is further supported by the presence of all stages of oocytes, from oogonia to ovulated oocytes, in the same ovary (Chapter 3), indicating that several batches of oocytes may ripen during one breeding season. Thus, female *C. nicholsii* have the potential to be sequentially polyandrous. In contrast, a male's egg mass is usually
Figure 5.3  Relationship between specific growth rate and fish size, in female and male *C. nicholsii* during the breeding season. Symbols are: females (●) and males (□). The regression lines are given for females (solid line) and males (broken line).
made up of clutches deposited by several females. Based on the mean female clutch size recorded in the laboratory, two females contributed to the average composite clutch defended by a male in the field. Further, larger males appear to monopolize matings: males smaller than 7.0 cm did not guard composite clutches, although they made up 33% of the male population during the breeding season (Chapter 3). Hence, larger males receive more matings than smaller males. However, whether size-specific fertility increases more rapidly for males than for females in *C. nicholsii*, as predicted by the size-advantage model ((Ghiselin, 1969; Warner, 1975a; Warner et al., 1975), is not clear.

A more robust test of the size-advantage model is to estimate whether potential reproductive output of similarly-sized females and males differ in the direction and magnitude predicted by the model (Warner, 1988). In my study, clutch size of the one 7.5 cm female did not differ from that of nine same-sized, nesting males (one-sample t-test: \( t = -0.004, p > 0.50 \); Figure 5.1a, b). Further, approximately 70% of the nesting males had smaller composite clutches than this single female (Figure 5.1a, b). Thus, reproductive output of this single large female is similar to, or even higher than in large males, and not lower as predicted by the size-advantage model. However, this result has to be treated with caution, since it is based on a clutch of only one female. A lack of both small males and large females made a more powerful comparison of size-specific reproductive output in both sexes impossible, a problem also faced in other studies on protogynous teleosts (Warner, 1988). To circumvent this problem, Warner (1988) suggested creating the missing sex-size classes through experimental manipulation, and introducing them into the natural population. An alternative approach would be to follow Warner's suggestion and raise numbers of large females in the field, but estimate their reproductive output in the laboratory as described in this study. In *C. nicholsii*, males smaller than 7.0 cm are abundant in the field, but do not obtain clutches under natural conditions. On the other hand, females larger than 7.0 cm are very rare in the field, and the difficulties of monitoring them continually makes it nearly impossible to accurately assess their reproductive output.
A second prediction of the size-advantage model is that the size-specific difference in female and male reproductive output determines the size at which sex change occurs (Ghiselin, 1969, Warner, 1975a; Warner et al., 1975). For an individual to maximize its lifetime reproductive output in the C. nicholsii population studied, it is most advantageous to change sex when large enough to compete for the nesting male status, i.e. 7.0 cm. Two of the sex changers detected in the field were 7.0 and 7.2 cm (Chapter 4), supporting this prediction of the size-advantage model. In contrast, the large number of males smaller than 7.0 cm does not support the idea that females change sex at this threshold. I will discuss this issue below.

Monopolization of Spawnings by Large Males

High reproductive output of some males in polygynous mating systems accrues from defence of resources, defence of a harem of females, or from the possession of attributes attractive to females (Emlen and Oring, 1977). In C. nicholsii, large males do not defend a harem, but defend a limited resource, namely suitable nest sites (Chapter 4). The defence of a nest site is needed for males to attract females and obtain clutches. Yet, despite the fact that males as small as 5.0 cm defended territories with a number of rocks providing suitable spawning surface (Chapter 4), males smaller than 7.0 cm were never observed nesting in the field. Thus, the possession of a nest site is not the only factor determining the reproductive output of male C. nicholsii.

This study showed that the size of a male C. nicholsii determines whether a male obtains a clutch: males ≥7.0 cm monopolized spawnings. However, among nesting males ≥7.0 cm, the size of a male’s egg mass was extremely variable. Further, 19% of the 26 territorial males ≥7.0 cm followed for six weeks were not seen guarding a clutch. Hence, other factors besides the possession of a nest site and male size must determine the spawning success of large, territorial males.
The quality of a territory or nest site may determine whether a male obtains a clutch. Territory size of *C. nicholsii* increases with male size (Chapter 4). Thus, the territories of males smaller than 7.0 cm may be too small to attract females and obtain clutches, as was observed for the three-spined stickleback *Gasterosteus aculeatus* (Van den Assem, 1967). I did not find that nest site perimeter was correlated with male size, however, other characteristics of the nest site may be important. For example, the available spawning substrate under a male's nest site largely determines male clutch size in the blenny *Ophioblennius atlanticus* (Côte and Hunte, 1989), and in the gobies *Padogobius martensi* (Marconato et al., 1989) and *Pomatoschistus minutus* (Lindström, 1992). In *C. nicholsii*, female preference for nest sites with more available spawning area, irrespective of the occupant's size, was documented both in the field (Chapter 4) and in the laboratory (De Graaf, 1995). Further, large males were much more successful at obtaining a nest site with a large spawning surface than small males (Chapter 4). This suggests that large males may prevent small, territorial males from obtaining a nest site with a large suitable spawning area, and acquiring clutches in the field.

The higher reproductive output of large males in *C. nicholsii* may also be a function of dominance relationships among territorial males: courtship behaviour of small, territorial males may be suppressed by dominant, larger males. In several teleosts, females prefer to spawn with the male that courts most vigorously (Schmale, 1981; Cole, 1982a; Keenleyside *et al.*, 1985; Torricelli *et al.*, 1988; Knapp and Kovach, 1991). In the laboratory, subordinate male *C. nicholsii* court only when the previously dominant male begins egg guarding (Cole, 1982a). In this species, the relative dominance status of a male is highly correlated with size (Chapter 4). Furthermore, in the field, small territorial males were chased more often within their home ranges than large males (Chapter 4). This high intrusion rate may restrict the courtship behaviour of these smaller males, and consequently limit the number of females they attract.
The Adaptive Significance of Early Sex Change.

The size-advantage model predicts that females change sex after becoming large enough to attain dominant or territorial male status (Ghiselin, 1969; Warner, 1975a; Warner et al., 1975). In *C. nicholsii*, the minimum size of nesting males was 7.0 cm at Bamfield (present study) and 6.9 cm SL at Naples Reef, California (Breitburg, 1987). Yet, at both locations, approximately one third of the male population is smaller than this (Chapter 3; Cole, 1983, Breitburg, 1987). Since all males are most likely derived from females (Chapter 3), early sex change takes place in *C. nicholsii*.

Early sex change may be adaptive if sexually active females suffer reduced growth rates or lower survivorship compared to similarly-sized males (Charnov, 1982; Iwasa, 1991). My study showed that, in *C. nicholsii*, mean growth rate of small, non-nesting males was twice that of similarly-sized, mature females. Higher growth rates for non-territorial, terminal phase males were also documented in the tropical, protogynous wrasse *T. bifasciatum* (Warner, 1984a) and parrotfish *S. viride* (van Rooij et al., 1995). Thus, early sex changers in these species may obtain territorial or nesting-sized male status more rapidly than females that change sex later in life. The rapid take-over of vacant territories by bachelor males in *S. viride*, led Cardwell (1989) to suggest that these males indeed have a higher probability of successfully acquiring territories than females that must first change sex.

