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## Reproductive biology of an odacine labrid, *Odax pullus*

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The sexual ontogeny of butterflyfish *Odax pullus* was examined in the Hauraki Gulf of New Zealand through histological analysis of gonad material, size and age information and seasonal patterns of sexual maturation. The patterns of gonad development and schedules of male recruitment were established and sexual ontogeny of *O. pullus* was diagnosed as monandric protogyny, with all males developing from mature females after female-to-male sex reversal. All individuals underwent an immature female phase before maturing as functional females at 228.7–264.8 mm fork length ( $L_F$ ) and at 1.1–1.5 years of age, and there was no evidence of a juvenile bisexual phase. Degrading mature oogenic elements were found in the gonad lumen of individuals with developing spermatogenic tissue, providing histological evidence for functional protogyny. Sex change was estimated to occur at 359–379 mm  $L_F$  and 2–3 years of age. The diagnosis of monandric protogyny for *O. pullus* coincided with the pattern of sexual ontogeny seen in the majority of labrids, particularly those of the same clade (tribe Hypsigenyini) and contrasted with that seen in a number of other temperate labrids. This study suggests that the protogynous mode of sexual development in *O. pullus* is likely to be lineage-specific, *i.e.* associated with the phylogeny of labrid sexual development, and is not constrained by environmental effects on the evolution of sex change in temperate regions.

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### INTRODUCTION

Teleosts in general, and perciform reef fishes in particular, are characterized by high levels of flexibility in their mode of sexual development (Mank & Avise, 2006; Kazancıoğlu & Alonzo, 2010). Within perciforms, a large number of species are sequential hermaphrodites (one individual functions first as one sex before changing sex to function as the other sex), with the most diverse representatives being the reef-associated perciform families Serranidae (groupers) and Labridae (wrasses, parrotfishes and odacines) (Sadovy de Mitcheson & Liu, 2008). Although the

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processes of male recruitment may vary among species of these diverse groups, the majority display a pattern of sex change from female to male (protogyny). The study of sequential hermaphroditism in these groups has had a long history (Reinboth, 1975) and includes analyses of the evolutionary factors driving sex change (Warner *et al.*, 1975). Over the past 10 years, phylogenetic reconstructions of the evolutionary history of these families (Westneat & Alfaro, 2005; Craig & Hastings, 2007; Alfaro *et al.*, 2009) have provided an opportunity to resolve issues concerning the evolution of sequential hermaphroditism in greater depth (Erisman *et al.*, 2009; Kazancıoğlu & Alonzo, 2010).

Accurate reconstructions of the evolutionary history of sequential hermaphroditism depend on the availability of species-specific detail of sexual ontogeny and mating systems within the different groups. For example, a comparative analysis of sex change within the Labridae based on 78 species provided strong support for the size-advantage hypothesis as the primary driver of sequential hermaphroditism in this group (Kazancıoğlu & Alonzo, 2010). Although the majority of the species reviewed showed evidence of sequential hermaphroditism (as opposed to gonochoristic sexual development, *i.e.* fixed separate sexes), the exceptions were associated with particular lineages, *i.e.* labrine wrasses and sparismatinine parrotfishes, and with some environments. In particular, many gonochoristic labrine wrasses occurred in northern hemisphere temperate environments. The odacine labrids (weed whittings and butterfishes), which are nested within the sister group to all other labrids (tribe Hypsigenyini), represent a monophyletic group at the base of the labrid tree (Clements *et al.*, 2004; Alfaro *et al.*, 2009). They are a highly distinctive morphological and ecological grouping and are restricted to the temperate waters of the southern hemisphere. Determination of the mode of sexual development within this distinctive basal group is of interest to the broader picture of labrid sexual evolution.

This study investigates the reproductive biology of an odacine labrid, the butterfish *Odax pullus* (Forster). The main aim was to determine the pathway of male recruitment (functional protogyny *v.* gonochorism) and to provide a diagnosis for the pattern of sexual ontogeny in this species. Although the majority of labrids are protogynous, there is considerable diversity in the ontogeny of male and female gonad development (Sadovy de Mitcheson & Liu, 2008). The diagnosis of sexual ontogeny is complicated by the presence within many labrid species of two pathways of male development, with some males developing directly from a juvenile gonad (primary males) and others from sex reversal of functional females (secondary males) (Reinboth, 1967, 1975; Munday *et al.*, 2006; Sadovy de Mitcheson & Liu, 2008). Moreover, the presence of a mismatch between gonad morphology and functional development renders the diagnosis of functional protogyny a challenging exercise as in a number of species of Serranidae and Labridae, where some testes are secondary in configuration, but do not necessarily pass through a functional female phase (Robertson *et al.*, 1982; Sadovy de Mitcheson & Liu, 2008).

Although much of the evidence for protogyny is based on the histological analysis of gonad material, establishing the occurrence of sex reversal (1) in relation to the age of female sexual maturation and (2) its timing with respect to spawning provides important additional evidence for the diagnosis of functional sex change. Accordingly, the following approach using both histological and demographic criteria was developed to investigate the sexual ontogeny of *O. pullus*. First, the full size and age distribution were sampled over two complete seasonal cycles. Second,

the different sexual identities were identified through histological analysis. Third, the size and age distributions of females and males and of individuals displaying both male and female elements within the gonad were determined. Fourth, detailed histological analyses were performed to determine (1) the morphological characteristics associated with each sexual identity, (2) the histological basis of maturation patterns in each sex, (3) the histological and morphological characteristics associated with the presence of male and female elements within the same gonad and (4) to provide a basis for determining the size and age of female maturation. Finally, the seasonal pattern of sexual maturation was analysed to determine the periodicity of spawning and the distribution of transitional gonads relative to the time of spawning.

## MATERIALS AND METHODS

### SAMPLE COLLECTION

A total of 309 individuals of *O. pullus* were collected for gonad histology in the Hauraki Gulf of New Zealand (35.9–36.6° S; 174.7–175.9° E). Fish were sampled monthly over 2.5 consecutive years between August 2005 and January 2008. An additional 108 individuals (total of 427) were collected for size and age data.

Fish were sampled by spearing and processed within 1 h of collection. Fork length ( $L_F$ ), total mass ( $M_T$ ) and gutted mass ( $M_{GU}$ ) were recorded for each fish to the nearest mm and 10 g, respectively. The sagittal pair of otoliths was removed for the estimation of age, following successful validation of daily and annual increment formation and of the position of the first annual increment (Trip, 2009). Annual increments were counted under a stereodissector with transmitted light, and daily rings were counted under a high-power microscope at  $\times 100$  oil magnification with transmitted light. Ages enumerated in days ( $A_D$ ) were consequently converted into years ( $A_Y$ ) as follows:  $A_Y = A_D \cdot 365.25^{-1}$ . Gonads were removed and placed in FAAC, a formalin-based fixative (formaldehyde 4%, glacial acetic acid 5%, calcium chloride 1.3%; Pears *et al.* (2006)). Fixed gonads were transferred into 70% ethanol before histological processing. The additional 108 individuals sampled were sexed macroscopically. The error associated with macroscopic identification of sexual identity was assessed from the proportion of individuals for which the histological diagnosis of sexual identity did not coincide with that estimated from macroscopic examination of the gonads.

### GONAD HISTOLOGY

Gonads were blotted dry and weighed whole (gonad mass  $M_G$ , g). All gonad samples were embedded in paraffin wax, sectioned at 7  $\mu$ m, mounted on glass slides and stained with Gill's haematoxylin and eosin stain (H&E).

