

Quality of Life and Management of Living Resources

Proposal Nr. QLRT-PL1999-01567

**Sexual Identification and Development in the Swordfish –
Improved Determination Tools for more Efficient Stock
Assessment and Implementation of Control Measures**

SIDS

FIRST PROGRESS AND INTERIM REPORT

**1.1.1 - 5. Sustainable agriculture, fisheries and forestry and
integrated development of rural areas including mountain areas**

**1.1.1- 5.1. 2 .Sustainable fisheries and aquaculture
1.1.1- 5. 4. 3. Monitoring and enforcement of the CFP**

Project Progress Summary

Section 1: PROJECT IDENTIFICATION		NOT CONFIDENTIAL
Information to be provided for project identification		
Title of the project: Sexual Identification and Development in the Swordfish – Improved Determination Tools for more Efficient Stock Assessment and Implementation of Control Measures		
Acronym of the project: SIDS		
Type of contract	RTD	Total project cost (in euro) 385,442 €
Contract number QLRT-PL1999-01567	Duration (in months) 24 Months	EU contribution (in euro) 344,465 €
Commencement date 01.03.2000	Period covered by the progress report (e.g. 1 February 2000 – 31 January 2001) 01.03.2000 – 01.03.2001	
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Key words (5 maximum - Please include specific keywords that best describe the project.). Swordfish, muscle biopsy, sex determination, steroids, vitellogenin		
World wide web address (the project's www address) http://www.uni-duesseldorf.de/WWW/MathNat/Zoophys/bridges/swordfish.htm		

List of participants Provide all partners' details including their legal status in the contract i.e., contractor, assistant contractor (to which contractor?).

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Section 2: Project Progress Report**NOT CONFIDENTIAL***(2 pages maximum.. Use short sentences. Be factual. Avoid technical terms as much as possible)***Objectives:**

To sample swordfish populations from the eastern, central and western Mediterranean Seas and develop methods to determine steroid hormone concentrations and vitellogenin concentrations in plasma and tissue of these fish over a seasonal reproductive cycle. These will then be used to determine sex and sexual maturity and compared with histological studies made at the same time. Using this study as a calibration a muscle biopsy test will be evolved for standard determination of sex and maturity from single muscle samples. This will be tested by simulated market conditions and “double blind” experiments under field conditions.

The objectives will be carried out over two fishing seasons with the second season serving as market testing. In the first season the main objectives are to determine the stability of the sampling methods and to develop the necessary methods for determining steroids and vitellogenin in swordfish plasma and muscle.

Results and Milestones:

From the first fishing season over 400 fish were examined throughout 118 fishing days. In total 345 plasma samples were taken; 368 muscle samples and 308 gonad samples. Although full sampling of plasma, gonads and muscle was obtained for only approximately 50% of the fish sampled. Due to problems with centrifugation and long absences from the port some plasma samples were lost. Difficulties arose also when fish were not gutted at sea preventing sampling. The fishing season extended from March through until December in some areas. Four milestones were successfully reached including the stability protocol which certified the use of the present sampling method as being adequate for steroid measurement storage at 10°C and room temperature. Vitellogenin was isolated from female fish and antibodies for an ELISA test have now been prepared. A standard ELISA to determine levels in plasma and muscle for the swordfish are now in progress. All steroid tests have been successfully applied to both plasma and tissue for the swordfish and gonad histology carried out. From early results it appears that some male swordfish have levels of vitellogenin in their plasma and these are correlated with presence of the female hormone estradiol further examination of the gonads of at least eight male specimens from the central Mediterranean confirm the presence of oocytes in the testis. This may be the first evidence of “endocrine disruption” in a top pelagic predator with the Mediterranean basin and requires further study

Benefits and Beneficiaries:

From the development of the Vitellogenin antibodies a full-scale test can now be developed for the benefit of fisheries scientists. The steroid tests have also been successfully adapted for swordfish and can be used in general by fisheries biologists throughout the Mediterranean.

The demonstration of sex-reversal in the male fish is an alarming result and indicates the need for detailed studies on the extent of the perturbations in the environment involving monitoring the frequency of occurrence and the presence of organic pollutants, heavy metals etc in both the environment and tissues of these fishes. The benefits of the present study as an “early-warning” for future trends should not be under-estimated.

Future Actions (if applicable):

1. Application for prolongation of the present study until May 31st 2002
2. Prolongation of the fishing season until December for Spanish and Italian partners
3. Deliverables delayed until all the data from the extended fishing season has been assessed including, steroids, vitellogenin and gonad histology
4. Redistribution of the sampling load and finances between partners if necessary. Determination of the distribution of sex-reversal in males within the Mediterranean basin.
5. Extend the sampling to an area in the Atlantic (Azores) as part of a pilot control project.
6. Prepare new application under the Auspices of the call for proposals under "Quality of Life " and "Sustainable Development" covering all aspects of the threat of "Endocrine Disruption" in large pelagic predators.

Progress Report

Title of the project <u>S</u>exual <u>I</u>dentification and <u>D</u>evelopment in the <u>S</u>wordfish – Improved Determination Tools for more Efficient Stock Assessment and Implementation of Control Measures		
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1. OBJECTIVES AND EXPECTED ACHIEVEMENTS

In the large, socio-economically important, Mediterranean marine fishery for the Swordfish (*Xiphias gladius*) the determination of sex and gonadal maturity of fishes at capture are important requisites for stock assessment and the controlled regulation of the fishery. This fishery accounts for a yearly financial turnover of well over 300 million EURO within the combined fisheries of the EU. In many cases sex determination and gonadal maturity of the swordfish cannot be determined due to the lack of external sexual dimorphisms between the sexes or the catch is either "gutted" at sea making sex and gonadal maturation determinations on the landing of the catch impossible. Therefore this data is mainly lacking from present stock assessment exercises. The use of fisheries observers "on board" may be costly, time-consuming and ineffective when the fishermen due to the high market value of intact fish forbid major invasive sampling.

In search of a more efficient, cost effective and less invasive assessment method of sex and gonadal maturation an innovative enzyme-linked immunosorbent assay (ELISA) mediated muscle biopsy sample (MBS) will be developed to establish the levels of the sex hormones 17β -estradiol (E_2), 17α - 20β -dihydroxy-4-pregnen-3-one ($17,20\beta$ P), 11-ketotestosterone (11-KT) together with the lipoprotein vitellogenin (Vtg) from Swordfish muscle biopsy samples (MBS). They have until now only been measured in the plasma of other fish species. These substances can be used as specific indicators of sex and spawning state of the fish. The changes in steroid hormones and vitellogenin can normally be correlated with GSI and oocyte diameter. The concentration of $17,20\beta$ P can be used to determine the time course of both egg and sperm release. The objective of the present study will involve the further development of established plasma ELISA methods for, E_2 , $17,20\beta$ -P and 11-KT for use in muscle tissue and the development of a new ELISA test for Vtg in both swordfish plasma and muscle. These will then be correlated with morphological parameters such as gonadal index, gonadal type and oocyte maturation stage. Through this correlation a standardised model with tables of values will be developed to identify either male or female fish and the reproductive state of the gonads i.e. pre-spawning, post spawning. At the same time the correlation between plasma and muscle samples will be established and the stability of both steroid hormones and vitellogenin levels in muscle and plasma under field sampling conditions on board ship and at the market determined. The new MBS method will not only be relevant for large pelagic species but also for other marine commercial species in general. It will also be valuable management tool for the CFP to oversee conservation measures such as a closed season by identifying fish in the market place in terms of species, sex and gonadal maturity from single muscle biopsy samples.

