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Cover Page Footnote

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Reproductive Morphology of Male Black Perch (*Embiotoca jacksoni*)

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The surfperches (Embiotocidae) are comprised of 24 species, all but one species is marine and they range from central Baja California to Alaska (not including the Aleutian Islands) and Japan in the Pacific Ocean (Baltz 1984; Holbrook et al. 1997; Longo and Bernardi 2015). Embiotocids occupy various marine habitats including sandy bottoms, rocky reefs, kelp forests, and seagrass beds (Allen and Pondella 2006). The tule perch (*Hypsterocarpus traskii*) is freshwater and occupies lakes and rivers in central to northern California (Baltz and Moyle 1982; Wang 1986).

The surfperches are among approximately 500 fishes that exhibit internal fertilization and bear live young (Contreras-Balderas 2006). During copulation, male surfperches utilize external copulatory structures (e.g., genital papilla, intromittent organs) to transfer sperm into the female reproductive tract (Blake 1868; Hubbs 1917; Tarp 1952; Gardiner 1978). Sperm are transferred in a mass, which contains spermatozoa from multiple lobules (Gardiner 1978; Grier et al. 2005). The sperm mass of surfperches has been referred to as a spermatophore in previous literature (Shaw and Allen 1977; Gardiner 1978). It should be noted however, that the term spermatophore refers to a package of sperm which is encapsulated by an extracellular capsule (Ginzburg 1968; Wourms 1981). In embiotocids, the spermatozoa come together to form large aggregates called spermatozeugmata, which are not enclosed by a membrane (Ginzburg 1968; Wourms 1981; Grier et al. 2005). Spermatozeugmata are common in internally fertilizing teleosts (Grier et al. 1978; Downing and Burns 1995; Fishelson et al. 2007; Uribe et al. 2014).

Previous studies on black perch (*Embiotoca jacksoni*) have described multiple paternities of offspring by a given female, sperm competition, and population genetics (Reisser et al. 2009; Liu and Avise 2011; LaBrecque et al. 2014; Longo and Bernardi 2015; Johnson et al. 2016), but the basic reproductive biology of the black perch remains poorly understood. The black perch intromittent organs were first described by Agassiz (1853) who documented a smooth patch located on the anal fin with a surrounding sheath with a burrow to house the intromittent organ. The specific aims of our research note were to increase our understanding of the black perch reproductive biology. We have documented the progression of spermatocyte development and formation of the spermatozeugmata within the testicular tissue. Additionally, we have used histology to detail the association of the intromittent organs with the anal fin.

We used paraffin histological techniques to describe the black perch spermatocyte and intromittent organ morphology. Male black perch (111 ± 30 mm SL; 70–157 mm; n = 8) were collected from June to November 2014 in eel grass beds in Los Angeles, Orange, and San Diego Counties with at least two sites per location using a beach seine and hook and line methods. Fish were euthanized using an overdose of tricane methanesulfonate (MS-222) of a purity of at least 98% (1 g: 5,000 ml seawater). The testes and anal fin were dissected and preserved in Bouin's fixative and 10% neutral-buffered formalin, respectively, then transferred to 70% ethanol. Tissues were processed for paraffin histology by dehydration in a series of graded ethanols, cleared with xylene, and infiltrated and embedded with paraffin wax. Tissues were sectioned using a rotary microtome to a thickness of 5µm and stained with hematoxylin and eosin or Mallory

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*Corresponding author: kforsgren@fullerton.edu trichrome stains. Tissues were examined using brightfield microscopy (Olympus BX60). Photos were taken using a digital camera (QICAM QImaging Fast 1394) and imaging software (Q-Capture Pro 7, QImaging, 2010). Fish were handled according to an approved protocol that followed the policies and guidelines of the California State University, Fullerton's Institutional Animal Care and Use Committee.