Gonad development was examined across the length of the gonad lobe and between gonad lobes. Two sub-sample procedures were as follows: (1) a sub-set of 39 gonad samples was serially sectioned in the anterior, medial and posterior regions of the tissue to examine the consistency of gonad development along the gonad length and (2) in a sub-sample of 10 gonads, both lobes were sectioned to examine the consistency of reproductive diagnosis between gonad lobes analysed. Analysis of the sub-sampled histological sections showed no variation in the diagnosis of sexual identity or reproductive status among gonad regions sampled and between lobes sectioned. Consequently, transverse sectioning of the medial region of one randomly chosen gonad lobe provided representative information for the diagnosis of reproductive developmental stage and sexual identity of each gonad.

All histological sections were examined under a high-power microscope with transmitted light. For each section, sexual identity, reproductive activity and spawning history were assessed. Examination of the slides was blind with respect to fish identity, age or size. Stages of oocyte development were classified by the latest non-atretic stage present in the

tissue (West, 1990). Oocyte atresia was identified following criteria presented in Hunter & Macewicz (1985), and the presence of  $\alpha$ -stage atretic vitellogenic oocytes and of brown bodies was recorded. The latest and most abundant spermatogenic stage present was used to identify the developmental stage of each testis (Grier, 1981). Gonads were classified as one of the following four categories: (1) oogenic tissue present throughout the section (female), (2) spermatogenic tissue observed throughout the section (male), (3) concomitant presence of primary oocytes and spermatogenic cysts and no signs of prior spawning as a female (developing primary males or late-transitional secondary males) and (4) concomitant presence of primary oocytes and developing spermatogenic tissue and signs of prior female function (atretic or degenerating vitellogenic, or later stages, oocytes) (transitional individuals, *i.e.* secondary males) (Sadovy & Shapiro, 1987; Sadovy de Mitcheson & Liu, 2008). The presence of intra-lamellar muscle bundles, atretic vitellogenic oocytes and postovulatory follicles in ovaries was recorded, and their incidence across months was established to examine their relationship to the timing of postspawning events. When appropriate, these criteria were used in the diagnosis of female-spawning history (Liu & Sadovy, 2004). Criteria used in the diagnosis of gonad sexual identity, reproductive activity, developmental stage and spawning history are summarized in Table I.

## DIAGNOSIS OF SEX CHANGE

The putative presence of protogynous sex change in *O. pullus* was assessed using criteria developed in Sadovy & Shapiro (1987) and Sadovy de Mitcheson & Liu (2008). First, transitional individuals were identified histologically based on the presence of atretic (or degenerating) vitellogenic (or later stage) oocytes (AVO). The staining properties of degenerating stage V oocytes (dO5) found in the lumen of transitional gonads were compared to that found in spawning, postspawning and mature resting females. These sections were unstained for H&E and re-stained for the identification of glycoproteins, a primary constituent of yolk, using periodic acid Schiff (PAS) and haematoxylin (counterstain) (Wallace & Selman, 1981). Second, the organizational microstructure of juvenile gonads was examined (Liu & Sadovy de Mitcheson, 2009), and testis microstructure was compared to that of ovaries (Shapiro & Rasotto, 1993). Third, male gonads were examined for: (1) the position of sperm sinuses (central duct situated dorsally *v.* peripheral ducts) and (2) the presence of a membrane-lined lumen (indicative of ex-ovarian lumen) and lamellar structure. Finally, size and age of individuals displaying simultaneous presence of oogenic and spermatogenic tissue including transitional individuals were plotted against those of immature individuals and size and age-at-female maturity. Size and age-frequency distributions of males and females were also established as a proportion of the total number of individuals collected.

## REPRODUCTIVE SEASONALITY AND TIMING OF SEXUAL MATURITY AND SEX CHANGE

Reproductive seasonality was assessed by examining variation in the proportion of ovary mass relative to body mass in mature females using a gonado-somatic index ( $I_G$ ). This approach estimates the developmental stage of the oocytes and indicates proximity to spawning (Morgan *et al.*, 2010). The formula for calculating  $I_G$  was not used in the sense of providing a measure of gonad mass that is independent of body mass but rather to display the proportional investment in reproductive *v.* somatic tissue or relative gonad mass ( $R_{MG}$ ), which was estimated as follows:  $R_{MG} = 100M_G M_{GU}^{-1}$ .  $R_{MG}$  was plotted with mean monthly sea surface temperature recorded *in situ* at the Leigh Marine Laboratory in the Hauraki Gulf across both years sampled. As the use of ratios in controlling for body size has been questioned (de Vlaming *et al.*, 1982; Packard & Boardman, 1988, 1999; Raubenheimer, 1995; Ebert *et al.*, 2010), the issues with respect to the use of gonad indices were considered.

Timing and duration of the spawning season were assessed over a two-year cycle of monthly sampling (from August 2005 to January 2008), using  $R_{MG}$ . The timing of the formation of intra-lamellar muscle bundles (ILMB), postovulatory follicles (POF) and AVO was also examined.

TABLE I. Description of histological features used in the diagnosis of sexual development in *Odax pullus*, including gonad sexual identity, reproductive activity and spawning history

Gonad tissue	Sexual identity	Reproductive activity	Most advanced gamete stage present	Spawning history
Oogenic tissue only	Female	Inactive	Pre-vitellogenic oocytes: chromatin nucleolar (stage I) or peri-nucleolar (stage II) oocytes	No AVOs: thin ovary wall, small cross-sectional area → immature; spawning history could not be diagnosed → undetermined AVOs present: ILMBs and BBs and thick ovary wall may be present → mature Mature
		Ripening 1 Ripening 2 Spawning	Cortical alveoli (stage III) oocytes Vitellogenic (stage IV) oocytes Hydrated oocytes (stage V), POFs, BBs and ILMBs may be present	
		Postspawning	AVOs present, BBs, degenerating hydrated oocytes (stage V) in ovarian lumen, all oocyte stages present	
Both oogenic and spermatogenic tissues present	Transitional	Inactive	Peri-nucleolar oocytes (stage II), spermatogenic crypts developing (spermatocyte is the most advanced stage present), degenerating vitellogenic (or later stage) oocytes, prominent central membrane-lined lumen	Signs of prior spawning as a functional female: AVOs, POFs

TABLE I. Continued

Gonad tissue	Sexual identity	Reproductive activity	Most advanced gamete stage present	Spawning history
	Male (late-transitional)	Inactive	Peri-nucleolar stage oocytes (stage II), spermatogenic crypts (spermatogonia and spermatocytes most abundant), central lumen, sperm sinuses may be present	No signs of prior spawning as functional female (no AVOs, no POFs)
		Active	Peri-nucleolar stage oocytes (stage II), all stages of spermatogenesis, spermatozoa abundant, sperm sinuses developed filling with spermatozoa, central lumen may be present	
Spermatogenic tissue only	Male	Inactive	Spermatogonia crypts abundant, spermatocytes and spermatides may be present, sperm sinuses empty	Remnant of central lumen may be present
		Ripening	Spermatides and spermatozoa most abundant, sperm sinuses filling with spermatozoa	
		Spawning	Spermatozoa most abundant, sperm sinuses packed with spermatozoa	

AVO, atretic vitellogenic oocyte; BB, brown body; ILMB, intra-lamellar muscle bundle; POF, postovulatory follicle.

The  $L_F$  and age at maturity were estimated using females collected during the spawning season (Pears *et al.*, 2006). The  $L_F$  and age at which 50% of females were mature (proportion of mature females relative to the total number of females within each  $L_F$  or age class) were calculated following Williams *et al.* (2008) and Trip (2009). For each female, information used was  $L_F$  (in mm, 50 mm increments), age (in years, 1 year increments) and maturity (immature or mature). The best-fit logistic model was estimated by minimizing the negative  $\log_{10}$  of the likelihood based on a probability density function with a binomial distribution (Haddon, 2001). The 95% c.i. were estimated using a bootstrapping procedure (Moore *et al.*, 2007) and calculated using the 2.5 and 97.5 percentiles of the bootstrap estimates (Haddon, 2001).

The  $L_F$  and age-at-sex change was estimated by calculating mean  $L_F$  and age of transitional individuals.