The greater and greater exploitation of large pelagic stocks (swordfish, bluefin tuna, albacore and bonito) in the Mediterranean over the past ten years spurred the scientific community (especially in EU countries) to focus on the biology of the species caught. Within this scenario, there was general agreement that such an extensive exploitation of this natural resource required a more in-depth knowledge of the biological parameters of each species, so that appropriate national and EU policies could be set up. Considering the magnitude of the catches and that catches have constantly increased, ICCAT recommended that immediate measures be taken for the conservation of the stocks of the species mentioned above. The implementation of such measures is predicated upon the acquisition of a certain amount of data, which should contain precise information on the statistics, the identity, and the biological characteristics of the stocks for their assessment. It is already quite clear that swordfish together with tuna are classified as over-fished in the Mediterranean (Call for Proposals 99/C 122 Annexe DG 14- Fisheries).

For a sustainable fishery a scientific basis must be present and this proposal provides an improvement of the existing management tools and methods for assessing both sex ratio and sexual maturity using modern molecular biology methods in areas where the relevant data is lacking or difficult to obtain by standard methods. This application therefore concurs with the priorities of this thematic part of the call for proposals and with the anticipated deliverables.

The innovative methodological development involved in this study supports the Common Fisheries Policies of the EU in both an up-stream and down-stream mode. Providing basic data without which research projects on Swordfish and the BluefinTuna cannot be carried out and also at the same time providing a tool for monitoring the CFP. Common market fisheries employment will be dependent upon sustainable management of the fisheries. Without the biological data provided by sex determination and gonadal maturity no assessment of fishing effort on the stock population can be made. In the global fisheries market a sound data base on which to base policy decisions is essential. Biologically vulnerable stocks with resource conservation requirements will need control measures for policing closed seasons. MBS offers the opportunity not only for species identification, since vitellogenin is species specific but also for sex and gonadal maturity from samples taken at the fish market or wholesaler. Thereby allowing fisheries regulations to be applied to the end of the retail chain.

Specific Objectives

- a) Identification and isolation of sex hormones and vitellogenin in plasma and muscle biopsy samples (MBS) taken from the swordfish. Confirm stability of steroids under field sampling conditions and their suitability as sex and gonadal development markers
- b) Development of an Elisa-mediated standard test to determine the concentrations of the above substances in both plasma and muscle biopsy samples (MBS).
- c) Verification of the correlation between sex hormone and vitellogenin levels in both plasma and MBS. Reciprocity of biometrics data on gonadosomatic indices and sex ratio with MBS determinations.
- d) Test and evaluate sex and maturity determination using MBS in both field (double blind) and market conditions.
- e) Provide a standard MBS method for fisheries stock assessment of sex ratio and gonadal maturity for "gutted" or "high market value" fisheries of the Mediterranean, North Atlantic and North Sea areas. Thus improving the efficiency of stock assessment methods by accurate determination of sex and gonadal maturity for a greater proportion of the fish catch at lower costs. This will extend our knowledge of the reproductive state of the fish for use in assessment models and at the same time serve as an implementation tool for the surveillance of control measures at the market place.

2. PROJECT WORKPLAN

2.1 Introduction

The workplan is structured around the methodology to be used in the project combined with the seasonal restrictions imposed by the fishing seasons in Years 1 and 2. The proposed two year research workplan is conditional on the successful findings of the steroid stability studies in the initial phase of the workplan. Two fishing seasons are essential in which comparisons and verification can be made. The necessity for such an extended sampling scheme is due to the first phase trials and establishment of the correlation between plasma and tissue samples and the need to examine gonad morphology and GSI. Thereafter in the second phase muscle biopsy samples can be directly used. Initially before fish sampling has occurred test samples from swordfish obtained in a EU funded Tuna programme in 1999 will be used to help swiftly set up the standard methodologies. The workplan timetable is shown in Figure 3 below with the dates of the numbered deliverables (D1-n) and also the numbered milestones reached (M1-n). Two fishing campaigns (I, II) will be carried together with test market sampling during the second campaign. All the data will be correlated after each fishing season and during the second year the analysis carried out as a double blind to assess the accuracy of our predictions.

Methodology

Catch Sampling Protocol (Partners 1, 3, 4 and 5)

- a) Fish will be marked with a serial Floy-Tag (a relatively non-invasive method which does not damage marketable tissue) in the dorsal fin on coming on board for later identification. This allows later checking of weight, length and hard-structure sampling for age analysis as fish weight and length are first assessed in Port.
- b) Plasma sampling - Blood sampling direct from the heart and separation from whole blood is achieved by centrifugation on board and identification of the sample will be via colour coding and according to Floy Tag number. Samples will then be stored on ice or dry ice.
- c) Removal of gonads – Gonads will be removed and fixed or frozen with colour coding/Floy Tag number used for later correlation with weight and length and hormone and vitellogenin levels.
- d) Muscle Sampling – Muscle samples will be taken using muscle biopsy needles either commercially available or modified for this study. This should ensure that the same tissue volume is taken each time. Samples will be stored with colour coding and also Floy Tag serial number.
- f) Stability and Verification Protocol and Standardisation.
 - Whole fish experiments (30 Fish - 15 Female + 15 Male):
 1. Choice of muscle to use/ amount required
 2. Live or dead fish ?
 3. Time of sampling ?
 4. Storage of fish before sampling ?
 5. Storage muscle sample after sampling ?
 - Plasma samples
 1. With or without phenylmethylsulfonylfluoride (PMSF) protease inhibitor (1%) added to syringe
 2. Direct freezing in liquid nitrogen
 3. Freezing in dry ice
 4. Unfrozen kept cool – time course experiments
 5. Room temperature stability.

Gonad Morphometrics (Partners 3, 4 and 5)

a)- Gonad maturity stage will be determined using the Mayer scale for partial spawning species

b)- Gonadosomatic-index using gonad weight and body weight. When body weight will not be available, gonad weight and body length will be used .

c) – Histological and histochemical analysis of the gonads. As soon as possible after the fish is captured, operators on board will take fragments from the gonads which will be fixed in buffered 10% formaline or Bouin's solution. These samples will be dehydrated and paraffin-embedded. Microtome sections will be stained with histological (Hematoxylin-Eosin) and basic histochemical methods (Alcian, Pas, Pyronine)

d) – oocyte morphometry. The size of oocyte populations of different maturity stage gonads will be evaluated on histological sections by an image analyser.

Biometrics Data (Partners 3, 4 and 5)

- LJFL (cm) measured from the tip of the lower jaw to the caudal fork;

- Eviscerated weight (g), when this will be possible.

- Age will be estimated using cross sections of the second spiniform ray of anal fin to construct a size-age key of sampled specimen.

Steroid Hormones (Partners 1 and 2)

I. Preparation for measurement - Plasma of animals, stored at -20°C , will be thawed and the steroids will be extracted twice with dichloromethane, the organic phase evaporated. The residue can be solubilized in the appropriate buffer for ELISA processing. The tissue of animals, stored at -20°C will be homogenized in buffer and centrifuged, the supernatant will be treated in the same way as plasma samples. Concentrations can then be related to the muscle protein concentration (Modified to our standard procedure).

II. Quantitative measurements - For detection and measurement of both 17β -estradiol, $17,20\beta$ -P, Testosterone and 11- ketotestosterone we will be able to use an available ELISA procedure elaborated for other fishes and presently in routine use in the laboratory of Partner 2. The hormones estradiol (female) and 11-ketotestosterone (male) have been found in almost all teleost fish examined and there is no reason to expect them not to be the hormones of swordfish.)

III. Steroid separation and isolation.-The assay will be fully validated by comparing the results obtained from plasma and tissue samples before and after purification by HPLC or TLC.

Vitellogenin (Partners 1 and 2)

I. Identification and purification - Plasma of female animals (with high gonado-somatic index), stored at -20°C will be centrifuged and applied to a Biogel column and then an ion-exchanger (Resouce Q). Adsorbed proteins will be eluted with a linear gradient of NaCl. Absorbance of the eluted fractions will be measured at 280 nm. Eluted fractions containing vitellogenin will be identified on SDS-PAGE and then concentrated using an Amicon cell to the desired protein concentration. The whole procedure will be performed at 4°C and the protein concentration will be determined by Bradford method. Molecular weight determination will be carried out both by gel filtration on Superose-6, and by SDS electrophoresis.