On the other hand, although I did not estimate survivorship, mortality rates are probably similar for similarly-sized female and male *C. nicholsii*. Breitburg (1987) speculated that both sexes are similarly susceptible to predation, because females and males overlap in size and have similar colouration. In my study, the recapture proportions for females (38%) and males (49%) did not differ significantly (G-test: G=0.61, 0.25<p<0.50), suggesting that female and male mortality is indeed similar. However, recapture proportions also include loss due to emigration and tag loss, and thus are not a powerful indication of mortality. In *T. bifasciatum*, survivorship did not differ between non-reproductive males and sexually active females of similar sizes (Warner, 1984a).
Finally, early sex change may be adaptive, if bachelor males obtain a higher fertilization success than similarly-sized females, by using an alternative mating tactic. In *C. nicholsii*, males smaller than 7.0 cm often had running milt, and were capable of spawning, and raising a clutch in the laboratory. This strongly suggests that these smaller males are reproductively active. However, I could not confirm sneaking in the field. Sneaking has been suggested to occur in gobies of the genus *Pomatoschistus*, where small males lack nuptial colouration, but possess very large testes and genital papilla during the breeding season (Miller, 1984). However, Miller (1984) presented no empirical evidence of sneaking. Thus, it is not clear if sneaking occurs in gobies. Nevertheless, the opportunity for sneaking in *C. nicholsii*’s environment seems considerable, since the habitat provides numerous hiding places for smaller males (Chapter 3) and close access to spawnings.

**Summary**

In summary, my study shows that size-specific reproductive output can increase more rapidly for males than for females, as indicated by the large size of male clutches relative to females’, and the rapid increase in male clutch size when attaining nesting male status. Large males most likely monopolize matings through the defence of large territories, encompassing nest sites with large spawning surfaces. However, reproductive output of a single large female was similar to, or even higher than in similarly sized nesting males, and not lower as predicted by the size-advantage model. Hence, a more powerful comparison of size-specific reproductive output in both sexes is desirable.

These results suggests that, for a female, it is most advantageous to change sex when large enough to compete for the nesting male status, i.e. 7.0 cm. Yet, the large number of males smaller than 7.0 cm indicates that many females change sex at smaller sizes. In *C. nicholsii*, early sex change is advantageous: small, non-nesting males have a higher mean growth rate than mature similarly-sized females. As a result, small males may obtain the nesting male status more rapidly than females that change sex later in life. Finally, the presence of early sex
changers in *C. nicholsii* is also most likely related to sneak spawning, although this has yet to be confirmed in the field.
CHAPTER SIX

The Role of Steroid Hormones in Protogynous Sex Change in Coryphopterus nicholsii

A. Introduction

Seasonal sex change has been documented in four temperate, protogynous teleosts, the wrasses Semicossyphus pulcher (= Pimelometopon pulchrum; Warner, 1975b), Labrus bergylta (Dipper et al., 1977), L. ossifagus (Dipper and Pullin, 1979), and Notolabrus (= Pseudolabrus) celidotus (Jones, 1980). Year-round gonadal inspections of four species revealed that sex change took place in the non-breeding season. A seasonal pattern of sex change suggests that seasonal cycles in endocrine profiles mediate sex change in these species.

In gonochoristic teleosts, the endocrine system mediates the response of the reproductive system to changing photoperiod or temperature (Fostier et al., 1983; Peter, 1983; Liley and Stacey, 1983; Kime, 1993; Borg, 1994). In males, plasma levels of the gonadal androgens testosterone (T) and 11-ketotestosterone (11-KT) are highest just before and during the breeding season. In male teleosts, 11-KT is the major androgen associated with spermiation (Fostier et al., 1983), and both 11-KT and T may regulate reproductive morphology and behaviour (Liley and Stacey, 1983). In females, plasma 17β-estradiol (17β-E$_2$), T, and 11-KT levels peak during the pre-spawning period. 17β-Estradiol is involved in vitellogenesis, oocyte maturation ((Fostier et al., 1983; Ng and Idler, 1983; Mommsen and Walsh, 1988; Nagahama et al., 1994), and may regulate female reproductive behaviour (Liley and Stacey, 1983). The role of 11-KT and T in females is not clear. Testosterone is a precursor in the formation of other steroids, such as 11-KT and 17β-E$_2$ (Kime, 1987).

Seasonal changes in female and male plasma steroid levels have also been documented in two protogynous teleosts, the black sea bass Centropristis striatus (Cochran and Grier, 1991) and the ricefield eel Monopterus albus (Yeung and Chan, 1987). Both species
change sex in the non-breeding season (Chan and Phillips, 1967; Cochran and Grier, 1991). In both species, the seasonal occurrence of sex change may be related to seasonal changes in endocrine profiles. However, whether this correlation is causal, or whether both events are the result of a common causal factor, such as seasonal changes in day length and temperature, is unknown. Hence, it is of interest to examine the role of hormones in protogynous sex change in teleosts.

In this study, I examine whole-body concentrations of T, 11-KT, and 17β-E₂ in the blackeye goby, Coryphopterus nicholsii. My objective was to detect and describe correlations between whole-body steroid concentrations and sexual function (female, transitional, and male). Despite extensive collections, only three immature transitional fish were captured (Chapter 3). Thus, natural levels of steroid concentrations were only measured in mature females and males, in both breeding and non-breeding seasons.

Hypotheses on the causation of sex change, arising from correlations between sex and steroid concentrations, were tested in hormone administration studies. I examine the effects of androgens on gonadal structures of mature females, through administration of 17α-methyltestosterone (17α-MT), 11-KT, 11-KT’s precursor 11-ketoadrenosterone (11-KA), and an aromatase inhibitor (Fadrazole; which blocks the conversion of T into estrogens).

Finally, I examine the effect of Gonadotropin Releasing Hormone (GnRH) administration on gonadal structures of mature females. In teleost, GnRH participates in the control of reproduction, and its' activity is related to the photoperiod (Peter, 1983). However, whether GnRH is involved in mediating sex change in teleosts is not clear.
B. Material and Methods

Whole-body Steroid Concentrations

General

The mean size ± SE, and sample sizes of extracted *C. nicholsii* (mature females and males, and immature females, transitionals, and males) are given in Table 6.1. Fish were collected and preserved as described earlier (Chapter 3). Sufficient blood could not be drawn from individual fish for steroid Radio-Immuno Assays (RIA's). Therefore, I measured steroids on whole-body extracts of mature females and males (i.e. fish ≥4.8 cm; Chapter 3), collected during both breeding (May-June collection) and non-breeding seasons (October collection). In addition, I compared whole-body steroid concentrations of three transitionals <4.8 cm captured in July and August (Chapter 3) with those of immature, similarly-sized females and males, obtained in collections at the same time of the year.