Spermatocytes were staged according to previously published literature on embiotocids and other fishes (Gardiner 1978; Grier 1981; Schulz et al. 2010; Grier et al. 2005; Uribe et al. 2014). Black perch have an unrestricted lobular testis type (Uribe et al. 2005). Lobules are formed by sperm cells synchronously developing (i.e., at the same stage of spermatogenesis; Fig. 1). Interstitial tissue was observed between the lobules (Fig. 1). Primary spermatocytes had an enlarged nucleus and were held tightly together in a cyst (Fig. 1A), whereas the nuclei of secondary spermatocytes stained lighter than primary spermatocytes and their arrangement was not as rigid (Fig. 1B). Spermatids were characterized by a change in cell shape associated with the elongation of the nuclei and were smaller than spermatocytes (Fig. 1C). Spermatozoa are arranged into packets within the lumen of the lobule with sperm heads located at the perimeter and the tails (i.e., sperm flagella) pointed towards the center of the lumen (Fig. 1D). A dense aggregation of spermatozoa packets within the efferent ducts form a spermatozeugmata (Fig. 1E).

Examination of testicular micrographs of black perch ≥ 90 mm SL had testes that contained all stages of sperm development and spermatozeugmata within the lobules (Fig. 2A), indicating that they sexually mature and in a spawning capable/spawning reproductive phase (Brown-Peterson et al., 2011). Black perch < 90 mm SL did not have spermatozeugmata within the testicular tissues but all stages of spermatocyte development were present (Fig. 2B). These males were in a developing reproductive phase (Brown-Peterson et al., 2011).

The spermatozeugmata are presumable transferred into the female reproductive tract during copulation via one of the intromittent organs. Black perch intromittent organs are located on both sides of the anterior portion of the anal fin. Black perch < 90 mm SL did not have externally visible intromittent organs, however a smooth patch was observed on the anal fin (Fig. 3A). Wourms (1981) also notes that the intromittent organ is not present during the non-breeding season (i.e., winter), which we have also observed. In this case, the anal fin has no modification and looks like the fin of a black perch < 90 mm SL (pers. obs.). Black perch ≥ 90 mm SL had intromittent organs housed within a sheath located on both sides of the anterior portion of the anal fin (Fig. 3B). The intromittent organs of the fluffy sculpin (*Oligocottus snyderi;* Family Cottidae), a viviparous marine species, have been described as having a modified anal fin ray with the ability to bend in order to enter the female reproductive tract (Morris 1955). This is similar to the gonopodium of freshwater poeciliids, which also have a modified anal fin ray with specialized muscles that allow the gonopodium to rotate during copulation (Rosen and Gordon 1953). The anal fin of the male black perch does not appear to be modified and fin rays do not appear to be involved in sperm transfer. The intromittent organs form on a smooth area of the anal fin, parallel to the body (Fig. 3A). A fleshy sheath develops that houses the intromittent organ, which make up the bulbous protrusions on both sides of the anal fin that can be observed externally during breeding season (Fig. 3B).

Examination of the tissue of the anal fin of black perch < 90 mm SL that had not yet developed intromittent organs revealed that tissue of the presumptive intromittent organ was present (Fig. 4). Surrounding the intromittent organ tissue was loose and dense connective tissues (Fig. 4). Fin rays were observed but are not modified. It should be noted that although it appears that the anal fin rays are interrupted, the intromittent organ develops on the surface of the fin, thus fin rays can be observed in the surrounding tissue but are continuous. Histological examination of