II. Immunological procedures -Antibodies.

The vitellogenin preparation will be mixed with complete Freund's adjuvant and injected subcutaneously in rabbits. After the immunisation procedures the serum will be harvested (approximately 8 weeks). To remove the antibodies which react to common serum proteins, anti-vitellogenin will be absorbed overnight at 4°C with male plasma. After centrifugation, this antibody can be stored at -20°C in glycerol.

Western Blotting.

Immunoblotting will be used to identify the vitellogenin and its specificity in plasma samples.

III. Quantitative measurements with ELISA

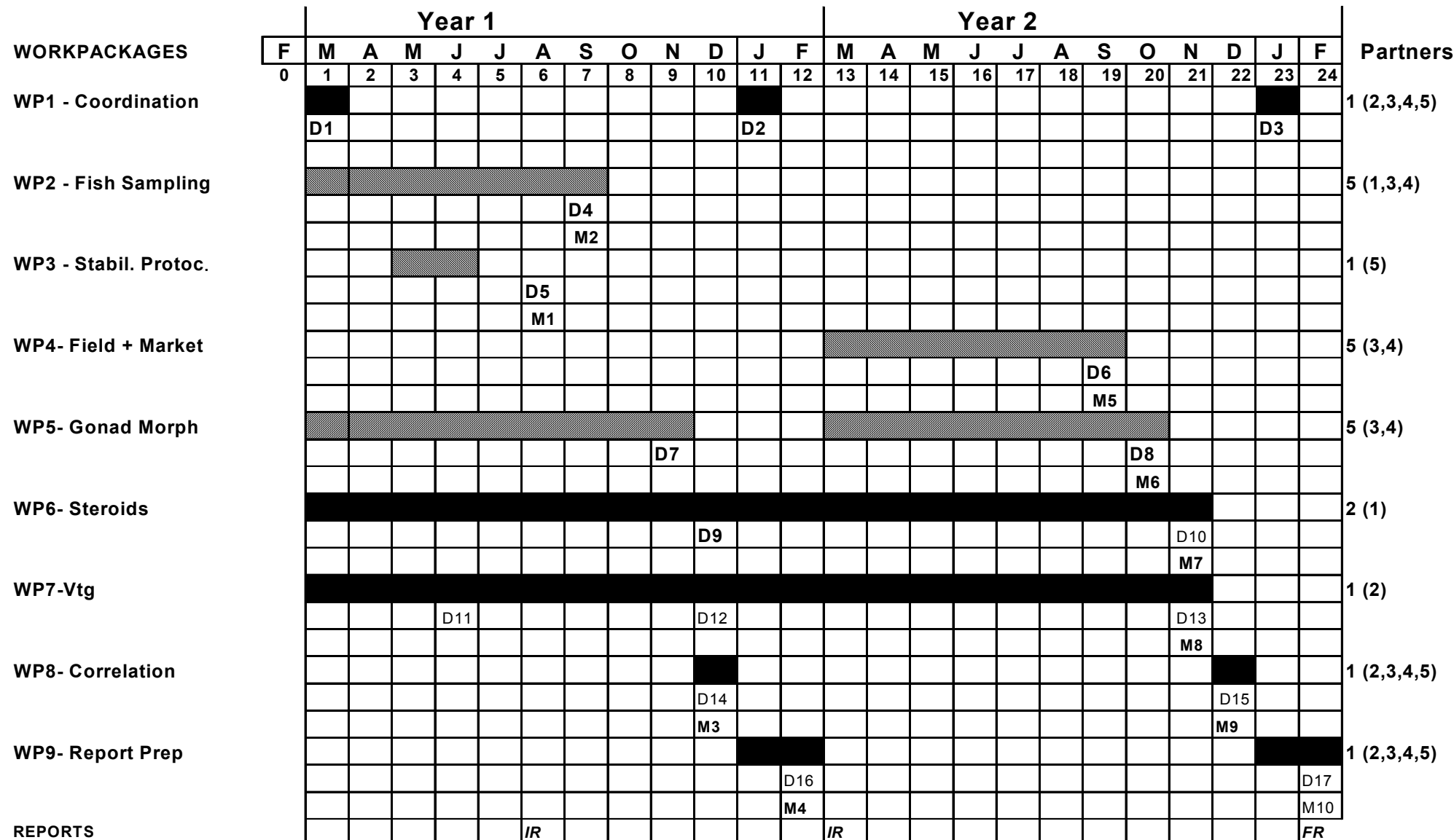
The immunoassay for the Swordfish vitellogenin will be developed and validated as for other fishes (and will be based on ELISA developed for salmonid and cyprinid fish the laboratory of Partner 2 and tested using plasma. Later the plasma results will be compared with the vitellogenin concentration in tissue.

IV. Measurement of Vitellogenin in tissue

Tissue of animals, stored at -20°C will be homogenized in buffer and centrifuged, the supernatant will be diluted in a coating buffer and directly incubated on micro-titration plates. For the quantitative measurements of vitellogenin the same ELISA procedure elaborated for plasma will be used. Concentrations can be related to the muscle protein concentration.

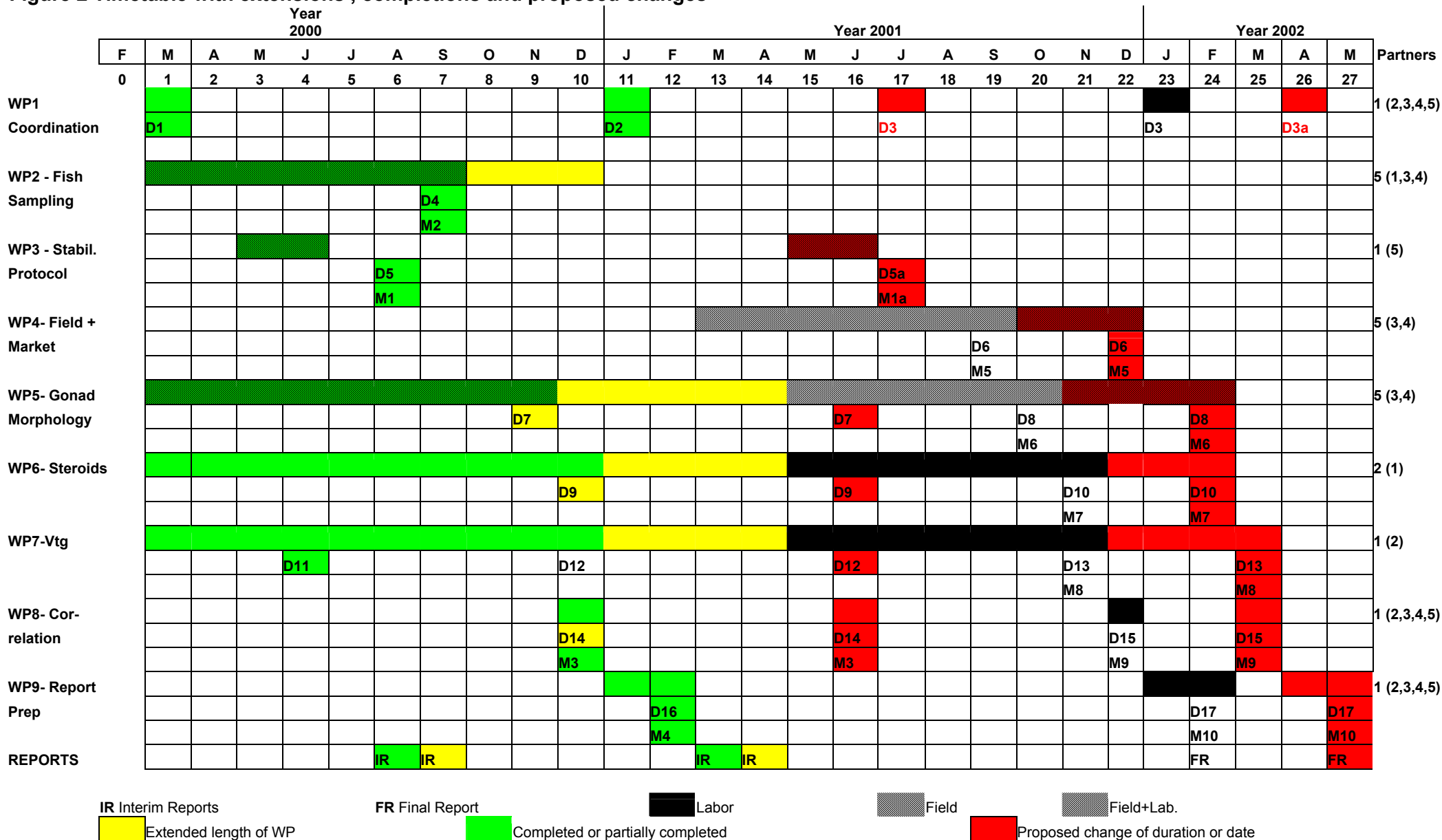
2.2 Project structure, planning and timetable

Figure 1 . Original Timetable for Workplan including the various field and laboratory phases defined for each workpackage. The deliverables are shown as a numbered D1-n and the numbered milestones as M1-n.