Steroid Extraction Procedure

I cut frozen samples into pieces (approximately 0.5 by 0.5 cm) and placed them in a glass Erlenmeyer in 5.0 ml 0.1 N NaOH at 4°C overnight, to soften bones and tissue (pers. comm. Dr. C. Schreck and M. Stratholt). The samples were then frozen with liquid N₂ and homogenized by hand with a porcelain mortar and pestle. The homogenate was transferred to a 200 ml glass Erlenmeyer, and the pestle was rinsed with 5.0 ml phosphate buffer-saline-gelatine (PBSG, at pH 7.0). The homogenate was vortexed with 10 volumes of di-ethyl ether for 1 minute, after which the organic and aqueous layers were allowed to separate. Subsequently, the aqueous phase was snap frozen in acetone and dry ice, and the ether solution including the steroids was transferred to a 200 ml glass Erlenmeyer and evaporated to dryness. This extraction was repeated twice for each homogenate. To remove final traces of fish tissue, I did a third extraction on the combined extract as described above, but this time with 7 ml ether and 1 ml PBSG in a 15 ml glass test tube. The final extract was reconstituted with 2 ml PBSG and
Table 6.1  Mean size ± SE, range, and sample sizes for extracted *C. nicholsii*: (A) mature (≥ 4.8 cm) females and males; (B) immature (<4.8 cm) females, transitionals, and males.

A) MATURE FISH

<table>
<thead>
<tr>
<th></th>
<th>Breeding Season</th>
<th>Non-Breeding Season</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SL ± SE</td>
<td>SL Range n</td>
</tr>
<tr>
<td>Females</td>
<td>6.1 ± 0.1</td>
<td>5.9 - 6.6 10</td>
</tr>
<tr>
<td>Males</td>
<td>6.3 ± 0.1</td>
<td>6.1 - 6.7 6</td>
</tr>
</tbody>
</table>

B) IMMATURE FISH

<table>
<thead>
<tr>
<th></th>
<th>SL ± SE</th>
<th>SL Range n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>4.0 ± 0.2 (4)</td>
<td>3.6 - 4.5 4</td>
</tr>
<tr>
<td>Transitionals</td>
<td>3.9 ± 0.3 (3)</td>
<td>3.6 - 4.5 3</td>
</tr>
<tr>
<td>Males</td>
<td>4.5 ± 0.0 (3)</td>
<td>4.4 - 4.5 3</td>
</tr>
</tbody>
</table>

stored at -20°C, for RIA's of testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (17β-E$_2$). I validated the extraction efficiency for each steroid by adding a known amount of labelled T, 11-KT, or 17β-E$_2$, to the frozen samples before extraction.

Radio-Immuno Assays

Testosterone, 11-KT and 17β-E$_2$ concentrations were measured on individual samples by specific RIA's, using the procedures described and validated by Van der Kraak *et al.* (1984) and Dye *et al.* (1986). $^3$H-T and $^3$H-17β-E$_2$ were obtained from Amersham Life Science; $^3$H-11-KT from Amersham International. Antibodies for T and 17β-E$_2$ were obtained from Chemical
Credential. The T antibody cross-reacts with T, 5α-testosterone, 11-oxotestosterone and 17β-E₂ at 100, 17, 4.5 and <0.01% levels. The 17β-E₂ antibody cross-reacts with 17β-E₂, 17α-estradiol, estrone, estriol and T at 100, 1.6, 1.4, 0.8 and <0.01% levels. The antibody for 11-KT was a gift from Dr. T.G. Owen (Helix Biotech Ltd., Richmond, B.C., Canada), and cross-reacts with 11-KT and T at 100 and 0.06% levels.

I measured the precision and replicability of the assays by assaying the same samples repeatedly within the same assay, and by assaying the same samples in separate assays. Intra- and inter-assay variability were calculated as coefficient of variation (CV = (SD / mean) x 100%).

I determined accuracy of the assays by assaying serial dilutions of extracted samples with high concentrations of endogenous steroids. Binding curves of these samples were compared with binding curves of serial dilutions of standard steroid to determine parallelism.

Concentrations of T, 11-KT, and 17β-E₂ were adjusted for extraction efficiency, and converted to 'ng/gram bodyweight' for each individual fish. In mature fish, whole-body concentrations of T, 11-KT, and 17β-E₂ were compared between females and males, within each season, by t-tests (Zar, 1984).

Hormone Administration

General

Mature females were captured in the field using SCUBA and hand nets, and measured and weighed as described earlier. During all experiments, I kept fish in separate compartments, to prevent visual, chemical, and physical contact with other fish. Individual compartments were made by dividing aquaria (60 x 30 x 30 cm or 50 x 25 x 30 cm) into two equal parts by opaque partitions, sealed with silicone sealant to prevent water mixing. All aquaria were kept indoors on daylight hours using artificial illumination, and were provided with two PVC tubes (10 x 2.5 x 5 cm and 10 x 5 x 5 cm). Within all experimental groups, sizes of fish were matched between tanks.
All hormone administration experiments were carried out during the non-breeding season, in 1994 and 1995. Hormones and hormone antagonist were administered before fish were established in their tanks. After six weeks, I measured, weighed, and sexed fish, and preserved whole abdomens for histological examination as described earlier. Gonads were analyzed in terms of cell types present (Table 3.2). Fish bodies were frozen at -70°C until hormone extraction. Body tissue (except for head) was extracted to determine T, 11-KT and 17β-E₂ concentrations by specific RIA's, as described above. Whole-body concentrations of T, 11-KT, and 17β-E₂ in the experimental groups were compared with those in the control groups by t-tests (Zar, 1984).

**GnRH injections**

Hormone injections were given peritoneally, on the ventro-lateral side, in front of the genital papilla and anus. Fish were not anaesthetized when receiving the injection. I administered two different forms of GnRH: chicken-GnRH-II analogue (dArg⁶-Pro⁹-Ne⁶, c-GnRH-II-A; a gift from Dr. N.M. Sherwood), and salmon-GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH₂, s-GnRH; Sigma Chemical Co., St. Louis). Of these two GnRH forms, c-GnRH-II-A is the most effective one in perciformes (pers. comm. Dr. N.M. Sherwood). Both c-GnRH-II-A and s-GnRH were administered in Cortland's saline and in coconut oil, at a concentration of 1μg GnRH/0.1 ml, per 10 g bodyweight. Coconut oil releases GnRH at a steady rate over a period of at least 6 weeks (pers. comm. Dr. N.M. Sherwood). Control groups received a saline or coconut oil injection. I tested six females in each group from November 19 - December 29 (1994), resulting in a total of 36 fish.

**GnRH, Androgen, and Aromatase Inhibitor Implants**

Elastomere implants were prepared by mixing the hormone with silicone elastomere (Silicone Elastomere Base 2186, Factor II, Lakeside, Arizona, USA), in a shallow glass container.
(25 x 5 x 1 mm). After the hormone was completely mixed, the glass container was left overnight at room temperature, to allow the silicone to set. The next day, I cut the implants to the desired size, and kept them at 4°C until future use. For control implants, the same procedure was followed, without the addition of the hormone.

To administer experimental and control implants, I anaesthetized fish with MS 222. A tiny incision on the ventro-lateral side, anterior to the genital papilla and anus, was made with spring scissors. Using fine forceps, the implant was gently slipped inside the abdominal cavity, and moved frontally as far as possible. The incision was closed with one stitch, using silicone treated silk (Silk 000, Davis and Geck Division, Danbury, Connecticut, USA). Fish were placed in plastic cups with fresh seawater to recover. After normal respiration had resumed, fish were returned to their individual compartments.