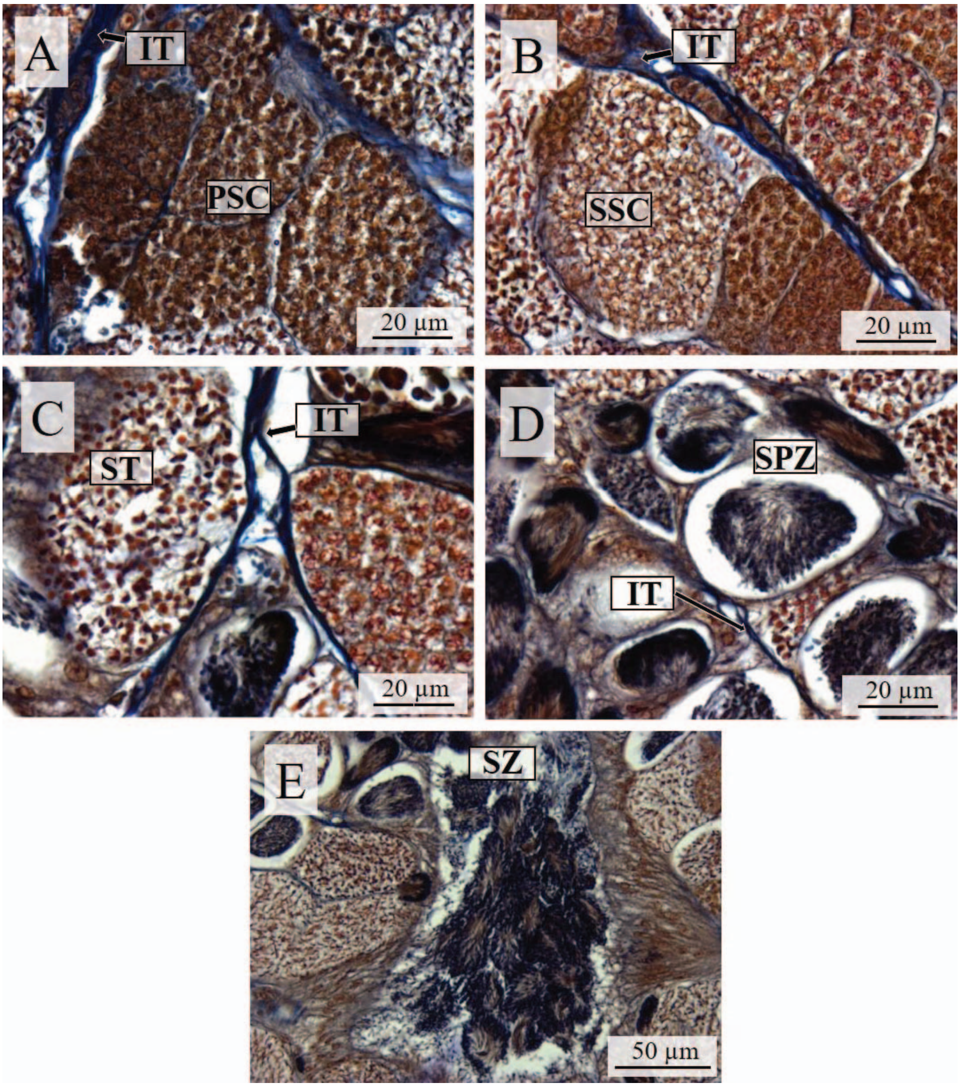

Fig. 1. Sperm cell developmental stages of black perch (*Embiotoca jacksoni*). A) Primary spermatocytes (40X); B) Secondary spermatocytes (40X); C) Spermatids (40X); D) Spermatozoa (40X); E) Spermatozeugmata (20X). All micrographs stained with Mallory trichrome. PSC = primary spermatocyte; SSC = secondary spermatocyte; ST = spermatid; SPZ = spermatozoa; SZ = spermatozeugmata; IT = interstitial tissue.

the intromittent organs of black perch ≥ 90 mm SL indicate they are surrounded by a protective sheath composed of smooth muscle (Fig. 5A). The outer layer of the sheath is composed of circular smooth muscle (Fig. 5A, B) with a layer of longitudinal smooth muscle (Fig. 5A, C). The smooth muscle layer is surrounded by an epithelial tissue (Fig. 5B). Dense fibrous connective tissue lies between the longitudinal smooth muscle and the intromittent organ (Fig. 5A, D). The intromittent organs are located on either side of a pair of fin rays (Fig. 5A, E). We hypothesize that the dense fibrous connective tissue surrounding the intromittent organs provides the structural