IR Interim Report *FR* Final Report Labor Field Field+Lab.

Figure 2 Timetable with extensions , completions and proposed changes



2.2.1 Progress during the first reporting period

In Figure 1 the original time table is shown and Figure 2 details the completion, changes or delays made to the original time table.

Altogether progress has been made on 8 of the 9 planned work packages with varying success and completion rates as detailed later under specific participants reports.

From the coordination progress problems have been encountered in the punctual transfer of return sheets to the coordinator. This has been remedied by the agreement of all participants to send their fishing return forms in by the 7th of each month (see minutes from first and second planning meeting Appendices I + II). Secondly the successful distribution from the sampling teams to the measurement teams has also been delayed and it has been decided (second planning meeting minutes) that the histological samples should be sent monthly to Bari and that the muscle and plasma samples be sent at 2 monthly interval via express carrier to Düsseldorf. To improve the overview of the number of samples taken in the second fishing season a third planning meeting is to be held in July in the UK.

Fish sampling has been successful in terms of the total number of fish samples as shown in Table 1. below. Although 426 fish were caught only a complete range of samples (plasma, muscle and gonads) were taken on 289 fish. The spread of samples over a whole month was difficult to achieve (see Athens results) and the numbers of fish per size range were 84 < 100 cm ; 204 <140 cm and 138 fish >140cm. The largest fish caught was 219 cm. In future fish sampling must be carried out on more than three day per month in Greek waters where the season is closed in October and the target number of fish increased to 150. Malaga and Bari were able to obtain fish even in October November and December and exceeded their 100 quota. The Malaga group should attempt to obtain more full samples at the expense of total numbers. In fishing season II both Malaga and Bari will attempt to obtain fish again up till December if possible therefore prolonging the phase of the WP.

Of the plasma samples problems were experienced by the Athens and Malaga groups in centrifugation (see comments of individual partners) of the samples for either plasma or serum. This led to approximately 40% of these samples to be discarded as they were unsuitable for measurements of steroids or vitellogenin. In both Spain and Greece the length of fishing trips is considerably longer than those experienced in Italy. This leads to problems of centrifugation if no battery driven centrifuge has been purchased.

To compensate for this the sampling protocol has been revised again (See Appendices I and II) and blood samples can be taken and allowed to clot without added heparin. The serum should then be pipetted off from the supernatant after centrifugation in port. All samples should be kept on ice while at sea (around 0°-2°C) and then frozen after centrifugation in port at -18°C. At the same time steps should be taken to obtain a battery-driven centrifuge for use at sea. Failing this trips should be limited to 24hrs.

Muscle samples did not appear to be a problem but the Spanish and Greek groups experienced difficulties with obtaining gonads (see individual reports). To remedy this they have been asked to purchase gonads when required from larger fish to the detriment of total numbers or to ensure that at least the sex of the fish is macroscopically determined. This may involve the use of a biopsy aspiration technique which takes small sample of gonad for microscopic verification of sex. Although muscle and plasma samples without sex can be analysed for steroids and vitellogenin, their use is however extremely limited as no base line determination for sex has been made.

Table 1. Statistical data on sampling programme during fishing season I

Partner	No. Days Fishing	No. Fish	Plasma	Muscle	Gonads	Incomplete
Athens	18	87	62	69	72	27
Malaga	50	167	117	128	74	97
Bari	50	172	166	171	162	13
Totals	118	426	345	368	308	137

The stability protocol (WP 3) was tested during a 4 week period in Italy together with the Bari Partners. A full report is given in Annex III along with a copy of the interim stability report submitted to Brussels. A successful sampling period was carried out on board a drift net fishing boat based in Campo Marina I Italy. Fishing was carried out over a 24hr period and then the fate of muscle sample steroids followed over a 48hr period at room temperature and at refrigerator temperature. The stability of Testosterone was found to be adequate when stored at room temperature or on ice. It was decided to extent this stability work with a more in depth study of all the steroids and vitellogenin in the next fishing season (Coordinators Note: this has now been carried out successfully).

Steroid measurements were successfully carried out in those plasma and serum samples which were useable. The following comments concerning the problems involved were made :

1. Plasma samples of poor quality– about 50% were whole blood and unusable, packaging was erratic, some vials had loose or separated tops – screw caps would be better. Snap-on caps must be firmly pressed in before despatch. All samples processed.

2. Tissue samples had both a tough skin and underlying muscle which had to be separated – generally there was enough muscle, but sampling with scalpel giving a V-shaped sampled had predominantly skin. Packaging was poor and erratic – plastic bags and vials with poorly fixed snap-on caps – screw caps would be better. Homogenisation with electric homogeniser is better than the glass pestle and mortar – practice runs done and now ready to process most samples.

These suggestions have now been put into place to provide better plasma/ serum samples through centrifugation. Packaging will be improved for the second fishing season by all partners. Partner 2 has given a detailed reported under the “Role of Participants” section.

From the gonad morphology work 309 gonads have been fixed approximately and until Febuary 189 specimens mostly from Italian waters had been examined and another 120 samples will be analysed by 30th June 2001 (for details see report from Bari on work package). There were numerous changes to the macroscopic sex determination and staging made at capture compared with the more accurate histological examination of the fish later. The monthly trend of large numbers of small fish is to be discouraged but it is difficult to avoid this as the majority of fish caught are below 140cms LJFL. Priority should be given to fish over 140 cm LJFL. If macroscopic gonad determination is not allowed it has been suggested that aspiration needles be use to aspirate a small sample of gonad into a biopsy needle for later histological examination. Aspiration biopsy needles can be provided by the partner in Düsseldorf.

Update of Work Packages, Deliverables and Milestones

The following pages show the description of the work packages with details of completions and extensions. These have been colour coded and have been summarized in Figure 2. Basically since the fishing season I was extended until December in both Italy and Spain samples to be analysed arrived in March 2001. In this case all the deadlines for deliverables and milestones have been shifted accordingly. It is hoped that by June 31st 2001 all the complete samples will have been measured for all parameters, thereby enabling a correlation to be made between, histology, steroids and vitellogenin. These will be discussed at the planning meeting in July and decisions made on the further sampling and time table.

2.2.1.1 Discussion-Conclusion

It has been successfully shown in the Bluefin tuna that the steroid sexratio formula can be used to determine sex and also the use of a "Dot-Blot" for Vtg will identify female fish (See Annex IV). The aim of the present study to develop such a system has met with both positive and negative results. On the positive side the assays for all the major components Steroids, gonad histology and vitellogenin have been developed successfully. On the negative side female hormones (estradiol) and vitellogenin have been found within male fish from the central Mediterranean. The results are analysed in detail below.

