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# Testing a non-lethal method for determining the sex of California halibut, *Paralichthys californicus*, in non-spawning condition

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The California halibut, *Paralichthys californicus* (Ayres), is the target of an avid recreational fishery and a nearly US $2 million commercial fishery. It is one of the State of California's highest priorities for developing a fishery management plan (California Department of Fish and Game 2001). A 2011 stock assessment conservatively found the southern population (from Point Conception, California to the USA-Mexico border) to be depleted to about 14% of its unfished biomass (Maunder *et al.* 2011). However, this stock assessment lacked the sex-specific data required to be reliable (MacCall *et al.* 2011). Sex-specific data are necessary because of the vastly different life-history parameters for female and male *P. californicus*. Most notably, females grow faster, are caught more frequently and mature significantly later than males (Maunder *et al.* 2011). The most robust study on the subject found mature females as small as 36 cm, 50% of individuals mature at 47 cm and 100% mature at 59 cm. In contrast, the same study found mature males as small as 19 cm, 50% of individuals mature at 23 cm and 100% mature at 32 cm (Love & Brooks 1990). The state's managing authority, the California Department of Fish and Wildlife (CDFW), plans to gather more data over the next several years to address data gaps and deficiencies identified by the stock assessment peer-review panel before pursuing a fishery management plan.

The method most widely used by CDFW to determine sex in *P. californicus* involves dissection and physical inspection of the gonad due to the lack of external, sexually dimorphic characteristics. However, dissection cannot be used to gather sex-specific landings data from the live commercial fishery or in tag–recapture studies. Other non-lethal techniques for determining sex that have been proven effective in similar species include observing the natural or forced discharge of gametes (St-Pierre 1984); cannulation (Nielsen *et al.* 2014); sonography (Loher & Stephens 2011); and genetic analysis (Galindo *et al.* 2011). The first two methods are limited to mature individuals during spawning activity, and the last method requires careful specimen collection and laboratory expenses. In contrast, sonography can be performed quickly on live fish, year-round, regardless of the fish's sexual development or spawning condition (Shields *et al.* 1993; Loher & Stephens 2011).

Here, the possibility of using veterinary ultrasound (i.e. sonography) to determine sex in *P. californicus* was tested following Loher and Stephens (2011). To limit the impact of the study on the wild *P. californicus* population, whole-fish samples were obtained from existing samples, recreational fishers and aquaculture facilities. The CDFW donated 34 frozen *P. californicus* that had been obtained as by-catch from purse seine vessels targeting sardines. These fish were used to optimise the

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sonographic method for *P. californicus*. Subsequently, saved images from 13 fish were unusable for analysis, primarily because distinguishing features were not captured in the frame. The remaining images from 21 fish were included in the analysis. Live test subjects were provided by Hubbs Sea World Research Institute (HSWRI) in San Diego ($n$ = 30) and the SEA Lab in Redondo Beach ($n$ = 17). Freshly caught *P. californicus* ($n$ = 13) were obtained from anglers competing in an annual halibut derby that occurs in Marina Del Rey, California.

*Paralichthys californicus* have a prolonged spawning period with activity occurring during December through April and June through August. Off southern California, there is a major spawning peak in February with two minor peaks occurring in April and June through August (Moser & Watson 1978). All fish in this study were sampled outside the peak spawning period, but moderate levels of spawning activity were possible. This was particularly evident among the Sea Lab fish, for which forced expulsion of gametes was necessary to validate the sonography results. This procedure was successful in 89% of the Sea Lab sample ($n$ = 19) of which 82% were confirmed males.

Sonography was performed using a refurbished Sonosite 180 (SonoSite, Inc., Bothwell, MA, USA) veterinary ultrasound equipped with a 10–5 MHz, 38-mm aperture linear transducer. Ultrasonic coupling gel was not used (Loher & Stephens 2011). The transducer was oriented longitudinally over the fish's eyed-side gonad. The transducer was centred on the primary mass of the gonad (immediately posterior to the visceral cavity) and centred on the gonad dorsoventrally (Fig. 1). The frequency (or scanning depth) was adjusted based on the size of the fish to ensure that both gonads could be seen. The primary mass of the ovaries are rounded, forming distinct sideways, double 'U' (UU) shape in the ultrasound image, while the primary mass of the testes are pointed and triangular (Fig. 2). Ovaries can be further distinguished by their homogeneous density and gradual posterior tapering. In contrast, the testes are of heterogeneous density and consist of multiple overlapping lobes that appear as irregular shapes in the ultrasound image. There was some variation among individuals for each of these features. For example, the double 'U' was not always possible to clearly image, and the texture of the testes varied greatly. For this reason, the three features were used in combination to determine sex.