## RESULTS

### SEX-SPECIFIC SIZE AND AGE

Size and age information were collected for 324 females and 91 males of *O. pullus* in the Hauraki Gulf, New Zealand. The error associated with macroscopic identification of sexual identity was calculated as 0.8% and considered negligible. The largest individual sampled was a male (531 mm  $L_F$ , 6 years old), while the oldest individual collected was a female (469 mm  $L_F$ , 11 years old). Females were distributed across the  $L_F$  and age ranges, from 87 to 499 mm and from 0.32 to 11 years. In contrast, males ranged from 367 to 531 mm  $L_F$  and from 3 to 9 years of age. Size and age of males overlapped with that of larger and older females. Males were absent, however, from the smaller and younger size and age classes, suggesting the presence of a differential pattern of recruitment between the sexes (Fig. 1).

### GONAD HISTOLOGY

A total of 250 gonad sections showed exclusively ovarian tissue (females), 51 sections displayed exclusively testicular tissue (males) and eight of the sections revealed the concomitant presence of spermatogenic and oogenic tissue. Three scenarios were considered: (1) males matured directly from an immature juvenile stage

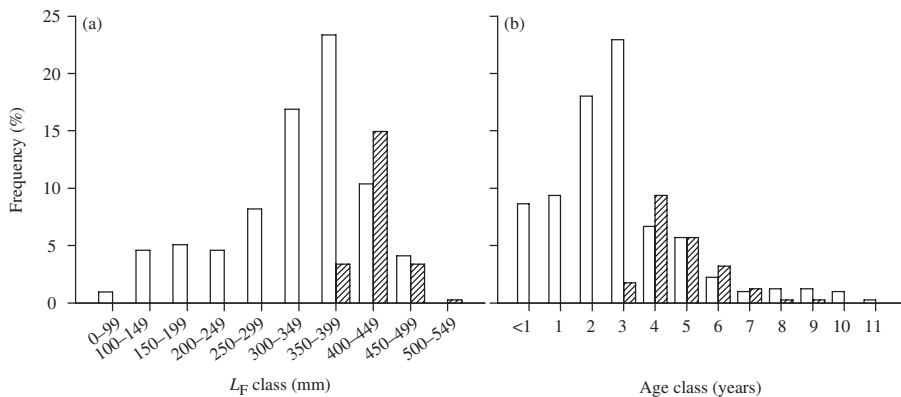


FIG. 1. (a) Fork length ( $L_F$ ) ( $n = 415$ ) and (b) age-frequency distribution ( $n = 405$ ) of male (▨) and female (□) *Odax pullus* in the Hauraki Gulf. Frequency (%) is relative to total number of individuals sampled.

(gonochorism or primary male development), (2) males matured from functional females (secondary male development) and (3) a combination of these, with both primary and secondary male development (diandric protogyny).

### *Females*

All transverse gonad sections of individuals <359 mm  $L_F$  and aged  $\leq 1$  years displayed exclusively ovarian tissue, and there was no evidence of spermatogenic cysts in those sections. Thirty-six per cent of females examined were diagnosed as immature (*i.e.* never spawned). Immature females ranged from 90 to 385 mm  $L_F$  and 0.3 to 3 years old [Fig. 2(a)]. Ovarian lamellae of immature females were packed with pre-vitellogenic oocytes (chromatin nucleolar and peri-nucleolar stages) [Fig. 3(a)]. Fifty-seven point six per cent of females were mature (*i.e.* spawning or showing signs of previous spawning episodes) [Fig. 3(b)–(e)]. Mature females ranged in body size from 215 to 499 mm  $L_F$  and from 1 to 10 years of age [Fig. 2(b)]. Spawning history of 6.4% of females (*i.e.* 16 females of the 250 individuals showing exclusively ovarian tissue) could not be reliably identified (undetermined inactive females); these females were sampled outside the spawning season and ranged from 288 to 426 mm  $L_F$  and from 1 to 5 years old.

All transverse sections of ovaries showed a lamellar structure [Fig. 3(a)–(e)]. Lamellae were tightly packed in immature and mature inactive females and filled the ovarian lumen in spawning females. Nests of germ cells and early meiotic primordial cells were commonly observed within the lamellar membrane of inactive ovaries. The ovarian lumen was lined by a membrane-like layer and was filled with stage V (hydrated stage) oocytes (O5) in spawning females and dO5 in postspawning and some mature resting females, suggesting that the ovarian lumen is used as an egress for oocytes during spawning. Pre-vitellogenic oocytes were present in ovaries at all stages of ovarian maturation. All stages of oocyte growth were present in ovaries of active spawning females, suggesting the presence of multiple spawning events for each active female during the spawning months (batch spawning).

### *Males*

The smallest individual found to display exclusively spermatogenic tissue was 376 mm  $L_F$  and the youngest was 3 years old [Fig. 2(d)]. Thirty-one per cent of testes showed a membrane-lined central lumen filled with connective tissue and brown bodies [Fig. 3(g)]; these individuals ranged from 393 to 455 mm  $L_F$  and from 3 to 6 years old. Testes were tightly packed with spermatogenic tissue and presented a lobular structure [Fig. 3(f)–(h)]. All testes were externally lined with a thick muscular wall. Sperm ducts were consistently distributed at the periphery of the testicular tissue in both inactive [Fig. 3(f)] and active males [Fig. 3(h)], and no dorsal sperm ducts were found. Sperm sinuses were filled with mature sperm in active males, suggesting that peripheral sperm sinuses are used for the egress of sperm during spawning [Fig. 3(h)].

### *Individuals with concomitant male and female gonad elements and transitional individuals*

Eight transverse gonad sections revealed the concomitant presence of spermatogenic and oogenic tissue. The dO5 were identified within the central lumen of two