Steroids

The steroid data obtained is consistent with previous reports on seasonal hormone levels in showing an increase of 11KT in males and of E2 in females during gonadal recrudescence . It was, however, apparent from the data that there were some stage 1 fish with exceptionally high GSI, while some at Stage 3 had GSIs more typical of Stage 1 fish. The steroid patterns observed support our suggestion that 11KT and estradiol might be suitable indicators of sexual and reproductive status. 11KT was high (>3 ng/ml) in Stage 3 males or those with GSI>4, while E2 was high (>5 ng/ml) in Stage 3 females and those with GSI> 1. Mean 11KT was below 3 in all except one female and mean E2 below 3 in all males.

Regression lines show a significant correlation between 11KT and GSI in males ($P<0.01$) but not females, and between GSI and E2 in females ($P<0.001$) but not males. Testosterone was not correlated with GSI in either sex and is probably not a useful indicator for the purpose of this project. There are few previous data for E2 in males or 11KT in females as these hormones are rarely measured due to the common conception that they are zero, but there is increasing evidence from other workers that significant amounts of E2 are found in some males and 11-ketotestosterone in females (personal communications). It is not clear whether such "abnormal" hormones are natural or the result of endocrine disruption. It may be relevant that some of the fish caught in this sampling season showed evidence of intersex gonads (See Work packages 5 and 7). All fish with E2 >6.16 ng/ml were females, while 9/10 of the fish with 11KT> 3.0 ng/ml were males according to macroscopic examination. There was an apparent shortage of sexually mature specimens in the fish so far analysed and at the moment maturity stage cannot be reliably assigned from the macroscopic data available.

The above data will be re-analysed when all samples have been assayed and when histological data becomes available.

Gonad Morphology

Female morphological traits were restricted to measurements on the Italian samples and Greek and Spanish will be analysed by June 31st 2001. As expected an increase in GSI for female fish occurred in June. The majority of the fish were in the mature stage (4). In July 50% were postovulatory and by August and September all were postovulatory. In male fish GSI was not marked maximum values only reaching 0.3-0.4 compared with 5.5 in females. An apparent double peak with a high GSI value in July and an autumnal peak in October were correlated with spermatogenesis in both cases. The cause of this remains to be investigated.

Abnormalities were observed in the Testes of 8 male specimens, which showed the presence of oocytes. So far this has only been observed in specimens from the central Mediterranean basin. These findings were correlated with other parameters (see following page).

Vitellogenin

The rapid identification of vitellogenin in female plasma samples using ion-exchange chromatography enables this to be isolated and sent for antibody preparation. Individual fish plasma measurements using ion-exchange chromatography revealed measurable amounts of vitellogenin in male fish. This was not the case in previous studies with Bluefin Tuna.

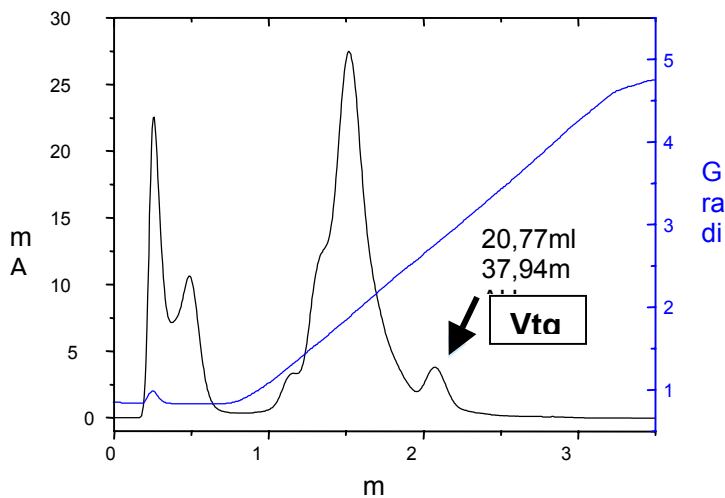
Figure 3 below illustrates the results for a specific male fish #621. It is clear that both steroid hormone patterns, Vitellogenin peaks and Testes histology clearly reveal the presence of endocrine disruption.

For the successful determination of sex this makes matters more complicated in the central Mediterranean area if this is a common phenomena. For Greek and Spanish sampling and in principle the Atlantic this may not be a problem. Using the full data base we should be better able to discriminate using steroids rather than Vitellogenin. If endocrine disruption is taking place then a reassessment will be required of the whole Mediterranean stock.

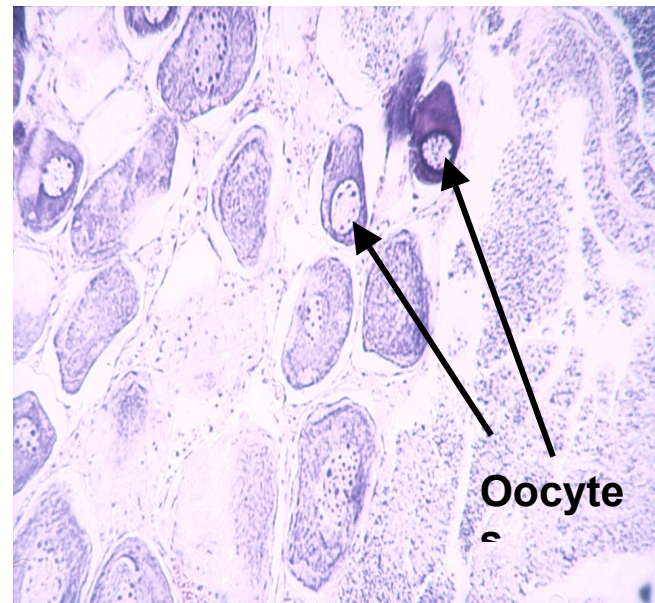
Further work is now required on the sexsteroid ratio to discriminate between males and females and perhaps other steroid markers specific to male fish.

Plasma Hormone Levels:

Sample	sex	GSI	KT	E2	T	KT/E2	E2/KT
XI 621	male	0,33	6,66	0,26	9,16	26,02	0,04
XI622	female	11,37	0,69	38,95	1,51	0,02	56,44



Xiphias gladius
621 Male



Section of the gonad
of # 621 Male

2.2.1.2 Future action

1. Application for prolongation of the present study until May 31st 2002 without added financial costs to the EU.

Considering the amount of material already collected and the delay in obtaining full samples it would be more realistic to prolong the present study without further costs to the EU until the 31st May 2002. This will provide enough time for a full analysis of all the data provided by the various facets of the programme.

2. Prolongation of the fishing season until December for Spanish and Italian partners

From the experience in the first season it would also be preferable for an extension to the sampling programme up until December if possible in Italy and Spain. The fishery is closed in Greece from the 1st October. An extension of the season in the first fishing season has led to delay in measuring samples and therefore from this experience an extension of the contract would again be beneficial.

3. Deliverables delayed until all the data from the extended fishing season has been assessed including, steroids, vitellogenin and gonad histology.

From the timetable of work laid down originally it is clear that deadlines were set to early considering the extension of the sampling season etc. In July 2001 all data from the previous fishing season will be correlated and a clear picture for deliverables for the next season laid down. We do not envisage any major delays in the second sampling period since new guidelines have been drawn up. This will enable the deadline of May 31st for the final report.

4. Redistribution of the sampling load and finances between partners if necessary.
Determination of the distribution of sex-reversal in males within the Mediterranean basin.

From the detailed accounts provided by both the Greek and Spanish groups there are severe difficulties in providing full samples from each fish. In part this is due to the long fishing trips with no access to centrifugation or freezing. To counteract this battery powered centrifuges should be purchased or trips limited to 24 hrs at the maximum. This is a technical problem which can be solved. Alternatively serum samples can be readily obtained provided the samples are centrifuged before freezing. The second cause of the lack of full sampling is that fish are not gutted at sea precluding the taking of gonad samples in the Spanish fishery. Here the tagging of fish with a simple Floy tag at sea and then following the fish at landing may help. Unfortunately few fish are landed on a daily basis but the Spanish group will be asked to use their fund to purchase gonads. In light of these experience a redistribution of funds amongst the sampling groups to ensure that at least 300 full samples from fish are sampled in total according to the schedule laid down in Appendix I should be considered. This may be at the expense of lack of geographical coverage and partners may consider subcontracting tasks with agreement of the EU to other sources who can provide full samples even on a limited basis. Careful screening of suitable plasma and serum samples must be done each month as a quality control to assure that the sampling programme is adhered to.