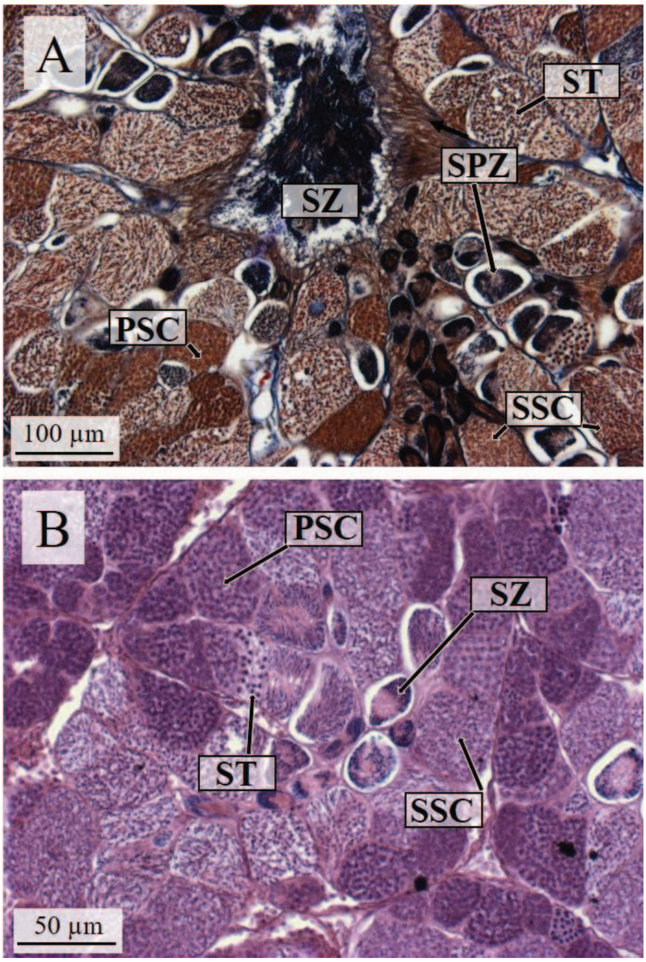

Fig. 2. Testicular micrographs of male black perch (*Embiotoca jacksoni)*. A) Testes from males ≥ 90 mm SL contained all stages of spermatocyte development, including spermatozeugmata (10X; Mallory trichrome stain); B) Testes from smaller < 90 mm SL contained all stages of spermatocyte development, but no spermatozeugmata (20X; hematoxylin and eosin). PSC = primary spermatocyte; SSC = secondary spermatocyte; ST = spermatid; SPZ = spermatozoa; SZ = spermatozeugmata.

support for the intromittent organ itself. Thus, smooth muscle contraction would act upon the intromittent organ forcing it out of the sheath.

The morphology of black perch intromittent organs is similar to the striped (*Embiotoca lateralis*) and pile perches (*Damalichthys vacca*; Blake 1868; pers. obs.) of the subfamily Embiotocinae. The shiner perch (subfamily Embiotocinae) have intromittent organs within a sheath similar to the black, striped, and pile perches and in addition have a single genital papilla (Fig. 6A; Wiebe 1968; Shaw 1971; Gardiner 1978). The barred (*Amphistichus argenteus)* and walleye *(Hyperprosopon argenteum*) surfperches (subfamily Amphistichinae) have been described as having only a genital papilla located on the ventral side of the body, anterior to the anal fin (Fig. 6B; Blake 1868; Carlisle et al. 1960; LaBrecque et al. 2014).