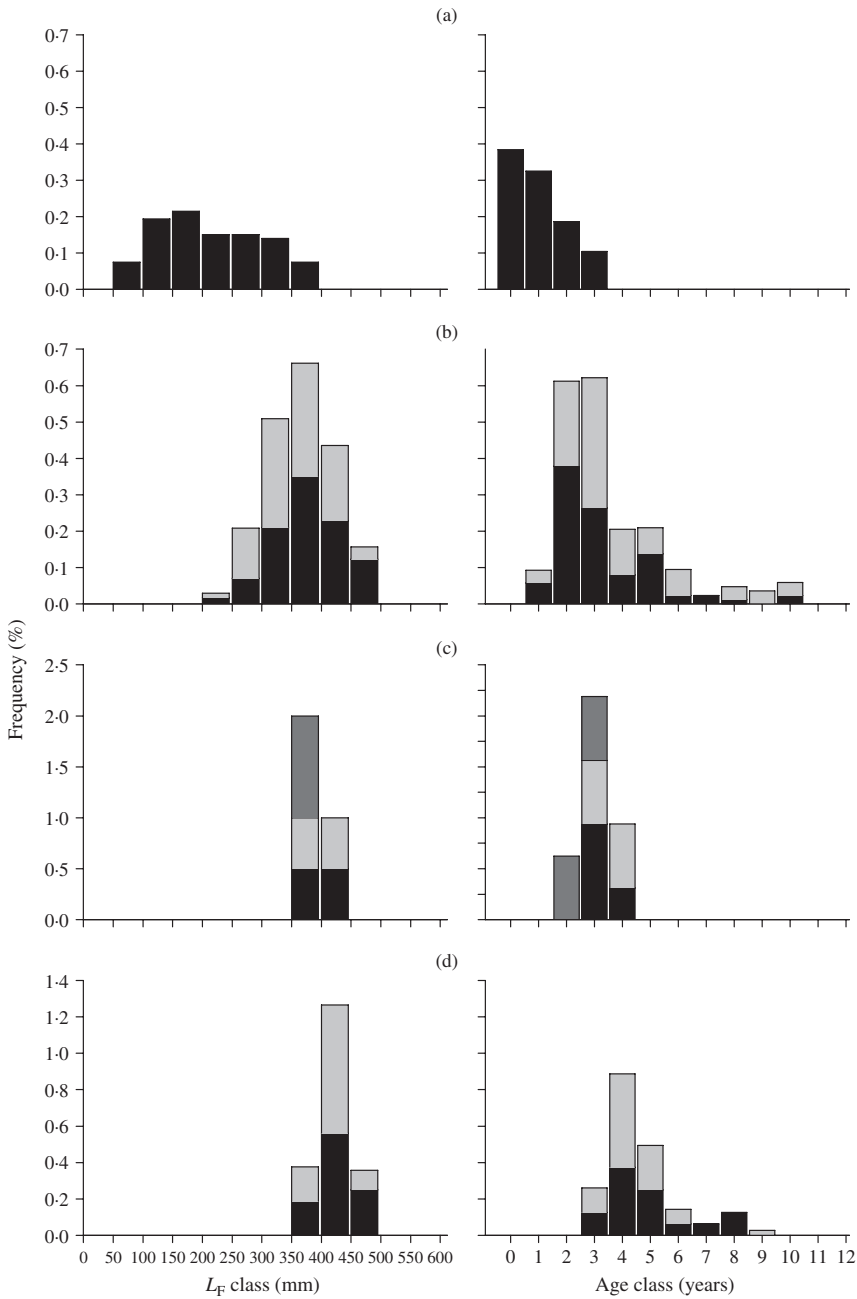


FIG. 2. Relative fork length ( $L_F$ ) size and age-frequency distributions of (a) immature females ( $n = 90$ ), (b) mature inactive (■) ( $n = 57$ ) and active (ripening, spawning or postspawning) (▒) ( $n = 87$ ) females, (c) individuals displaying concomitant oogenic and spermatogenic cysts [inactive males,  $n = 4$  (■), active males,  $n = 2$  (▒)] and individuals undergoing functional female-to-male sex reversal (transitional) (▓) ( $n = 2$ ) and (d) males [inactive males,  $n = 14$  (■) and active males,  $n = 37$  (▒)] of *Odax pullus* in the Hauraki Gulf. Frequency is relative to the total number of individuals sampled within each developmental stage. Note differences in scale along y-axis.

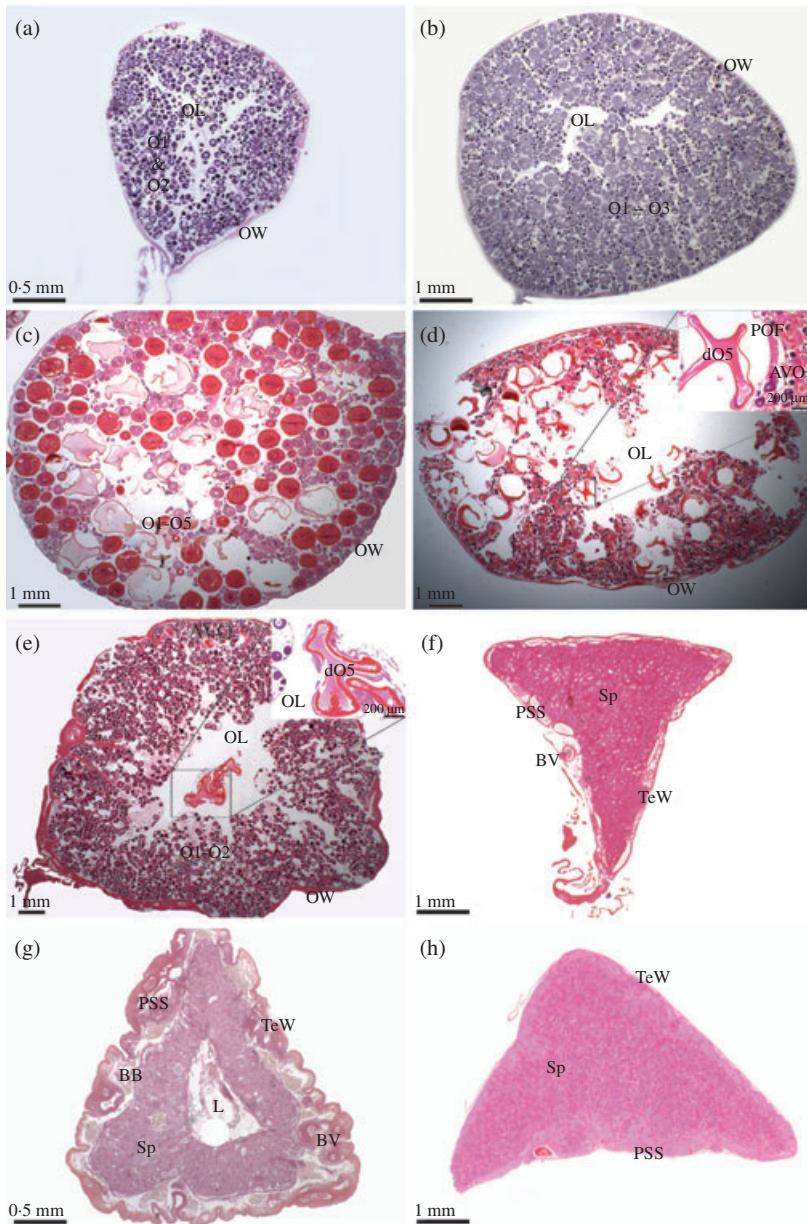


FIG. 3. Photomicrographs of transverse histological sections of (a)–(e) ovaries and (f)–(h) testes of female and male *Odax pullus* from the Hauraki Gulf at different stages of development: (a) immature female, (b) mature ripening female, (c) mature spawning female, (d) mature postspawning female, (e) mature inactive (resting) female, (f) inactive male, (g) active male showing remnant central lumen and (h) active (spawning) male. AVO, atretic vitellogenic oocyte; BB, brown bodies; BV, blood vessel; dO5, degenerating hydrated (stage V) oocyte; ILMB, intra-lamellar muscle bundle; L, central lumen; O1, chromatin nucleolar (stage I) oocyte; O2, peri-nucleolar (stage II) oocyte; O3, cortical alveoli (stage III) oocytes; O4, vitellogenic oocyte; O5, hydrated oocyte; OL, ovarian lumen; OW, ovarian wall; POF, postovulatory follicle; PSS, peripheral sperm sinus; Sp, spermatogenic tissue; TeW, testis wall. Sections were stained with haematoxylin and eosin.