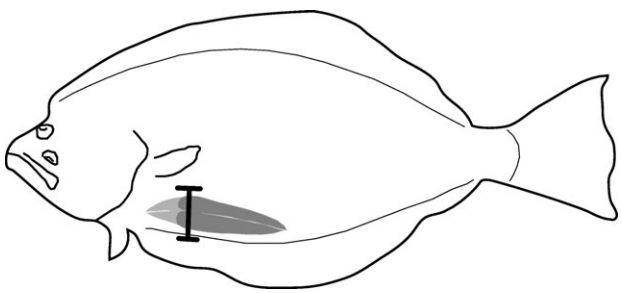

**Figure 1.** The position and orientation of the transducer over the primary mass of the gonad (ovaries shown here) are depicted by the bar.

The sex-identification protocol was then tested on live, freshly caught fish by first making a determination using sonography and then confirming it using one of three accepted methods: past-spawning records of pit-tagged fish (from HSWRI), forced expulsion of gametes from 'ripe' fish (from SEA Lab) and visual identification, via dissection, of landed fish (Derby-caught). The same person performed sonography on all fish. A different person performed the confirmation. Live fish were anesthetised with tricaine methanesulfonate (MS-222), weighed to the nearest hundredth of a gram wet weight using a tared flat scale, measured to the nearest millimetre total length (TL), returned to a tub of fresh sea water to be scanned with the ultrasound and returned to their tank. Landed fish were weighed using an International Game Fish Association certified scale to the nearest tenth of a pound and the weight converted to kilograms. Visual confirmation of sex was conducted by making an incision starting at the cloaca and cutting approximately 5 cm towards the tail along the ventral side of the fish to expose the gonad.

To establish the effect of reviewer experience on the performance of the technique, four reviewers of varying experience with sonography were shown a training slide show that described the protocol, discussed potential variations in gonad morphology and included sample images. The reviewers were then given a randomised set of test images, consisting of images from 63 of the 81 sample fish. None of the images used in the training slides were used in the test set. Reviewers had three choices for scoring based on the images provided: male, female or unclear. The accuracy of each reviewer was evaluated individually. Sexing accuracy with saved images using three-of-four and four-of-four reviewer agreement was also determined. In the latter case, any disagreement among the four reviewers' scores was scored as unclear for that fish.

The fish in the sample ranged from 401 to 1090 mm TL and included 44 confirmed females and 37 confirmed males. While all males sampled, ranging in size from 401 to 683 mm TL, were likely mature (>230 mm), 10 of the females, ranging in size from 404 to 460 mm TL, were likely immature (<470 mm) (Love & Brooks 1990). Differences in gonad morphology between the wild and captive *P. californicus* were not expected, nor were they observed. Sex was correctly identified using sonography in 80 of the 81 fish (98.8% accuracy). The