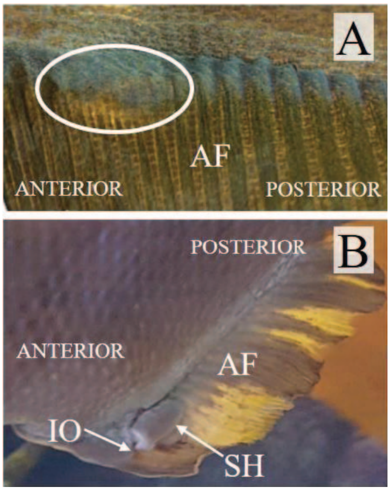

Fig. 3. Left, lateral view of the anal fin of male black perch (*Embiotoca jacksoni*). A) Anal fin of black perch < 90 mm SL; white circle represents location of developing intromittent organ and surrounding sheath; B) Anal fin of black perch ≥ 90 mm SL indicating the surrounding sheath that houses the intromittent organ. AF = anal fin; IO = intromittent organ; SH = sheath.

Our research note is the first to document the formation of black perch spermatozeugmata within the testis. Additionally, we have characterized the tissues of the intromittent organs and its muscular sheath which reside on an unmodified anal fin. We have identified several curious differences in male external reproductive structures between black perch and other embiotocids. We are conducting a more comprehensive study of the intromittent organs of the embiotocid family. We are also describing the functional anatomy of the female, particularly acceptance of the copulatory structure into the reproductive tract.

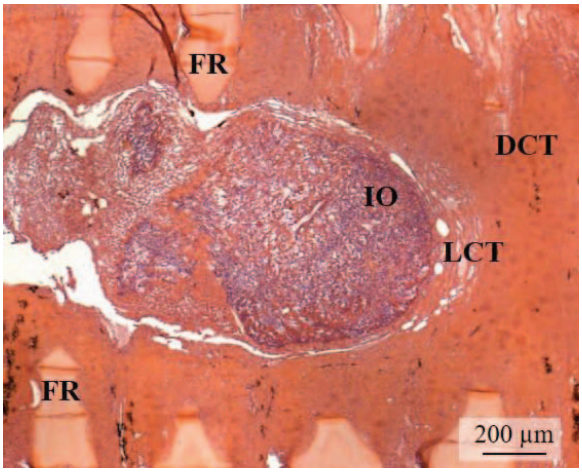

Fig. 4. Histological sagittal section of the anal fin of a black perch (*Embiotoca jacksoni*) < 90 mm SL stained with hematoxylin and eosin (4X). IO = intromittent organ; DCT = dense connective tissue; LCT = loose connective tissue; FR = fin ray.