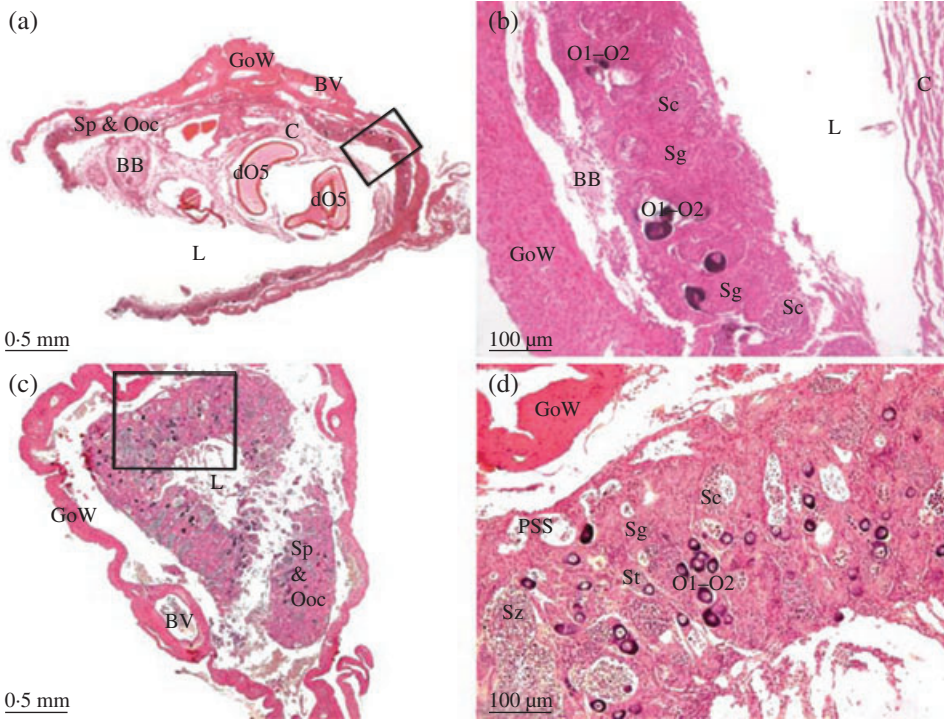


FIG. 4. Gonads of *Odax pullus* from the Hauraki Gulf displaying concomitant male and female gonad elements. Photomicrographs of transverse histological sections of: (a), (b) a transitional individual undergoing functional female-to-male sex reversal and (c), (d) a functional male following sex reversal (late-transitional). C, connective tissue; GoW, gonad wall; Ooc, oogenic tissue; Sc, spermatocyte; Sg, spermatogonia; St, spermatid; Sz, spermatozoa (see Fig. 3). Sections were stained with haematoxylin and eosin.

individuals that displayed primary oocyte growth within developing spermatogenic cysts [Fig. 4(a), (b)]. Following histological validation of the dO5, these two individuals were diagnosed as undergoing functional sex reversal (*i.e.* transitional individuals). Transitional individuals were collected in October and November, displayed  $L_F$  of 359 and 379 mm and were aged 2 and 3 years [Fig. 2(c)]. In the remaining six sections, no signs of prior female function were found. Oocytes observed within the developing spermatogenic cysts were pre-vitellogenic oocytes including chromatin nucleolar (stage I) and peri-nucleolar (stage II). The most advanced stage of spermatogenesis present varied across individuals; however, spermatogonia and spermatocytes were the most abundant stages. Peripheral sperm sinuses were absent or developing, and a prominent central membrane-lined lumen containing abundant connective tissue and brown bodies was observed in the majority of these sections. These individuals were identified as either inactive (sperm sinuses not developed, spermatocytes as most abundant stage) or active males (sperm sinuses developed and filled with spermatozoa, spermatozoa as most abundant stage) [Fig. 4(c), (d)]. Their size and age ranged from 367 to 418 mm  $L_F$  (mean size of 396 mm  $L_F$ ) and from 3 to 4 years old (mean age of 3.3 years) [Fig. 2(c)].

The central lumen of the two transitional individuals identified displayed distinct dO5-like structures [Fig. 4(a)]. Similar structures were found in the ovarian lumen of mature resting females [Fig. 3(e)] and resembled the structure of hydrated oocytes and degenerating hydrated oocytes observed in the ovarian lumen of spawning and postspawning females [Fig. 3(c), (d)]. The cytoplasm and envelope of the dO5-like structures found in transitional individuals [Fig. 5(a), (b)] and mature resting females [Fig. 5(c), (d)] and of the O5 and dO5 found in spawning [Fig. 5(e), (f)] and postspawning females [Fig. 5(g), (h)] stained positively with PAS. These results indicated the presence of glycoprotein elements, which are a primary component of the female-specific yolk-precursor vitellogenin and are characteristic of the chorion (zona pellucida) that develops specifically in vitellogenic oocytes (McMillan, 2007). These results suggest that the structures found in the lumen of the transitional individuals are related to female postspawning events and hence to prior female function, indicating the presence of functional protogynous sex change in *O. pullus* in the Hauraki Gulf.

Examination of the  $L_F$  and age of individuals showing concomitant oogenic and spermatogenic tissue and no signs of prior spawning as a female showed that: (1) these individuals were absent from the smaller  $L_F$  and younger age classes, (2) they occurred in  $L_F$  and age classes where immature females were absent and (3) they overlapped in  $L_F$  and age with that of transitional individuals and of the smallest males present (Fig. 6), indicating that the presence of concomitant male and female gonad elements in these individuals was likely to be related to sexual transition (*i.e.* late-transitional individuals). Examination of the  $L_F$  and age of transitional individuals in relation to that of immature females, mature females and males showed that (1) transitional individuals occurred after the  $L_F$  and age at which 50% of females were sexually mature and (2) that  $L_F$  and age of transitional individuals overlapped with that of the smallest males sampled (Fig. 6).

#### REPRODUCTIVE SEASONALITY AND TIMING OF SEXUAL MATURITY AND SEX CHANGE

The  $R_{MG}$  of mature females increased between July and November and peaked in August in both years examined, suggesting that spawning of *O. pullus* in the Hauraki Gulf took place during the austral winter and spring months from July to November, peaking in August. This timing coincided with that of the coldest mean sea surface temperatures (Fig. 7).

Vitellogenic oocyte growth started in March with the appearance of cortical alveoli stage oocytes (stage III) [Fig. 8(a)]. Vitellogenesis was initiated at the start of the spawning season in July, when oocytes of all mature females had reached the vitellogenic (stage IV) or hydrated (stage V) stages. From September to November, a gradually increasing percentage of mature females showed receding ovaries, with an increase in the frequency of ovaries showing postovulatory follicles, atretic vitellogenic oocytes and intra-lamellar muscle bundles [Fig. 8(b), (c)].

Not all sexually mature females participated equally over the duration of the spawning season. Twenty-three per cent of active females collected during the spawning months showed receding ovaries with atretic vitellogenic oocytes between September and November, suggesting that some females ceased spawning after peak spawning activity in August. Females showing receding ovaries after peak spawning