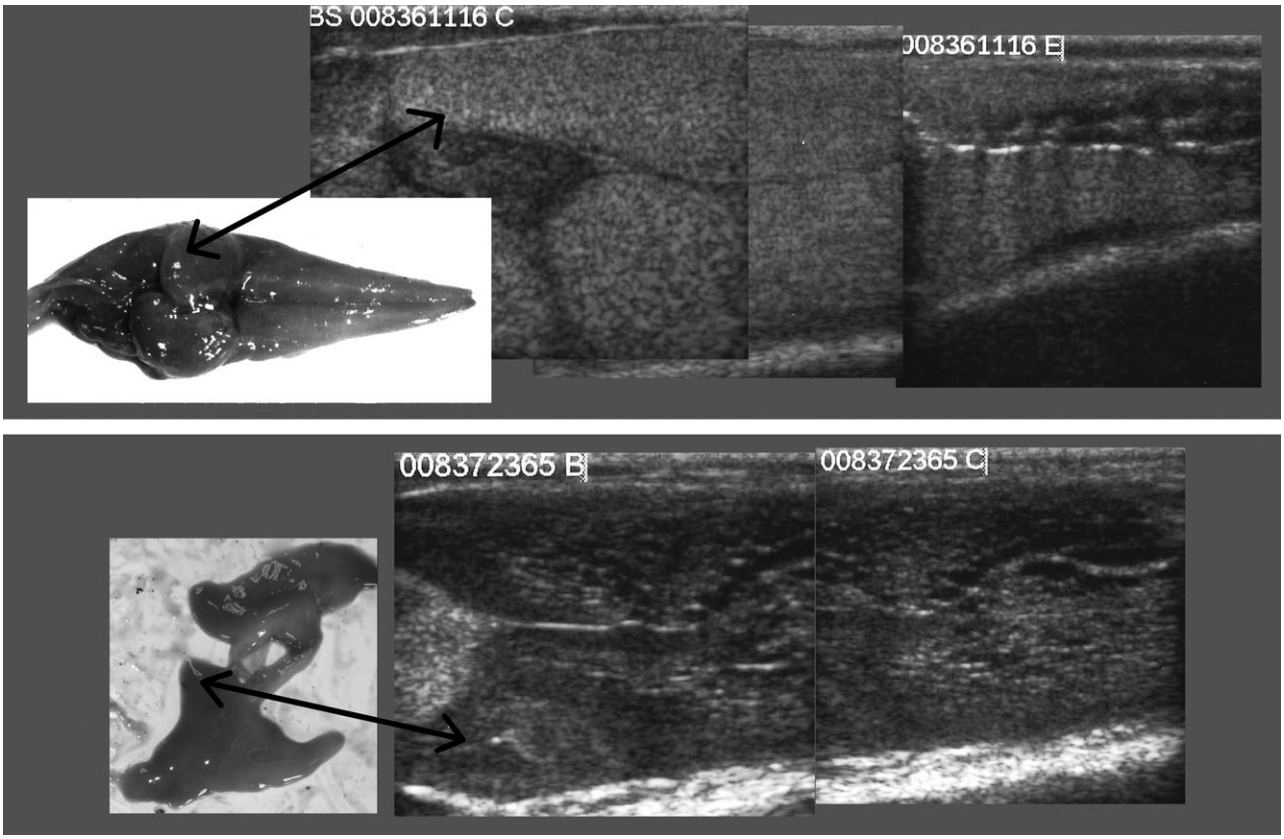


**Figure 2.** Ultrasound and actual images of *Paralichthys californicus* ovaries and testes. Ovaries (top) are distinct from testes (bottom) in the shape of the primary mass (rounded vs pointed, see arrows), their density (homogeneous verses heterogeneous) and form (two uniform lobes vs varied and overlapping lobes).

single incorrect sample was a female *P. californicus* from HSWRI and likely mature (540 mm TL). Interestingly, this fish was correctly sexed in the subsequent assessment of reviewer accuracy.

When comparing the performance of the technique among reviewers, accuracy increased with experience from 90.2% to 96.8%. The reviewer who only had experience with the training slides was the least accurate, while the reviewer who had conducted all of the real-time ultrasounds (96 prior readings) was the most accurate. There was no evidence for a sex bias in the misidentification or non-identification across all four reviewers (chi-squared test, 1 d.f., $P = 0.269$). However, the two reviewers with the least experience only misidentified or failed to identify male fish, suggesting that the smaller male gonads take more skill in identifying accurately. The number of fish the reviewers coded as unclear ranged between 0 and 8.6%, but this did not appear to be related to experience. Three-of-four agreement and four-of-four agreement resulted in 100% accurate sex determination (57/57 and 47/47 correct, respectively). However, significantly more fish were coded as unclear using four-of-four agreement (16/63 or 25.4%) than when using three-of-four agreement (6/63 or 9.5%) (chi-squared test, 1 d.f., $P = 0.018$). Therefore, three-of-four agreement is sufficient to obtain 100% accuracy using this sex determination protocol while maximising the number of useable samples. Notably, the reviewer who conducted all the real-time ultrasounds had slightly higher accuracy when scoring in real-time vs saved images (98.8% and 96.8%, respectively).

These results show that sonography is a highly accurate method for determining sex in *P. californicus* >400 mm TL by a single reviewer working in the field. In addition, experience improves accuracy, and real-time sonography is easier to read (the ability to manipulate the probe greatly aides in reading the image) and therefore more accurate than saved images. Furthermore, when working with inexperienced technicians or saved ultrasound images, three-of-four reviewer agreement is sufficient to obtain accurate results while maximising sample size. Given these results, a standard protocol for saving images to confirm findings in the field in the case of uncertainty or to perform quality control checks should be implemented. This technique will enable CDFW to expand its sex-specific data set

on *P. californicus* and will allow other researchers to conduct sex-specific studies using live individuals, such as tag–recapture studies. Ultimately, the data from such studies will improve the reliability of the next California halibut stock assessment and contribute to more effective fishery management.

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