Salmon-GnRH (0.04 mg/fish, n=9), 17α-methyltestosterone (17α-MT, 1 mg/fish, n=10), and 11-KT's precursor, 11-ketoadrenosterone (11-KA, 0.3 mg/fish, n=10) (all from Sigma Chemical Co., St. Louis) were administered in elastomere implants (1 x 1 x 5 mm) on August 3, 1995. In addition, 11-KT (0.26 mg/fish, 2 x 2 x 3 mm implant, n=6; Sigma Chemical Co., St. Louis) was administered on September 22, 1995. Finally, the aromatase inhibitor Fadrazole (Al, kindly provided by CIBA-GEIGY Corporation, New Jersey) was administered in 1 x 1 x 5 mm elastomere implants in low (0.1 mg/fish, n=8; September 22, 1995), medium (1 mg/fish, n=10; August 3, 1995), or high (5 mg/fish, n=8; September 22, 1995) doses. Control fish received a control elastomere implant (1 x 1 x 5 mm).

C. Results

Whole-body Steroid Concentrations

Validation of Extraction Procedure and RIA's

Recovery rates in the extraction procedure were 57% ± 4 SE (n=6) for T, 62% ± 3 SE (n=6) for 11-KT and 60% ± 3 SE (n=6) for 17β-E₂. Intra- and inter-assay variability showed that
RIA's for T, 11-KT, and 17β-E₂ were precise and replicable. Intra-assay coefficient of variance was 9.0% (n=4) for T, 9.2% (n=4) for 11-KT, and 0.6% (n=4) and 1.4% (n=4) for 17β-E₂. Inter-assay coefficient of variance was 4.8% (n=8) for 17β-E₂. In addition, the binding curves of serial dilutions of extracted samples were parallel to the standard curves (Figure 6.1a, b, c). Minimum detectable concentrations in the assays were 78 pg/ml for T and 11-KT and 39 pg/ml for 17β-E₂.

Concentrations of T, 11-KT and 17β-E₂

In the breeding season, mean whole-body concentration of T in females was two times higher than in males (t-test: t=2.70, p=0.03; Figure 6.2a). In contrast, in the non-breeding season, whole-body concentration of T in males was twice that of females (t=-2.08, p=0.06; Figure 6.2a).

Mean whole-body concentrations of 11-KT in females and males were very similar in the breeding season (t=0.81, p=0.43; Figure 6.2b). However, in the non-breeding season mean whole-body concentration of 11-KT in males was approximately 40% higher than in females, although the difference was not significant (Mann-Whitney test: U=82, p>0.1; Figure 6.2b).

In the breeding season, mean whole-body concentration of 17β-E₂ in females was approximately 4 times higher than in males (t=6.00, p<0.001; Figure 6.2c). In the non-breeding season, mean whole-body concentration of 17β-E₂ in females was still significantly higher than in males (U=41, p=0.002), although the difference was less pronounced (Figure 6.2c). In immature fish, whole-body concentrations of 17β-E₂ were similar to those measured in mature females in the breeding season. Mean whole-body concentration of 17β-E₂ in small males was approximately twice as high as in immature females and transitionals (Figure 6.3).
Figure 6.1  Binding curves of serial dilutions of extracted samples and standards, for (a) testosterone, (b) 11-ketotestosterone and (c) 17β-estradiol. Symbols for each graph are: standards (●), and extracted samples (■, ▲).
Figure 6.2 Whole-body steroid concentrations (mean ± SE) in mature female and male *C. nicholsii*, in the breeding and non-breeding season; (a) testosterone, (b) 11-ketotestosterone and (c) 17β-estradiol. * indicates a statistically significant difference (p<0.05) between females and males within each season, n.s. = non-significant; (n) = sample size.
Figure 6.3 Whole-body 17β-estradiol concentrations (mean ± SE) of immature *C. nicholsii* females, transitionals, and males; (n) = sample size.
Hormone Administration

GnRH Administration

Two out of 12 females had changed completely into males, one after treatment with c-GnRH-II-A and one after treatment with s-GnRH (Table 6.2). The gonads of these two fish lacked any ovarian tissue, and consisted of well-formed seminiferous tubules, lined with spermatocytes (Figure 6.4a). A thick layer of connective tissue was visible in the s-GnRH treated female, but was absent in the c-GnRH-II-A treated female. The other 9 females (one female died), and the 12 having received s-GnRH or c-GnRH-II-A injections dissolved in coconut oil, showed no signs of transformation. Twenty of these females had gonads with previtellogenic oocytes, very few yolk vesicle stage oocytes, and few atretic bodies. One female, which had received a c-GnRH-II-A coconut oil injection, showed progressive signs of atresia, with numerous pink bodies in its gonad.

Table 6.2 The effects of treatments with GnRH: (s) = saline injection, (c) = coconut oil implant, both 1 μg GnRH/0.1 ml, per 10 gr bodyweight; (e) = elastomere implant, 0.04 mg s-GnRH/fish. The results of each experiment are given as the number of fish that remained female (F), partly transformed into a male (T), or fully transformed into a male (M); n (†) = sample size (number of fish that died during experiment).

<table>
<thead>
<tr>
<th></th>
<th>n (†)</th>
<th>SL (cm) ± SE</th>
<th>SL range</th>
<th>Days</th>
<th>Period</th>
<th>F</th>
<th>T</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-GnRH (s)</td>
<td>6 (1)</td>
<td>5.8 ± 0.3</td>
<td>5.0 - 7.2</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>s-GnRH (c)</td>
<td>6 (0)</td>
<td>5.9 ± 0.2</td>
<td>5.2 - 6.6</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s-GnRH (e)</td>
<td>9 (0)</td>
<td>6.4 ± 0.2</td>
<td>5.8 - 7.4</td>
<td>42</td>
<td>Aug. 2/3-Sept. 14, '95</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>c-GnRH-II-A (s)</td>
<td>6 (0)</td>
<td>5.7 ± 0.3</td>
<td>5.0 - 6.8</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>5</td>
<td>0</td>
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</tr>
<tr>
<td>c-GnRH-II-A (c)</td>
<td>6 (0)</td>
<td>5.7 ± 0.2</td>
<td>5.2 - 6.5</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 6.4 Gonadal structure of female C. nicholsii treated with GnRH; (a) complete reversed gonad, s-GnRH saline injection; (b) early transitional stage gonad, s-GnRH elastomere implant. Abbreviations: pvo=previtellogenic oocyte, L=lumen, sc=spermatocytes, ct=connective tissue. Magnifications: (a) 100X and (b) 100X.
After s-GnRH administration in elastomere implants, two out of nine females showed signs of sex change (Table 6.2). The cells of these gonads formed a loose mass, and little clumps of spermatocytes lined the disintegrating ovarian lumen (Figure 6.4b). Ovarian cells consisted mainly of previtellogenic oocytes, and numerous atretic cells and pink bodies. The gonads of the other seven females showed no changes.

Androgen Administration

Both 11-KT and 11-KA administration induced almost complete sex change in all females (Table 6.3). The gonads of 4 11-KT and 9 11-KA treated females showed no signs of ovarian tissue (Figure 6.5). Thick layers of connective tissue surrounded the testes. However, most of these testes did not have fully developed seminiferous tubules, but rather showed clumps of spermatocytes. The gonad of one 11-KA treated female showed numerous spermatozoa. In one 11-KA treated female, gonadal structures consisted of both ovarian and testicular cell types.