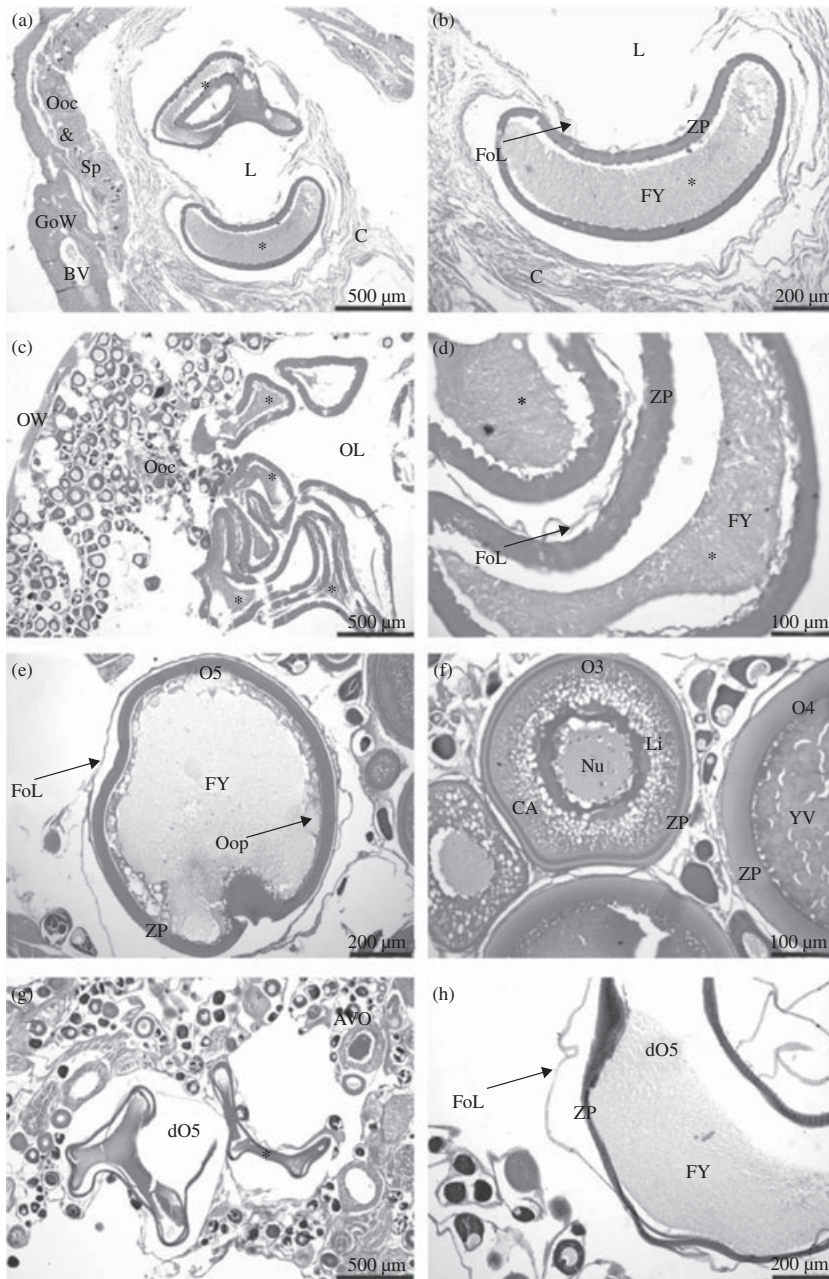


FIG. 5. Histological identification of structures found in the central lumen of transitional *Odax pullus* individuals and mature resting females (\*) from the Hauraki Gulf. Photomicrographs of transverse gonad sections of: (a), (b) a transitional individual; (c), (d) a mature resting female; (e), (f) a spawning female showing vitellogenic and hydrated oocytes and (g), (h) a postspawning female showing degenerating hydrated oocytes and atretic vitellogenic oocytes. CA, cortical alveoli vesicle; FoL, follicle layer; FY, fluid yolk; Li, Lipid vacuoles; Nu, nucleus; Oop, ooplasm; YV, yolk vacuole; ZP, zona pellucida (chorion) (see Figs 3 and 4). Sections were stained with periodic acid Schiff (PAS) and haematoxylin.

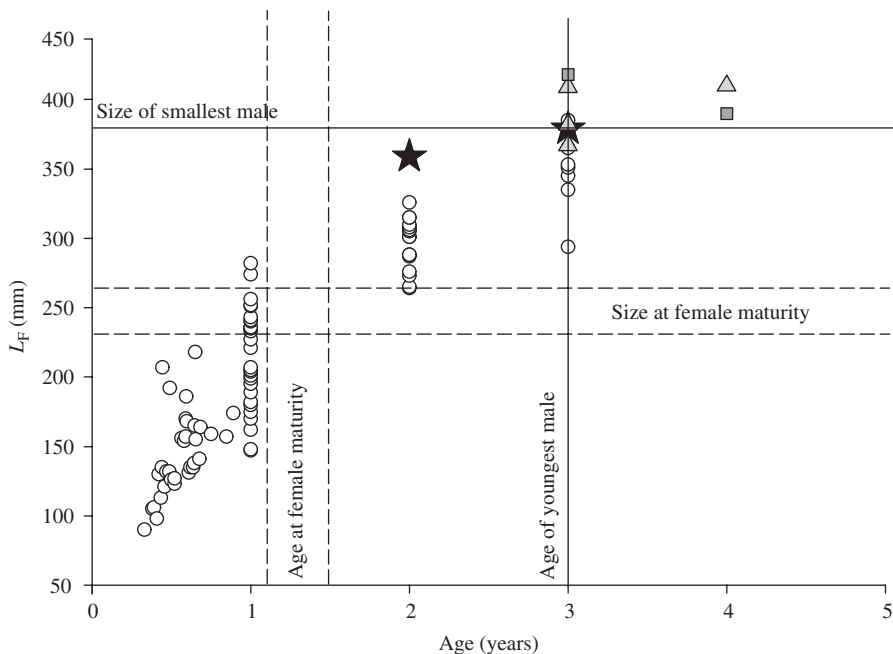


FIG. 6. Relationship between fork length ( $L_T$ ), age, gonad morphology and reproductive function in *Odax pullus* from the Hauraki Gulf. The  $L_F$  and age of immature females ( $n = 90$ ) (○) are compared to that of individuals with both male and female gonad elements, including transitional ( $n = 2$ ) (★) and late-transitional inactive males (△) and active males (■) ( $n = 6$ ), and to the timing of female sexual maturity (228.7–264.8 mm  $L_F$  and 1.1–1.5 years) and of sex reversal (359–379 mm  $L_F$  and 2–3 years). The 95% C.I. of estimated  $L_F$  and age-at-50% female maturity (‡) and  $L_F$  and age of the smallest male sampled (‡) are indicated.

were those displaying a relatively smaller mean  $\pm$  S.E.  $L_F$  ( $329.7 \pm 12.4$  mm) and younger mean  $\pm$  S.E. age ( $2.4 \pm 0.2$  years) as compared with that of females that continued spawning ( $416.2 \pm 10.1$  mm and  $4.6 \pm 0.6$  years), suggesting that larger older females contributed to a comparatively longer period of time than smaller younger females over the length of the spawning season.

Transitional individuals were found in October and November, suggesting that sex reversal may start during the second half of the spawning season after peak spawning activity. Mean  $\pm$  S.E. age of transitional individuals ( $2.5 \pm 0.5$  years) coincided with that of females showing receding ovaries during the spawning months ( $2.4 \pm 0.2$  years). These results suggest that younger mature females may participate in spawning until peak spawning is achieved, after which some of these females may undergo ovary regression before changing sex.

Half the females were sexually mature at 240.7 (95% C.I. 228.7–264.8) mm  $L_F$  and 1.2 (95% C.I. 1.1–1.5) years old, and 95% of females were sexually mature at 297.9 (95% C.I. 280.3–317.2) mm  $L_F$ , and 1.9 (95% C.I. 1.6–2.2) years old (Fig. 9). The smallest mature female was 215 mm  $L_F$ , and the youngest mature female was 1 year old. In contrast, the smallest male recorded was 367 mm  $L_F$  and the youngest male was 3 years old (Fig. 2).

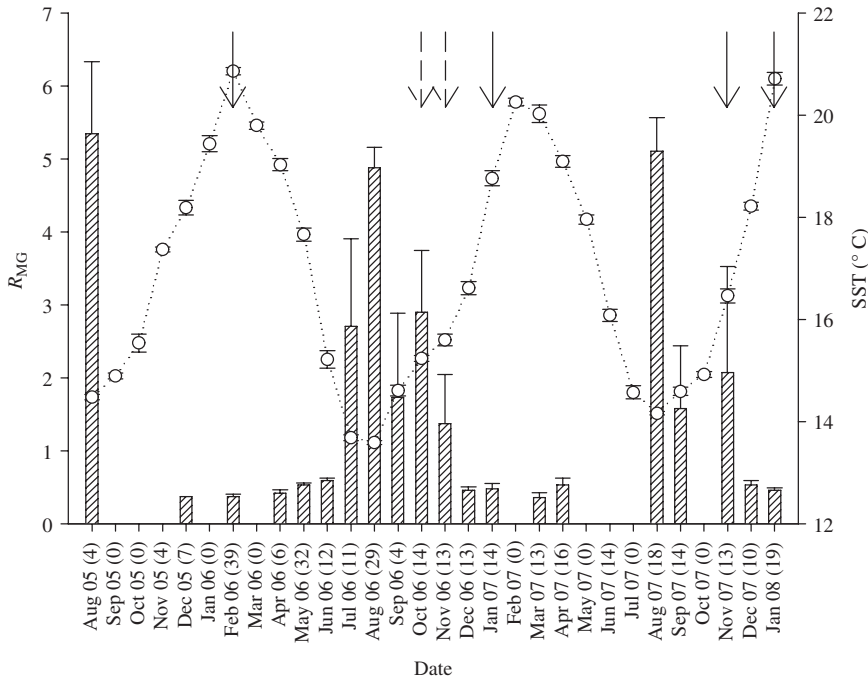


FIG. 7. Mean  $\pm$  S.E. monthly variation in relative gonad mass ( $R_{MG}$ ) of mature female *Odax pullus* from the Hauraki Gulf ( $n = 138$ ) and local mean  $\pm$  S.E. sea surface temperature (SST) recorded across months sampled (August 2005 to January 2008). Monthly sample sizes are presented in parentheses along the  $x$ -axis. Timing of female-to-male sexual transition: dates at which transitional individuals were found ( $\downarrow$ ) and dates at which individuals with concomitant male and female gonad elements (late transitionals) ( $\uparrow$ ) were found are indicated.