5. Extend the sampling to an area in the Atlantic (Azores) as part of a pilot control project.

As pointed out in the results above there are indications that swordfish in the Mediterranean may be exposed to "endocrine disruption". Evidence from the presence of Vitellogenin and

Estradiol in the plasma of large males and histology on male testes have shown that oocytes are present. We therefore plan to carry out (with permission of the EU) a small pilot project on samples taken in the Swordfish population around the Azores. This will involve the sampling of approximately 30 large male fish within the next few months through a collaboration with the University of the Azores. The costs will be approximately 1000€ for 1 months sampling.

6.Prepare new application under the Auspices of the call for proposals under “Quality of Life “ and “Sustainable Development” covering all aspects of the threat of “Endocrine Disruption” in large pelagic predators

To investigate the presence and danger of “Endocrine Disrupter” in top pelagic predators of the Mediterranean food chain a large scale project will be drawn up involving environmental chemists, fisheries biologists, fish physiologists and geneticists to look at the distribution of inorganic and organic pollutants within the tissues of Swordfish and Tuna and the distribution of endocrine disruption within the Mediterranean basin. This will be coupled with the monitoring of environmental stress not only on the reproductive system but also through stress hormones such as catecholamines and also molecular markers such as heat shock proteins.

The continued feasibility of developing a sex determination tool will depend upon the extent of the distribution of “endocrine disruption” within the Mediterranean. After July 2001 we should have some idea of its occurrence within the Mediterranean basin as a whole and its relevance in Atlantic populations. In either case the results of the second fishing season together with control population data are extremely important for socio-economic decisions to be made in the future.

2.3 Description of the Workpackages

New additions are shown in red, uncompleted work in orange and completed deliverables in green

WP1	Workpackage description
	<p>Workpackage number: 1 Start date or starting event: Beginning Fishing Season ; Month 1 Completion Date: Prolongation until May 1st 2002 Current Status : In progress N° of the partner responsible 1 N°s of other partners involved: 1, 2, 3, 4, 5 Person-months per partner: 1 (5), 2(1), 3(1), 4(1), 5(1): Total = 9 Devoted Person Months 1(2,5), 2(0,5), 3(0,5), 4(0,5), 5(0,5) Total= 4,5</p>
	<p>Objectives Coordination and Management Meetings 1. Initial set-up and co-ordination of the project with standardised methodology. Preparation for WP2 with the organisation of sampling kits by partner 1 and 2 for partners 3, 4, and 5. Distribution of Swordfish test samples from Tuna fishing 1999 to partners 1 and 2 2. Co-ordination meeting for WP4 and preparation for interim report 3. Co-ordination and discussion meeting for final report</p>
	<p>Description of work During the first month of the project a meeting of the participating staff of all five groups will be held in the laboratory of the Co-ordinator to discuss the organisation of the project. The compilation of kits for "on board" sampling will be previously initiated by partners 1 and 2 for partners 3, 4, 5. Partner 1 will purchase an ELISA reader and computer system and partners 1 and 2 exchange Swordfish test samples from the 1999 Tuna fishing campaign. The second co-ordination meeting will be held 10 months later to discuss findings of previous fishing season and laboratory work and planning of the WP4- Field and Market sampling. The third co-ordination meeting will take place after a further 12 months. Progress during first Reporting Period: Two coordination meetings have been held and the results are summarized in the section on Project management and in Interim Report Appendices I and II.</p>
	<p>Deliverables D:1 Co-ordination & Standardised Protocol A standardised sampling protocol will be determined and circulated to all participants D:2 Planning Programme for Field & Market Detailed Plan for the implementation of the field and simulated market sampling together with draft of interim report. D3: Co-ordination of Final Report and discussion draft of final report D3 will be delayed and broken up into D3 and D3a. The former taking part in July 2001 and the latter in April 2002.</p>
	<p>Milestones and expected results No direct milestones but full co-ordination of methodology, fishing programmes, No's of fish and preliminary report drafts.</p>

WP2	Workpackage description
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Workpackage number: 2
Start date or starting event: Beginning Fishing Season; Month 1
Completion Date: December 2000
Current Status : Completed
N° of the partner responsible 5
N°s of other partners involved: 1, 3, 4
Person-months per partner: 5(5), 1(3), 3(7), 4(5): Total = 20
Devoted Person Months: 5(5), 1(3), 3(7), 4 (5)

Objectives

Fish Sampling in first Season

- a) Procurement of Biometrics Data
- b) Procurement of Biological samples

Fish Campaign I will take place from March 99 through until October 99 based in the Mediterranean area fishing ports. Partner 5 will be responsible for organising this part of the study. The object will be to obtain initial plasma samples in the first half of the season together with preliminary muscle biopsy samples (MBS) which will all be rapidly frozen for maximum protection. In this fishing campaign complete samples of all parameters from 300 fish (175 female + 125 male) will be obtained. Partners 5, 3 and 4 are expected to deliver data on 100 fish each with a distribution of 2/3 females to 1/3 males. Solitary muscle samples from the landed catch may be collected where ever possible.

Description of work

Fish Sampling

Biometrics Data

- LJFL (cm) measured from the tip of the lower jaw to the caudal fork;
- Eviscerated weight (g), when this will be possible.

Catch Sampling Protocol

- a) Fish will be marked with a serial Floy-Tag (a relatively non-invasive method which does not damage marketable tissue.) in the dorsal fin on coming on board for later identification.
- b) Plasma sampling – Blood sampling direct from the heart and separation from whole blood is achieved by centrifugation on board and identification of the sample will be via colour coding and according to Floy Tag number. Samples will then be stored on ice or dry ice.
- c) Muscle Biopsy Sampling (MBS) – Muscle samples will be taken using muscle biopsy needles either commercially available or modified for this study.
- d) Whenever possible extra muscle samples alone from landed fish should be taken and stored for future analysis.

Progress during first Reporting Period:

A total of 426 were sampled but only a full sampling profile was available from approximately 190 fish of which some plasma samples were not usable. See previous table in Part 2.2.1
See detailed Participant 3, 4 and 5 report in Section 2

Deliverables

D4: Biological samples of both plasma and muscle together with biometric data for 100 fish each will be delivered by Partners 3, 4 and 5 to Partners 1 and 2 for further analysis. Stored muscle samples from any other landed fish will also be provided.

Number of usable samples less than predicted due to sampling problems,

Milestones and expected results

M2: End of fish sampling programme I with successful delivery of biological samples to other partners. Full data sets on biometrics of all fish sampled together with part of the gonad morphometrics data.

WP3	Workpackage description
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Workpackage number: 3
Start date or starting event: Month 3
Completion Date: Prolongation until July 31st 2002
Current Status : Phase 1 completed in 2000 to be repeated in 2001
N° of the partner responsible 1
N°s of other partners involved: 5
Person-months per partner: 1 (3), 5(1): Total = 4
Devoted Person Months : 1(2) , 5(1) Total =3

Objectives

To determine in the first phase the best method of taking and storing plasma and muscle samples from swordfish in the field. Determine the stability of both steroid hormones and vitellogenin in plasma and muscle during May and June when steroid levels and vitellogenin levels are high. Provide a basis for the full funding of the project through an interim report at the end of month 6.

Description of work

Stability and Verification Protocol and Standardisation

Muscle sampling:

-Whole fish experiments (30 Fish - 15 Female + 15 Male):

1. Choice of muscle to use/ amount required?
2. Live or dead fish ?
3. Time of sampling ?
4. Storage of fish before sampling ?
5. Storage muscle sample after sampling ?

-Plasma samples

1. With or without phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (1%) added to syringe

1. Direct freezing in liquid nitrogen
2. Freezing in dry ice.
3. Unfrozen kept cool – time course experiments.
5. Room temperature stability.