The androgen 17α-MT had no visible effect on gonadal structures (Table 6.3). Gonads of all females lacked any form of testicular tissues, but showed various stages of atresia. One of these ovaries showed large areas of flattened spindle-shaped cells, surrounding degenerating oocytes.

Table 6.3 The effects of treatments with androgens: 17α-MT = 1 mg/fish, 11-KA = 0.3 mg/fish and 11-KT = 0.26 mg/fish; all in elastomere implants. The results of each experiment are given as the number of fish that remained female (F), partly transformed into a male (T), or fully transformed into a male (M); n (†) = sample size (number of fish that died during experiment).

<table>
<thead>
<tr>
<th></th>
<th>n (†)</th>
<th>SL (cm) ± SE</th>
<th>SL range Days Period</th>
<th>F</th>
<th>T</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-MT</td>
<td>10 (3)</td>
<td>6.2 ± 0.2</td>
<td>5.4 - 7.3 43 Aug. 2/3-Sept. 14, '95</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11-KA</td>
<td>10 (0)</td>
<td>6.4 ± 0.2</td>
<td>5.5 - 7.5 42 Aug. 2/3-Sept. 14, '95</td>
<td>0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>11-KT</td>
<td>6 (2)</td>
<td>6.0 ± 0.2</td>
<td>5.4 - 6.4 43 Sept. 22-Nov. 4, '94</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 6.5 Gonadal structure of complete reversed gonad of female *C. nicholsii* treated with 11-KA. Abbreviations: sc=spermatocytes, ct=connective tissue. Magnification: 50X.
Aromatase Inhibitor Administration

The effect of Fadrazole increased with increasing concentration, with complete sex change in some individuals at the highest dose (Table 6.4). At the lowest dose, only one female showed signs of transformation, with a few clumps of spermatocytes lining the ovarian lumen. The gonads of the other six females in this group showed no signs of testicular tissue development. At the medium dose, one female had an advanced ovotestis, with only a few oocytes left and numerous clumps of spermatocytes and a thick layer of connective tissue surrounding the ovotestis. In addition, gonadal structures of two other females in this group showed numerous atretic oocytes, and a few clumps of spermatocytes (Figure 6.6). The gonads of the other four females lacked any testicular tissue. The highest dose of AI administered induced complete sex change in two out of eight females. Their gonads consisted of well-developed seminiferous tubules, lined with spermatocytes and partly filled with spermatids. In addition, gonads of five other females in this group contained both testicular and ovarian cell types. Only one female in this group had no testicular tissue in the gonad.

Table 6.4 The effects of treatments with the aromatase inhibitor, Fadrazole: low = 0.1 mg/fish, medium = 1 mg/fish, high = 5 mg/fish; all in elastomere implants. The results of each experiment are given as the number of fish that remained female (F), partly transformed into a male (T), or fully transformed into a male (M); n (†) = sample size (number of fish that died during experiment).

<table>
<thead>
<tr>
<th></th>
<th>n (†)</th>
<th>SL (cm) ± SE</th>
<th>SL range</th>
<th>Days</th>
<th>Period</th>
<th>F</th>
<th>T</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>low</td>
<td>8 (1)</td>
<td>5.9 ± 0.2</td>
<td>5.2 - 6.5</td>
<td>43</td>
<td>Sept. 22-Nov. 4, ’95</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>medium</td>
<td>10 (3)</td>
<td>6.4 ± 0.2</td>
<td>5.4 - 7.1</td>
<td>42</td>
<td>Aug. 2/3-Sept. 14, ’95</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>high</td>
<td>8 (0)</td>
<td>5.9 ± 0.2</td>
<td>5.2 - 6.4</td>
<td>43</td>
<td>Sept. 22-Nov. 4, ’95</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 6.6  Gonadal structure of early transitional stage gonad of female *C. nicholsii* treated with 5 mg/fish of the Aromatase Inhibitor, Fadrazole. Abbreviations: pvo=previtellogenic oocyte, ato=atretic oocytes, L=lumen, sc=spermatocytes. Magnification: 25X.
Control Treatments

The gonads of the females receiving a control treatment lacked any sign of testicular tissue (Table 6.5).

<table>
<thead>
<tr>
<th></th>
<th>n (†)</th>
<th>SL (cm) ± SE</th>
<th>SL range</th>
<th>Days</th>
<th>Period</th>
<th>F</th>
<th>T</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline</td>
<td>6 (0)</td>
<td>5.7 ± 0.2</td>
<td>5.0 - 6.6</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>coconut oil</td>
<td>6 (1)</td>
<td>6.1 ± 0.4</td>
<td>5.2 - 8.1</td>
<td>40</td>
<td>Nov. 19-Dec. 29, '94</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>elastomere</td>
<td>10 (2)</td>
<td>6.4 ± 0.2</td>
<td>5.5 - 7.1</td>
<td>43</td>
<td>Aug. 2/3-Sept. 14, '95</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>elastomere</td>
<td>6 (1)</td>
<td>6.2 ± 0.1</td>
<td>5.8 - 6.5</td>
<td>43</td>
<td>Sept. 22-Nov. 4, '95</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Concentrations of T, 11-KT, and 17β-E₂

Whole-body steroid concentrations were only determined in the 11-KA, 11-KT and Fadrazole (5 mg/fish) experimental groups, because only these treatments resulted in complete gonadal sex change. Sample sizes vary among the different assays, due to insufficient extract and to loss of samples.

Mean whole-body concentrations of T were similar in the 11-KA and control group (Figure 6.7). In contrast, mean whole-body concentration of T in the control group was approximately 5 times higher than in the 11-KT group (Mann-Whitney test: U=3, p=0.01; Figure 6.7a). On the other hand, mean whole-body concentration of T in the Fadrazole group was 4 times higher than in the control group, but the difference was not significant (U=28, p>0.1; Figure 6.7a).
Figure 6.7 Whole-body steroid concentrations (mean ± SE) of 11-KA, 11-KT, AI and control treated female *C. nicholsi*; (a) testosterone, (b) 11-ketotestosterone and (c) 17β-estradiol. * indicates a statistically significant difference (p<0.05) in whole-body steroid concentrations; n.s. = non significant; (n) = sample size.
Mean whole-body concentrations of 11-KT in both the 11-KA and the Fadrazole groups were approximately twice that of the control groups (Figure 6.7b). However, neither difference was significant (11-KA: U=36, p>0.1; Fadrazole: t=-2.02, p=0.08). In contrast, mean whole-body concentrations of 11-KT in the 11-KT and control groups were almost equal (Figure 6.7b).

Mean whole-body concentrations of 17β-E₂ in all three experimental groups were approximately 1.5 times lower than in the control groups (Figure 6.7c). However, this difference was only significant in the 11-KA group (t=2.75, p=0.02).