Mean  $\pm$  S.E. and age-at-sex change were estimated at  $369 \pm 10$  mm and  $2.5 \pm 0.5$  years ( $L_F$ ), which correspond to 82% of adult  $L_F$  and 23% of maximum age.

## DISCUSSION

The combined histological and demographic evidence strongly support a conclusion of monandric protogyny in *O. pullus*, indicating that all males of this species are the result of functional sex change of mature females (single pathway of male recruitment). A particular emphasis of this study was the histological analysis of juvenile gonad ontogeny, and all juvenile individuals were found to be immature females with no evidence of a juvenile bisexual phase (Hamilton *et al.*, 2008). Rather, all individuals go through an immature female phase before maturing as females. Individuals displaying both male and female gonad elements were all larger and older than the mean  $L_F$  and age-at-female sexual maturity. These included transitional, with an average  $L_F$  and age of 369 mm and 2.5 years, and late-transitional individuals, with a mean  $L_F$  and age of 396 mm and 3.3 years, respectively. Oogenic elements suggesting recent female maturation were identified in the lumen of transitional individuals, providing histological evidence of functional female-to-male sex reversal in this species.

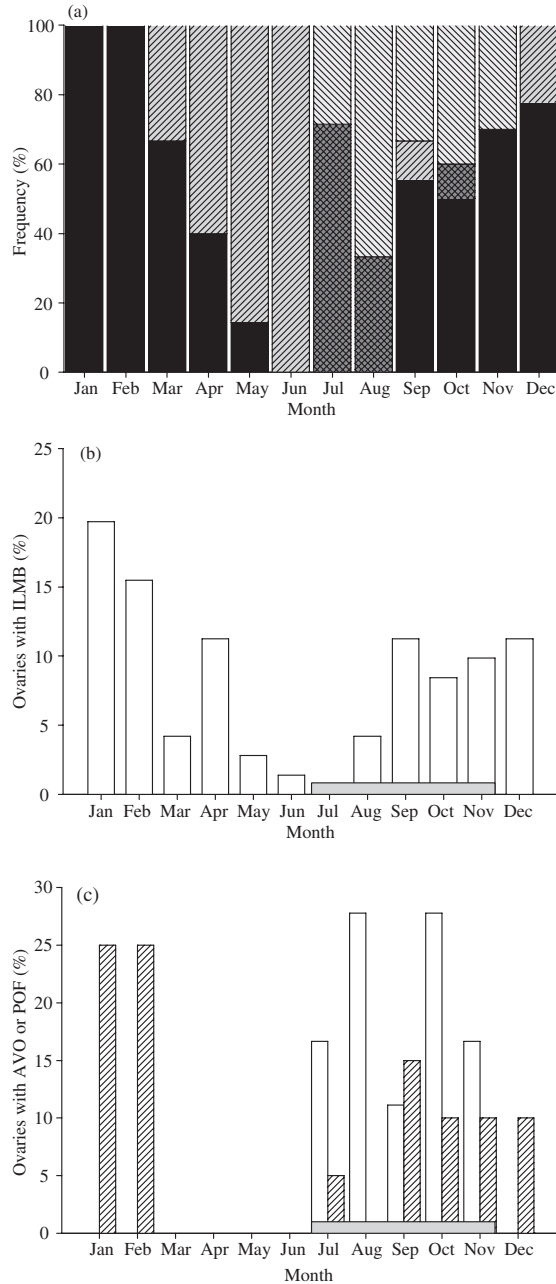


FIG. 8. Monthly progression of (a) oocyte development [based on the most advanced stage present; ■, stage II (peri-nucleolar); ▨, stage III (cortical alveoli); ▩, stage IV (vitellogenic); ▤, stage V (hydrated)] in mature ovaries ( $n = 144$ ) and (b), (c) of signs of spawning in mature female *Odax pullus* from the Hauraki Gulf: (b) intra-lamellar muscle bundles (ILMB) ( $n = 71$ ) and (c) postovulatory follicles (POF) ( $n = 18$ ) (□) and atretic vitellogenic oocytes (AVO) ( $n = 20$ ) (▨). y-Axes: (a) per cent of maturation stages across months and (b), (c) per cent of mature ovaries with ILMB, POF or AVO across months. Horizontal bars (▬) show timing and duration of spawning (as estimated from months with highest female gonado-somatic index).