Progress during first Reporting Period:

In May 2000 a 1month period was used to carry out a stability protocol. This will be repeated in May 2001. A stability report has been submitted see Interim Report Appendix III.

Deliverables

D5: Provide stability protocol to determine the best sampling method for plasma and tissues and also the most stable method of storing probes after sampling. Determine the breakdown rate of steroids and vitellogenin in plasma and muscles under various storage conditions. Provide first interim report to confirm the suitability of the methods used after 6 months to confirm further funding.

D5 converted to D5 and D5a which will be produced in July 2001.

Milestones and expected results

M 1: Provide clear guidelines for future sampling within fishing campaign II . Provide correction factors for the appraisal of probes taken within the first fishing campaign I. The successful completion of WP3 with corresponding positive results and the submission of the first interim report will make the full funding of the project possible.

M1 converted to M1 and M1a to be completed by July 31st 2001.

WP5	Workpackage description
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Workpackage number: 5
Start date or starting event: Beginning Fishing Season ; Month 1
Current Status : Analysis continuing
N° of the partner responsible 5
N°s of other partners involved: 3, 4
Person-months per partner: 5 (4), 3(10), 4(3)
Devoted Person Months : 5(2), 3 (4.3), 4(1.5)

Objectives

Gonad Morphometrics

To obtain complimentary data on GSI, gonad maturity, gonad histology and oocyte morphometry on the 600 fish from which plasma and muscle samples have been taken.

Description of work

- a) Removal of gonads – Gonads will be removed and fixed or frozen with colour coding/Floy Tag number used for later correlation with weight and length and hormone and vitellogenin levels.
- b)- Gonadosomatic-index using gonad weight and body weight. When body weight will not be available, gonad weight and body length will be used.
- c)- Gonad maturity stage will be determined using the Mayer scale for partial spawning species.
- d) - Histological and histochemical analysis of the gonads. As soon as possible after the fish is captured, operators on board will take fragments from the gonads which will be fixed in buffered 10% formaline or Bouin'solution. These samples will be dehydrated and paraffin-embedded. Microtome sections will be stained with histological (Hematoxylin-Eosin) and basic histochemical methods (Alcian, Pas, Pyronine)
- e) – Oocyte morphometry. The size of oocyte populations of different maturity stage gonads will be evaluated on histological sections by an image analyser.

Progress during first Reporting Period:

Not all samples have been analysed due to the late arrival of the Spanish samples in March and the Greek samples in January. Extended analysis through until 1st March 2002 March. See detailed Participant 3, 4 and 5 report in Section 3

Deliverables

D7: Partners 3,4, and 5 will each deliver full gonad morphometrics data for 100 fish caught in fishing season I

D7 has been delayed until July 2001 to enable samples to be examined thoroughly.

D8: Partners 3,4, and 5 will each deliver full gonad morphometrics data for 100 fish caught in fishing season II

D8 will be delayed until February 2002 to enable winter samples to be analysed.

Milestones and expected results

M6: Completion of all gonad morphometric data collection including laboratory histology. Provision of data on sex and gonad maturity from 600 fish in total. For comparison and tests of accuracy it is essential that data be collected over 2 fishing seasons.

M6 will be delayed until February 2002 to enable winter samples to be analysed

WP6	Workpackage description
<p>Workpackage number: 6 Start date or starting event: Beginning Fishing Season ; Month 1 Current Status: Measurements continuing N° of the partner responsible 2 N°s of other partners involved: 1 Person-months per partner: 2(17) 1 (4) Devoted Person Months : 2(7) , 1 (2)</p>	
<p>Objectives Development of ELISA test for steroid hormones in the swordfish Measurement of steroid hormone concentrations in plasma and tissues</p>	
<p>Description of work I. Preparation for measurement Plasma of animals, stored at -20° C, will be thawed and the steroids will be extracted twice with dichloromethane, the organic phase evaporated. After freezing at -20° C, the organic phase will be collected and evaporated using a Speed-Vac apparatus. The residue can be solubilized in the appropriate buffer for ELISA processing, or with methanol for injection on HPLC column. The tissue of animals, stored at -20° C will be homogenized in buffer and centrifuged, the supernatant will be treated in the same way as plasma samples. Concentrations can then be related to the muscle protein concentration.</p> <p>II. Quantitative measurements For detection and measurement of both 17β-estradiol, $17,20$-P, Testosterone and 11- ketotestosterone we will be able to use an available ELISA procedure elaborated for other fishes</p> <p>Progress during first Reporting Period: Almost all plasma samples have been measured and tissue samples are being prepared from the first fishing season. For detailed report see Participant 2 under section 3:</p>	
<p>Deliverables D9: Provide concentration measurements for all sex hormones for tissue and plasma samples collected in fishing campaign I D9 has been delayed until July 31st 2001 due to late delivery of sampling materials. D10: Provide concentration measurements for all sex hormones for tissue and plasma samples collected in fishing campaign II D10 : will be delayed until March 1st 2002 to allow time for full analysis.</p>	
<p>Milestones and expected results M7: Successful establishment of all ELISA mediated test for all the sex hormones to be studied. Provision of all hormone data for both plasma and MBS from the complete fishing and market campaigns. M7 : will be delayed until March 1st 2002 to allow time for full analysis.</p>	

WP7	Workpackage description
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Workpackage number: 7
Start date or starting event: Beginning Fishing Season ; Month 1
Current Status: Measurements continuing
N° of the partner responsible 1
N°s of other partners involved: 2
Person-months per partner: 1 (16), 2(5)
Devoted Person Months : 1(8), 2 (2)

Objectives

1. Isolation of Vitellogenin in Swordfish plasma
2. Development of Anti-serum for Swordfish Vtg
3. Development of Vtg ELISA
4. Measurement of Vitellogenin in plasma and muscle samples

Description of work

I. Identification and purification

Plasma of female animals (with high gonado-somatic index), stored at -20° C will be centrifuged and applied to a Biogel column and then an anion-exchanger column (Resource Q). Adsorbed proteins will be eluted with a linear gradient of NaCl. Absorbance of the eluted fractions will be measured at 280 nm. Eluted fractions containing vitellogenin will be identified on SDS-PAGE. Then concentrated using an Amicon cell to the desired protein concentration. The whole procedure will be performed at 4° C and the protein concentration will be determined by Bradford method. Molecular weight determination will be carried out both by gel filtration on Superose 6, and by SDS electrophoresis.

II. Immunological procedures

Antibodies.

The vitellogenin preparation will be mixed with complete Freund's adjuvant and injected subcutaneously in rabbits. After the immunisation procedures the serum will be harvested (approximately 8 weeks). To remove the antibodies which react to common serum proteins, anti-vitellogenin will be absorbed overnight at 4° C with male plasma. After centrifugation, this antibody can be stored at -20° C in glycerol (Bon *et al.*, 1997).

III. Quantitative measurements with ELISA

The immunoassay for the Swordfish vitellogenin will be developed and validated as for other fishes and will be based on ELISA's developed for Tuna vitellogenin developed in the laboratory of Partner 1 and salmonid and cyprinid fish the laboratory of Partner 2 and tested using plasma. Later the plasma results will be compared with the vitellogenin concentration in tissue.

IV. Measurement of Vitellogenin in tissue

Tissue of animals, stored at -80° C will be homogenized in buffer and centrifuged, the supernatant will be diluted in a coating buffer and directly incubated on micro-titration plates. For the quantitative measurements of vitellogenin the same ELISA procedure elaborated for plasma will be used. Concentrations can be related to the muscle protein concentration.

Progress during first Reporting Period:

Parts I and II have been completed and III and IV are in progress see detailed Participant 2 report in section3

Deliverables

- D11: Isolation of vitellogenin from swordfish plasma**
D12: Develop ELISA Test for Vtg determination and assay all fish samples from fishing campaign 1.
D 12 Completion of D12 will be delayed until July 1st 2001
D13: Deliver all data on Vtg in plasma and MBS samples from fishing campaign II and market MBS
D13: Delayed until the 1st April 2002

Milestones and expected results

M8: Successful establishment of Vtg ELISA for swordfish samples. Completion of all measurements of Vtg in plasma and MBS.