D. Discussion

My study showed that T, 11-KT and 17β-E₂ are naturally occurring steroids in both female and male *C. nicholsii*. The range of whole-body concentrations of T and 11-KT were similar to whole-body concentrations of androgens documented in male swordtails, *Xiphophorus helleri* (Hannes et al., 1984). Whole-body concentrations of 17β-E₂ have not been measured previously in mature fish. Further, mine is the first study to demonstrate the presence of 11-KT in a goby species. Thus far, studies on the reproductive endocrinology of gobies have been restricted to *in vitro* incubations of gonadal tissues of *Gobius jozo* (Colombo et al., 1977; Colombo and Colombo-Belvédère, 1977), *G. paganellus* (Colombo et al., 1970), and *Glossogobius olivaceus* (Asahina et al., 1985). Both T and 17β-E₂ were detected, but 11-KT was not isolated in either study.

Seasonal Patterns of Whole-Body Steroid Concentrations in *C. nicholsii*

The seasonal patterns and sex differences in whole-body concentrations of 17β-E₂ in *C. nicholsii* suggest that 17β-E₂ influences vitellogenesis and oocyte maturation. Whole-body
concentrations of 17β-E₂ in females were higher in the breeding than in the non-breeding season, and higher than those measured in males.

In contrast, whole-body concentrations of T and 11-KT in *C. nicholsii* do not suggest that 11-KT promotes spermiation, or that T or 11-KT regulate male reproductive behaviour. In the breeding season, concentrations of T were higher in females than in males, whereas 11-KT concentrations did not differ between the two sexes. Perhaps whole-body concentrations of T and 11-KT do not reflect circulating levels of these steroids in *C. nicholsii*. In male *X. helleri*, whole-body androgen concentrations showed similar changes over time as those observed in plasma androgen levels (Hannes *et al.*, 1984). Hence, it is likely that whole-body concentrations of T and 11-KT in *C. nicholsii* correlate with circulating levels of these androgens, although measurement of plasma T and 11-KT levels are necessary to confirm this. High concentrations of T in females in breeding condition have been detected before in teleosts (Fostier *et al.*, 1983; Kime, 1993; Borg, 1994). Testosterone is a precursor for 17β-E₂, and in female teleosts may function as such (Fostier *et al.*, 1983). Indeed, in the breeding season concentrations of T and 17β-E₂ are strongly correlated in female *C. nicholsii* (Pearson correlation: r=0.95, n=3, p=0.21).

### Hormonal Induction of Sex Change

My results strongly suggests that 11-KT is involved in the mediation of natural sex change in *C. nicholsii*. 11-Ketotestosterone is a naturally occurring steroid in both female and male *C. nicholsii*. In addition, complete sex change was induced in mature females by administration of 11-KT, and its precursor 11-KA. Administration of 11-KT to females also induced sex change in the protogynous parrotfish *Sparisoma viride* (Cardwell and Liley, 1991b) and wrasse *Thalassoma bifasciatum* (Grober *et al.*, 1991). Further, 11-KT has been documented in plasma samples of *S. viride* (Cardwell and Liley, 1991b).

On the other hand, 11-KT administration failed to induce sex change in the protogynous rice-field eel *Monopterus albus* (Tang *et al.*, 1974). Also, in the protogynous black sea bass
Centropristis striatus, plasma 11-KT levels in females were highest during the breeding season, whereas transitionals were mainly detected in the non-breeding season (Cochran and Grier, 1991). This suggests that sex change in C. striatus is not under control of 11-KT, although this was not tested experimentally. Thus, 11-KT may not be involved in natural sex change in all protogynous teleosts.

In addition to suggesting a principal role for 11-KT in protogynous sex change in C. nicholsii, the results of my study suggest that 17β-E₂ is also involved in the mediation of sex change. Sex change was induced in mature females by administration of Fadrazole. Fadrazole inhibits the conversion of T into 17β-E₂, thereby increasing the levels of T and decreasing the levels of 17β-E₂. Whole body concentrations of T and 17β-E₂ in females treated with Fadrazole indeed show this trend (Figure 6.7). Given Fadrazole's effect, I hypothesize that either an increase in T, a decrease in 17β-E₂, or a combination of these, causes sex change in Fadrazole treated females.

A role of T in mediating sex change is suggested by the higher concentrations of T in males than in females, measured both in C. nicholsii (non-breeding season: Figure 6.7), and in S. viride (Cardwell and Liley, 1991b). However, these elevated T levels in S. viride may be a function of an increase in territorial, agonistic behaviour, and may not be related to sex change (Cardwell and Liley, 1991a). No differences in plasma T levels were detected among female, transitional, and male T. dupperey (Nakamura et al., 1989), and between female and male C. striatus (Cochran and Grier, 1991), and M. albus (Yeung and Chang, 1987). Further, administration of the androgen 17α-MT to female C. nicholsii had no effect on their gonads. Administration of T or methyltestosterone to female M. albus (Tang et al., 1974) did not result in sex change either. The lack of sex change in both studies may be due to aromatization of the administered androgens, resulting in a decrease in androgen levels, and an increase in estrogen levels. However, administration of the non-aromatizable androgen dihydrotestosterone to female T. bifasciatum (Kramer et al., 1988) did not result in sex change either. Overall, these studies
suggests that in female *C. nicholsii* treated with Fadrazole, an increase in T is not responsible for sex change.

In contrast, 17β-E₂ is involved in maintenance of the female state in teleosts. Thus, a reduction in 17β-E₂ levels may stop vitellogenesis and oocyte maturation, two characteristics of the early stages of sex change (Chan and Yeung, 1983; Reinboth, 1970), and permit male development. In all experimental groups, whole-body concentrations of 17β-E₂ were lower than those in the control groups (Figure 6.7). Further, decrease of plasma 17β-E₂ levels during sex change have been measured in *T. dupperey* (Nakamura *et al.*, 1989) and *S. viride* (Cardwell and Liley, 1991b). Finally, 17β-E₂ levels were higher in females than in males in both *C. nicholsii* (present study) and *C. striatus* (Cochran and Grier, 1991). In only one study, on *M. albus*, 17β-E₂ levels did not differ between females and males (Yeung and Chan, 1987). Overall, these observations support the involvement of a decrease in 17β-E₂ levels in mediating sex change in teleosts.

Both c-GnRH-II-A and s-GnRH administration had negligible effects on the gonads of female *C. nicholsii*. Administration of LHRH-A to female *Monopterus albus* also failed to produce sex change (Yeung *et al.*, 1993). These failure to obtain consistent sex change suggests that it is unlikely that GnRH is involved in sex change. Alternatively, the dosage administered may not have been appropriate. Although the GnRH concentrations injected were similar to those used in studies to induce spawning of sexually mature teleosts (see review Crim *et al.*, 1987), this possibility cannot be ruled out until natural levels of GnRH are known. Also, GnRH administration may have been carried out in a refractory period, during which gonads are non-responsive to stimuli, presumably mediated by GnRH, that normally trigger gonadal recrudescence. In the winter flounder, *Pleuronectes americanus*, females were responsive to GnRH-A treatment throughout the year, except for a brief period after the reproductive season (Harmin *et al.*, 1995).
Summary

In summary, my study showed that T, 11-KT and 17β-E₂ are naturally occurring steroids in both female and male *C. nicholsii*. The results further suggest that both a decrease in 17β-E₂, and an increase in 11-KT concentrations are involved in mediating sex change in *C. nicholsii*. Whole-body concentrations of 17β-E₂ were lower in males than in females, and were also lower in the hormone administration groups than in the control groups. Finally, complete sex change was induced in mature females by administration of 11-KT, its precursor 11-KA and the aromatase inhibitor Fadrazole.
CHAPTER SEVEN

General Discussion

My major objective was to examine the occurrence of protogynous hermaphroditism in a temperate reef fish, the blackeye goby, *Coryphopterus nicholsii*. In this discussion, I focus on the three major questions I have addressed:
i) What is the adaptive significance of protogyny?
ii) Is sex change the direct result of changes in social conditions, or a result of changes in resource availability?
iii) What are the endocrine mechanisms mediating sex change?
I discuss these issues in relation to the strong disparity in the geographical distribution of protogyny in teleosts.