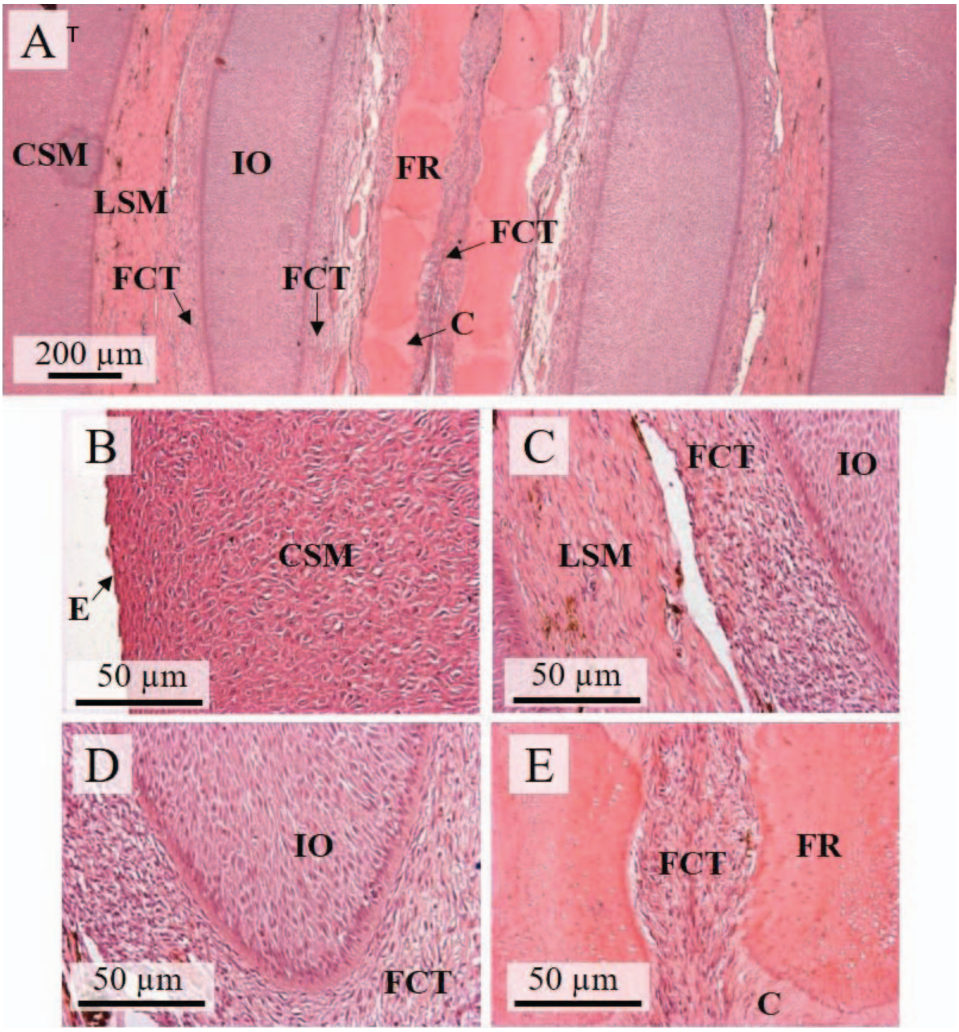

Fig. 5. Histological cross-section of the anal fin of a black perch (*Embiotoca jacksoni*) ≥ 90 mm SL stained with hematoxylin and eosin. A) Micrograph of the anal fin (4X); B) Circular smooth muscle (20X); C) Longitudinal smooth muscle and dense fibrous connective tissue (20X); D) Intromittent organ (20X); E) Fin rays and dense fibrous connective tissue (20X). CSM = circular smooth muscle; LSM = longitudinal smooth muscle; FCT = dense fibrous connective tissue; IO = intromittent organ; E = epithelium; FR = fin ray; C = cartilage.

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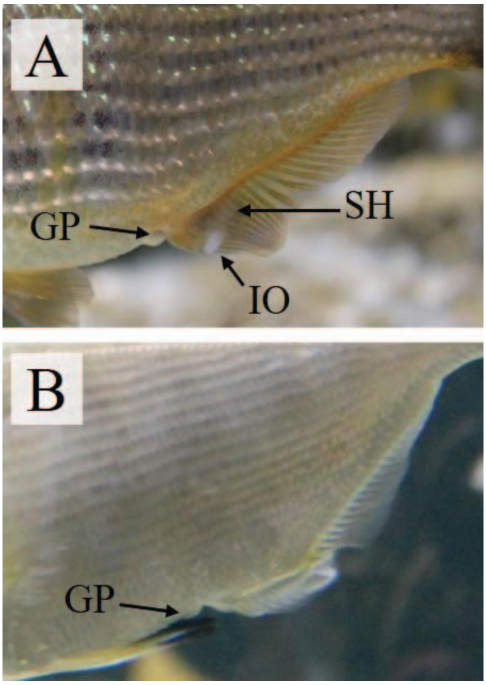

Fig. 6. Copulatory structures of two species of Embiotocids. A) Shiner perch (*Cymatogaster aggregata*) paired intromittent organs are housed within a surrounding sheath on the anterior portion of the anal fin and a single fleshy genital papilla located ventrally on the abdomen anterior to the anal fin (left side of body shown in figure); B) Walleye surfperch (*Hyperprosopon argenteum)* single genital papilla located ventrally on the abdomen anterior to the anal fin. IO = intromittent organ; SH = sheath; GP = genital papilla.

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