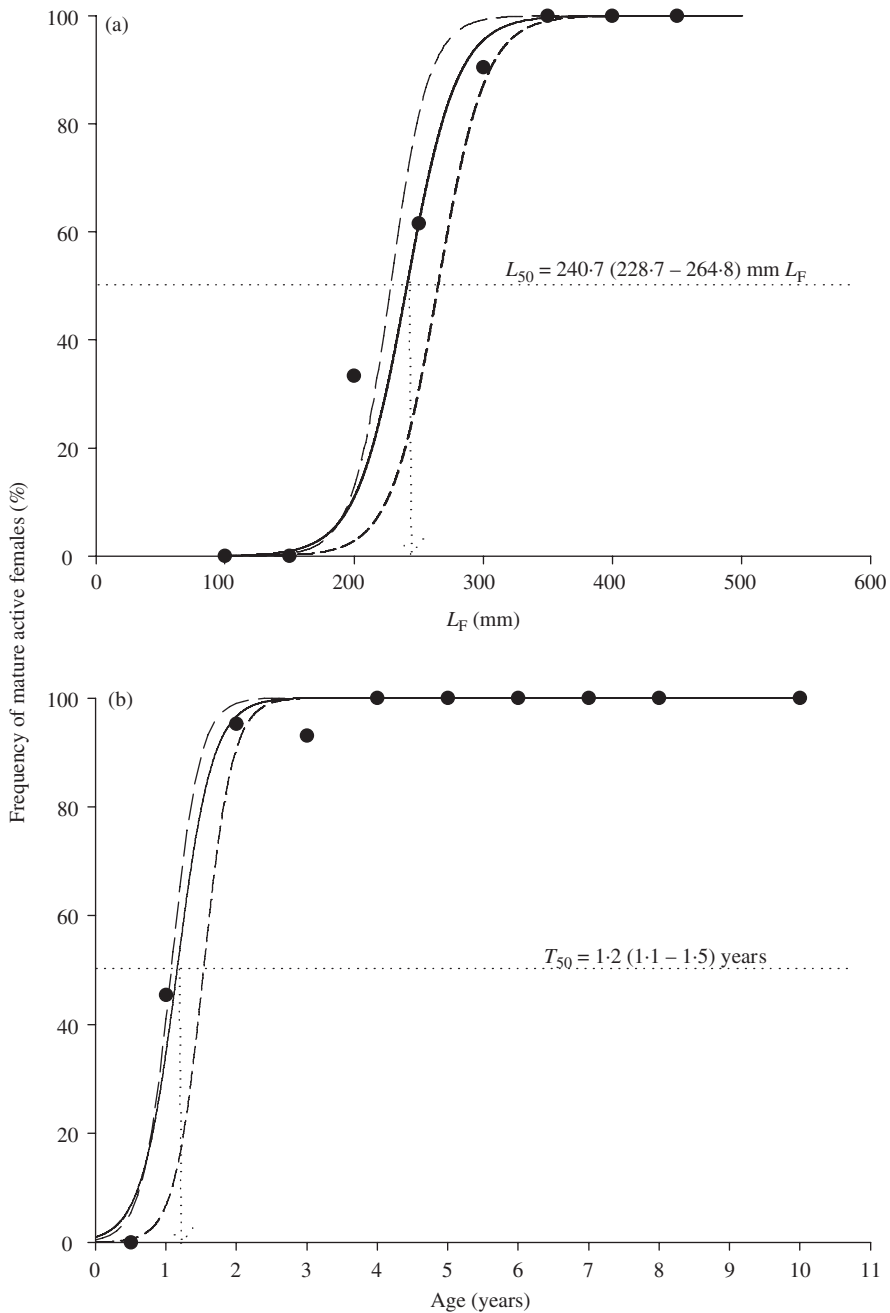


FIG. 9. (a) Fork length ( $L_F$ ) ( $n = 97$ ) and (b) age ( $n = 96$ ) at female maturity of *Odax pullus* in the Hauraki Gulf showing the frequency of mature active females collected during the spawning season (July to November inclusive). The logistic curves (continuous lines) are shown with 95% c.i. (---) and observed frequency data (●). The  $L_F$  ( $L_{50}$ ) and age ( $T_{50}$ ) (with 95% c.i.) at which 50% of females are sexually mature are indicated (.....).

In terms of gonad morphology, all testes of *O. pullus* displayed peripheral sperm sinuses that were used in the egress of spermatozoa. The testes of late-transitional males also showed a central membrane-lined lumen. The latter was absent, however, in older reproductively active males, and all testes showed a lobular structure as opposed to a lamellar structure that is characteristic of a prior ovarian form. Peripheral sperm sinuses, a membrane-lined central lumen and a lamellar structure are characteristic traits of the secondary development of a testis (Sadovy & Shapiro, 1987), and these traits are displayed by a majority of protogynous and gonochoristic (in the case of pre-maturational sex change) labrids (Sadovy de Mitcheson & Liu, 2008). In *O. pullus* males, the distribution of testis morphology with age and the presence of peripheral sperm sinuses argue strongly for secondary testis morphology, suggesting that all males are likely to have passed through a functional female phase. In contrast, the absence of both a central lumen and of an associated lamellar structure in older reproductively active *O. pullus* males suggests that there may be some elements of the structure of the testis of odacine fishes that are specific to this lineage within the labrid clade.

Examination of the age distribution of males and of individuals with transitional gonads in relation to that of female maturation further provides strong support for a diagnosis of functional protogyny. Sex change was estimated to occur in *O. pullus* at mean  $\pm$  S.E. of  $2.5 \pm 0.5$  years, while female maturity occurred at 1.2 years. If males are not recruited into the reproductive population until an age which is on average 1 to 2 years greater than that of female maturation, it appears unlikely that, in evolutionary terms, males would forego 1 to 2 years of reproductive activity as females before contributing to future generations. In addition, examination of the seasonal pattern of reproductive activity suggests that sexual transition, with its inevitable disruption of female reproduction, occurs over the months immediately following peak spawning activity, indicating that sex reversal has been displaced to a post-reproductive period that will not disrupt spawning.

There was a seasonal pattern of proportional somatic investment in female reproductive tissue, indicating that spawning of *O. pullus* occurs over the austral winter months (July to November). Reproductive activity is traditionally measured using  $I_G$  to control for differences in body size among individuals sampled, as it is generally assumed that  $I_G$  provides a measure of  $M_G$  that is independent of  $M_T$ . The use of ratios in controlling for body size has however, been questioned, and uncertainties have been raised as to the reliability of  $I_G$  (de Vlaming *et al.*, 1982; Packard & Boardman, 1999; Tomkins & Simmons, 2002; Ebert *et al.*, 2010; Morgan *et al.*, 2010). The primary issue underlying these uncertainties is that the assumption of both a linear and isometric (passes through the zero intercept) relationship between the numerator ( $M_G$ ) and denominator ( $M_T$ ) is rarely satisfied, thereby generating biased estimates of  $I_G$ , particularly in smaller sized individuals (Packard & Boardman, 1999). An alternative method is the use of ANCOVA to compare  $M_G$  across months by accounting for the effect of body size (covariate), provided that the slope of the relationship between  $M_G$  and  $M_T$  is consistent across groups examined. The slope of the relationship between  $M_G$  and  $M_T$  may, however, vary with oocyte development (de Vlaming *et al.*, 1982). Here, mean  $M_T$  of *O. pullus* individuals collected differed significantly across months sampled (ANOVA,  $F_{1,150} = 1.9$ ,  $P < 0.05$ ), and *post hoc* comparisons indicated no pattern of variation in mean  $M_{GU}$  across months

of sampling, *i.e.* that differences in mean  $M_T$  were randomly distributed across seasons over the 2.5 years of sampling. Moreover, transformation of  $M_G$  data failed to produce an isometric relationship, and the slope of the relationship between  $M_G$  and  $M_T$  of *O. pullus* differed between reproductively active and mature inactive females. The  $I_G$  was therefore used to generate an index of visual representation of the proportion of  $M_T$  assigned to reproductive growth. Although  $I_G$  may not be used in the usual sense of removing the effects of  $M_T$  from  $M_G$ ,  $I_G$  allows displaying the proportional investment in reproductive *v.* somatic tissue (oocyte development), described here as an index of relative gonad mass ( $R_{MG}$ ).

The preliminary investigation of the sexual ontogeny of odacine labrids suggests that this distinctive lineage, which is confined to temperate coastal environments, shares a protogynous mode of sexual development with the majority of other labrid taxa investigated to date. In particular, the odacines share a similar mode of sexual ontogeny with other members of the Hypsigenyine clade at the base of the labrid phylogeny. Examples of protogynous Hypsigenyine species include the large labrids of the genera *Semicossyphus* (Warner, 1975), *Lachnolaimus* (McBride & Johnson, 2007) and *Achoerodus* (Coulson *et al.*, 2009). In addition, labrids of the genus *Chorodon*, which forms the sister group to odacines within the Hypsigenyine clade, are also protogynous hermaphrodites (Fairclough, 2005). Further examination of the reproductive biology and sex-specific demography of odacine labrids should extend to include the closely related species residing in temperate Australian waters, *e.g.* *Heteroscarus acroptilus* (Richardson) and *Olisthops cyanomelas* Richardson.

In contrast, a number of other temperate labrids display a gonochoristic pattern of sexual development. This mode of sexual ontogeny is prevalent in labrine wrasses, including species of the genera *Centrolabrus*, *Symphodus* and *Tautoga* (Dipper & Pullin, 1979; Hostetter & Munroe, 1993; Raposeiro & Azevedo, 2009). These genera, however, are confined to the temperate waters of the northern hemisphere (Kazancıoğlu & Alonzo, 2010). The results of the present study and the prevalence of protogyny in temperate water Hypsigenyine labrids indicate that the presence of a protogynous sexual ontogeny in *O. pullus* is likely to be a lineage-specific characteristic and may not be constrained by environmental effects on the evolution of sex change in temperate regions (Sadovy de Mitcheson & Liu, 2008).

The mating system of *O. pullus*, and especially the role of older male individuals in the reproductive process, is currently unclear. Given the fact that *O. pullus* occupies a shallow, and frequently turbid and turbulent coastal environment (Meekan & Choat, 1997), examination of the behavioural aspects of the mating system will be difficult, although estimates of local movement patterns indicate high levels of male activity (Choat & Clements, 1993) and suggest the presence of a harem mating system typical of protogynous labrids. The most appropriate approach for future investigation of the mating system in this taxon appears to be a more detailed analysis of the relative demography of males and females, with a focus on male growth rates, size structure, longevity and investment in gonad tissue.

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