The Distribution of Protogyny: Effects of Male Mating Investment and Biogeography

The ability of males to control resources and monopolize spawnings influences whether protogyny is likely to occur in any given species (Warner, 1984b; Ross, 1990). My study has shown that, in *C. nicholsii*, the social and mating system is based upon the year-round defense of a territory by large individuals of both sexes. A territory always includes one or more shelter rocks. These are used as refuges by both sexes, and, as nest sites by males during the breeding season. Suitable refuge and nest site rocks are a limited resource and intraspecific competition determines which individuals gain access to them. Since the outcome of competition for shelter rocks is strongly size-specific, large males can monopolize spawnings.

It is surprising that protogyny is not more common among temperate, marine teleosts, since polygyny does not appear to be rare amongst them (Breder and Rosen, 1966; Potts, 1984). Examples of temperate species with resource defense polygyny are the wrasses *Tautogolabrus adspersus* (Pottle and Green, 1979a, b) and *Crenilabrus melops* (Potts, 1984), and the gobies

...
*Pomatoschistus minutus* (Hesthagen, 1977; Lindström, 1988) and *P. microps* (Vestergaard, 1976). In all these species, larger males monopolize spawnings, and reproductive output of males relative to females is similar or greater than I estimated for *C. nicholsii*. Yet, none appear to be protogynos (Policansky, 1982). This is surprising, especially since protogyny is common among tropical wrasses and gobies (Warner and Robertson, 1978; Thresher, 1984; Warner, 1984b). Why is this the case?

I speculate that a difference in the occurrence of certain mating systems in tropical and temperate waters partly explains the strong disparity in the geographical distribution of protogyny. In coral reef fish, the taxonomically most widespread mode of spawning is pelagic spawning (see Johannes (1978) and Thresher (1984) for discussions on potential reasons). In contrast, in temperate reef fish, demersal spawning, including paternal care, is the more common spawning mode (Gibson, 1969; Russell, 1976; Potts, 1984). The increase in male mating investment, associated with paternal care, most likely limits the reproductive advantage associated with polygyny, thereby reducing the selective advantage of protogyny. As a result, this may reduce the likelihood of the evolution of protogyny in temperate waters.

A comparison of several protogynous species supports the hypothesis that an increase in male mating investment results in a decrease in male reproductive output. In the tropical, protogynous wrasses *Thalassoma bifasciatum* and *Bodianus diplotaenia*, large males defend reproductive territories only during the daily spawning period. In both species, these males can obtain 50 pair spawnings a day, compared to one pair spawning for large females (Warner et al., 1975a; Hoffman et al., 1985). In contrast, in protogynous species in which large, territorial males defend a harem of females throughout the day (i.e. harem-polygyny), males obtain a lower number of daily pair spawnings, ranging from 6 times in the wrasse *B. rufus* (Hoffman et al., 1985), 10 times in the tilefish *Malacanthus plumierie* (Baird, 1988), 6 to 8 times in the parrotfish *S. viride* (Cardwell, 1989; Van Rooij et al., 1996c), to 11 times in *S. radians* (Farn, 1993).

Parental care further restricts the differential between female and male reproductive output: males of the tropical protogynous gobies *C. glaucofraenum* and *C. personatus* spawn year-round,
but spend 4 days and 5 to 6 days, respectively, guarding a clutch (Cole, pers. comm. in: Sponaugle and Cowen, 1994; Cole and Robertson, 1988). The results of the present study suggest that the ecological conditions in temperate regions further restrict the opportunities for males to acquire clutches of eggs. In *C. nicholsii*, an average of two females contributed to a male clutch in the field. In addition, at Bamfield a male *C. nicholsii* can only mate once every 14 to 33 days (present study; Cole, 1982a) for five months, and not every day year-round as in pelagic spawning species.

Similar results were obtained by Warner and Lejeune (1985), who compared mating success of females and males in four species of the wrasse genus *Symphodus*. The four species differed in the extent to which males provide parental care, and thus in the time males can spend mating. They demonstrated that in the species with little or no paternal care, the spawning rates of large, territorial males are relatively high compared to large females, and protogyny is common. In contrast, in the species with more extensive paternal care, including nest building and egg ventilation and guarding, large territorial males only have a slight reproductive advantage over large females. Protogynous sex change is absent in these species.

A similar argument may hold for the more frequent occurrence of protogyny among coral reef fish than among tropical freshwater fish. Parental care is much more common in freshwater than in marine teleosts, with 60% of freshwater families showing some form of parental care, compared with only 16% of marine families (Baylis, 1981). In teleosts, parental care by males alone is more prevalent than any other form of parental care (Blumer, 1979; Gross and Sargent, 1985). Hence, in tropical regions, frequent paternal care in freshwater teleosts may reduce selection for protogynous sex change, and protogyny may not evolve as frequently in freshwater as in marine environments.

Why do males provide more parental care at higher latitudes? The explanation may lie in latitudinal effects which limit the extremes of the potential reproductive output of temperate,
male teleosts. A reduction in male reproductive output with increasing latitude may force males to enhance their reproductive success through other means, i.e. by increasing their levels of paternal care. A likely limiting factor here is the reproductive output of females.

In teleosts, female fecundity decreases with increasing latitude. Such a decrease occurs in the freshwater gonochorines the sculpin *Cottus gobio*, the loach *Noemachilus barbatulus*, and the minnow *Phoxinus phoxinus* (Mills and Mann, 1983; Mann et al., 1984; Mills and Eloranta, 1985; Mills, 1988). In addition, the anadromous cisco, *Coregonus artedii*, and the lake whitefish, *C. clupeaformis*, exhibit a decrease in female fecundity with increasing latitude, independent of adult size (Morin et al., 1982). A similar decrease in female fecundity occurs in the American shad, *Alosa sapidissima* (Leggett and Carscadden, 1978). Transplantation experiments with *C. gobio* revealed that female fecundity was primarily controlled by environmental factors, possibly temperature or food availability (Mann et al., 1984).

To my knowledge, the reproductive output of male teleosts at different latitudes has only been estimated in *C. nicholsii*. In this species, the mean size of clutches guarded by similarly-sized males at high latitude (8050 eggs ± 2979 SE; 49°49' N, 125°10' W; Chapter 5) was approximately 20% smaller than at low latitude (9761 eggs ± 1572 SE; Naples Reef, 34°25' N, 119°57' W; Breitburg, 1987), but did not differ significantly (ANCOVA: \( \bar{F}_{1.43} = 1.35, p = 0.25 \)). However, at Naples Reef, males mate every 10 to 24 days for seven months (Breitburg, 1984), whereas at Bamfield they mate every 14 to 33 days for five months (Chapter 3, 5; Cole, 1984). This decrease in the duration of a breeding season, and increase in the period devoted to clutch guarding with increasing latitude, is most likely related to a decrease in water temperature (Figure 7.1). A similar decrease in the duration of a breeding season, and in the number of spawnings within a season, has been documented for other temperate gobies (see references in Miller, 1984). Thus, northern male *C. nicholsii* are likely to obtain fewer clutches in a breeding season than southern males. Consequently, for male teleosts to enhance their reproductive output with increasing latitude, they may increase their levels of parental care.
Figure 7.1  Temperature profiles at Bamfield (1994) and Laguna Beach (1966-1967) relative to the five (Bamfield) and seven (Laguna Beach) breeding season of *C. nicholsii*. Data on length of breeding season at Bamfield were obtained from present study and Cole (1982); at Laguna Beach from Wiley (1973).
Future research should compare reproductive strategies in protogynous and gonochoristic teleosts across a range of latitudes, as well as in detail in a single population. For example, at the northern most limits of the range of *C. nicholsii*, the Queen Charlotte Islands (B.C.), protogyny may have disappeared altogether.

**Proximate Factors Controlling the Onset of Protogynous Sex Change**

In protogynous teleosts with social control of sex change, the proximate factors mediating sex change are primarily determined by the type of social and mating system (Ross, 1990). In harem-forming species with linear, size-based dominance hierarchies, such as the wrasses *Labroides dimidiatus* and *Bodianus diplotaenia*, and the seabass *Anthias squamipinnis* (= *Subanthias squamipinnis*), the removal of the male resulted in sex change by the largest, dominant female in the harem (Fishelson, 1970; Robertson, 1972; Hoffman, 1983). In species with large, permeable social groups lacking rigid dominance hierarchies, it is thought that sex change may be induced by specific changes in the social group (Ross, 1990), such as changes in sex ratio (Shapiro, 1979; Shapiro and Lubbock, 1980) or size distribution (Ross, 1990). No experimental data are available to support the sex ratio model. On the other hand, in the protogynous *Thalassoma duperrey* an increase in the relative numbers of smaller conspecifics in a social unit can induce sex change (Ross *et al.*, 1983).

In *C. nicholsii*, changes in the social group, such as changes in sex ratio or size distribution, rather than changes in dominance status, are likely to induce sex change. At first sight, the strong size-dependent dominance hierarchy of *C. nicholsii* suggests that removal of the dominant male may cause the largest, dominant female to change sex. However, sex change in the presence of a dominant male is observed in *C. nicholsii*, and also in *C. personatus* (Cole and Robertson, 1988) and *C. glaucofraenum* (Cole and Shapiro, 1992, 1995). The lower incidence of sex change in mixed-sex groups than in all-female groups in the laboratory in both *C. personatus* (Cole and Robertson, 1988) and *C. glaucofraenum* (Cole and Shapiro, 1992, 1995), suggests
that the presence of large males has an inhibitory effect on sex change in these species. In *C. nicholsii*, the incidence of sex change was low in both mixed-sex and all-female groups.

My results suggest that the local size distribution affects the timing of sex change in *C. nicholsii*. There are two possible versions of this hypothesis. First, females may change sex as a function of their size relative to the size distribution of the population. My data suggest that such a mechanism operates in *C. nicholsii*: when several females were housed together in the laboratory, the largest usually changed sex. In contrast, the smallest female never changed sex, as was also observed in all-female groups of its congeners *C. personatus* (Cole and Robertson, 1988), *C. lipernes, C. dicrus* (Cole and Shapiro, 1990), and *C. glaucofraenum* (Cole and Shapiro, 1992, 1995). Thus it would appear that sex change in this species is mediated by the social environment with the specific cue being a female’s size relative to the population.

Second, females may change sex as a function of competition for nest sites. For *C. nicholsii*, limited availability of suitable nest sites may shape the mating system by affecting the intensity of competition among males for nest sites. Given similar nest site densities, an increase in population density will increase competition for nest sites, resulting in only the largest males being able to breed. Because fish that change sex when too small will be unable to obtain nest sites under such conditions, competition for nest sites may affect the rate and timing of sex change. However, a number of observations argue against this second suggestion. First, the size at sex change was similar in a number of local populations studied over an interval of 15 years (Chapter 4; Cole, 1983). Second, the minimum size at which males were observed to guard clutches was similar across these populations (Chapter 5), and in populations up to 1500 km apart (Chapter 5; Breitburg, 1987). In addition, the similar number of sex changers in laboratory tanks with different numbers of shelter rocks contradicts this second version. On the other hand, the more female-biased sex ratios in areas with few suitable nest sites support the competition version (Chapter 3; Breitburg, 1987; however, see Chapter 4: local sex ratios did not differ significantly in two habitats with different shelter rock densities). Further, Breitburg (1987)
showed that the number of suitable nest sites affected the proportion of males guarding clutches in the *C. nicholsii* population at Naples Reef.

With the available data I cannot distinguish these two versions of the size distribution mechanism. However, a combination of both versions may regulate sex change in *C. nicholsii*, since changes in the social environment will affect both relative size of females and competition for nest sites.

**Hormonal Control of Protogynous Sex Change**

In teleosts, as in most other vertebrates, sexual development is largely determined by reciprocal interactions in the brain-pituitary-gonadal (BPG) axis. The three components of the BPG axis are interdependent in their synthesis and release of hormones. The hormonal component of the BPG axis in the brain is Gonadotropin Releasing Hormone (GnRH), which is synthesized in various structures throughout the brain. This hormone stimulates the release of pituitary gonadotropin (GtH) into the blood. Gonadotropin stimulates gametogenesis and steroidogenesis in the gonad. The gonadal steroid hormones, of which testosterone (T), 11-ketotestosterone (11-KT) and 17β-estradiol (17β-E₂) are the most studied, are carried in the blood, and play an important role in gametogenesis, and regulating reproductive behaviour and development of secondary sexual characteristics (Fostier *et al.*, 1983; Liley and Stacey, 1983). In addition, gonadal steroids generally exert a negative feedback control over GtH release (Fostier *et al.*, 1983).

The importance of the BPG axis in the initiation of protogynous sex change in teleosts has been stressed by several authors (see reviews Reinboth, 1988; Shapiro, 1990; Ross, 1990; Francis, 1992). In species with social control of sex change, the brain is hypothesized to determine the gonadal sex, because social events can affect the gonads only through the brain. However, despite numerous physiological studies on protogynous hermaphroditic teleosts, no general endocrine mechanism mediating sex change has emerged.
This may be because, until recently, 'classical' teleost hormones, such as T, 11-KT, and 17β-estradiol, were the most studied gonadal steroids in investigations of the reproductive physiology of teleosts (Kime, 1993). However, Kime (1993) emphasizes the enormous diversity of steroids and steroid metabolites in teleosts. A variety of steroids, other than the ones measured above mentioned, may have important functions in the endocrinology of sex change. Hence, future research should be directed towards quantification of other known steroids, purification and identification of unknown steroids, and determination of their effects.
CHAPTER EIGHT

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