M8: Delayed until the 1st April 2002

WP8	Workpackage description
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Workpackage number: 8
Start date or starting event: December 2000
Current Status:
N° of the partner responsible 1
N°s of other partners involved: 2, 3, 4, 5
Person-months per partner: 1(5), 2(1), 3(1), 4(1), 5(1)
Devoted Person Months: 1 (0.25), 2 (0.25), 3(0.25), 4(0.25), 5(0.25)

Objectives

1. Establish correlation between hormone and Vtg data for plasma and MBS
2. Establish correlation of hormone and Vtg data with sex ratio and gonadal maturity data
3. Establish correlation of the double blind and market MBS
4. **Compare available results of existing projects on swordfish fisheries in the Mediterranean**

Description of work

All partners will prepare their data in the form of spreadsheets and graphical presentations in Excel. These data packages will then be exchanged via email + attachments

1. Partners 1 and 2 will establish correlation between concentration measurements of all steroid hormones and their corresponding levels in muscle tissue.
2. Partners 3, 4 and 5 will correlate their gonad morphometric data with their biometrics data.
3. Partner 1 will then combine the two parts of the of the study to give the overall correlation and distribute these results to all partners. A standard set of table will then be produced defining sex ration and gonad maturity from MBS.

4. Correlate data with existing projects and results from Swordfish fisheries

Progress during first Reporting Period:

Some correlations have been made but a complete correlation is only possible after all the samples have been analysed.

Deliverables

D14: Correlation relationship between steroid and Vtg concentrations in plasma and MSB for the first fishing campaign. First estimate of correlation between hormone and Vtg levels and Gonad morphometrics and biometrics

D14: Delayed until the 1st July 2001 when all data should be available

D15: Further correlation of steroid and Vtg conc. In plasma and MSB. Comparison of Double blind results and the accuracy of MSB for determining sex ratio and gonadal maturity.

D15: Will be dalyed until 1st April 2002

Milestones and expected results

M3: Completion of first phase appraisal of determining sex ratio and gonad maturity from MSB and measurement of steroid hormones and Vtg.

M3: Full results delayed until the 1st July 2001.

M9: Completion of Final correlation between MSB determined sex ratio and gonad maturity with conventional methods of gonad morphology and biometrics.

M9: Will be delayed until 1st April 2002

WP9	Workpackage description
<p>Workpackage number: 9 Start date or starting event: January 2001 Current Status: Interim report completed N° of the partner responsible 1 N°s of other partners involved: 2, 3, 4, 5 Person-months per partner: 1 (5), 2(1), 3(1), 4(1), 5(1) Devoted Person Months: 1(2), 2(0.5), 3(0.5), 4 (0.5), 5(0.5)</p>	
<p>Objectives Preparation of Interim and Final Reports</p>	
<p>Description of work Initially prepare draft proposal of interim and final reports. This includes all text, tables and graphic work for the two reports. Interchange of drafts via E-mail + attachment system so that at the co-ordinating meeting these can be assessed and changes made. Preliminary publications will be drafted for presentation at international meeting or in refereed scientific journals. Compare results from the present study with available data Progress during first Reporting Period: The initial stability report was completed and also the interim report.</p>	
<p>Deliverables D:16 Interim Report Delayed until the 1st May 2001 D:17 Final Report D17 will be delayed until the 31st May 2002</p>	
<p>Milestones and expected results M 4: Successful delivery of interim report. Preparation of publications and presentation of results at international conferences M10: Completion of project . Publication of methodology and results in international scientific journals Ratify market suitability, range and accuracy of MSB as a fishery management tool. Make suggestion for use of MSB in enforcing CFP. M10 Delayed until 31st May 2002</p>	

Table 1: Workpackage List

Workpackage list

Workpackage N°	Workpackage Title	Responsible participants n ^{o1}	Person-months	Start month	End month	Deliverable n°
1	Co-ordination + Methodology	1, 2, 3, 4, 5	9	1	23	1, 2, 3
2	Fish Sampling and Biometrics	5, 1, 3, 4	20	1	7	4
3	Stability verification protocol	1, 5	4	3	4	5
4	Field and Market Verification	5, 3, 4	21	13	19	6
5	Gonad Morphometrics	5, 3, 4	17	1	20	7, 8
6	Laboratory- Steroid Hormones	2, 1	21	1	21	9, 10
7	Laboratory – Vitellogenin	1, 2	21	1	21	11, 12, 13
8	Correlation of Biometrics GSI, Steroids and Vtg Data	1, 2, 3, 4, 5	9	10	22	14, 15
9	Report Preparation	1, 2, 3, 4, 5	9	11	24	16, 17
TOTAL			131			

¹ Workpackage leader listed first

Table 2 List of Milestones

List of Milestones

Milestone N°	Title	Delivery date	Participants	Description
1	Establish Guidelines and Correction Factors	6	1, 5	Provide clear guidelines for future sampling within fishing campaign II . Provide correction factors for the appraisal of probes taken within the first fishing campaign I. The successful completion of WP3 with corresponding positive results will make the full funding of the project possible. This will be the subject of the first interim report
M1a		17	1	Second stability test for Vitellogenin
2	End of Fishing Programme I	7	5, 1, 3, 4,	End of fish sampling programme I with successful delivery of biological samples to other partners. Full data sets on biometrics of all fish sampled together with part of the gonad morphometrics data
3	First Phase Appraisal of MSB	10	1, 2, 3, 4, 5	Completion of first phase appraisal of determining sex ratio and gonad maturity from MSB and measurement of steroid hormones and Vtg
M3		16	1, 2, 3, 4, 5	Extended until all measurements complete
4	Interim Report	12	1, 2, 3, 4, 5	Successful delivery of interim report. Preparation of publications and presentation of results at international conferences
5	End of Fishing Programme II	19	5, 3, 4	Successful completion of field fishing campaigns and market trials and the transfer of biological samples to the partners 1 and 2. Complete biometrics data collected and some of the field gonad morphometric data.
M5		22	5, 4	Extended until December

7	Completion of Steroid Hormone Data Collection	21	2, 1	Successful establishment of all ELISA mediated test for all the sex hormones to be studied. Provision of all hormone data for both plasma and MBS from the complete fishing and market campaigns.
M7		24	2, 1	Extended to allow completion of Data Collection
8	Completion of Vitellogenin Data Collection	21	1, 2	Successful establishment of Vtg ELISA for swordfish samples. Completion of all measurements of Vtg in plasma and MBS.
M8		25	1, 2	Extended to allow completion of Data Collection
9	Final Correlation	22	1, 2, 3, 4, 5	Completion of Final correlation between MSB determined sex ratio and gonad maturity with conventional methods of gonad morphology and biometrics.
M9		25	1, 2, 3, 4, 5	Extended to allow completion of Data Collection
10	Project Completion	24	1, 2, 3, 4, 5	Completion of project . Publication of methodology and results in international scientific journals Ratify market suitability, range and accuracy of MSB as a fishery management tool.
M10			1, 2, 3, 4, 5	Extended to allow completion of Data Collection

Table 3: List of deliverables

List of Deliverables

Deliverable N°	Title	Delivery date	Nature	Dissemination level ¹	Dissemination target ²
1	Co-ordination & Standardised Protocol	1	O	PU	Partners
2	Planning Programme for Field & Market	11	O	PU	Partners
3	Co-ordination of Final Report	24	O	PU	Partners, EC, Research. Jour. WWW
4	Biological Samples I	7	O	PU	Partners
5	Stability Protocol and Report	6	O, R	PU	Partners, EC, Research. Jour. WWW
6	Biological Samples II	19	O	PU	Partners
7	Gonadal Morphometrics I	9	O	PU	Partners
8	Gonadal morphometrics II	20	O	PU	Partners
9	Steroid Hormone Conc. I Plasma + MBS.	10	O	PU	Partners
10	Steroid Hormone Conc. II Plasma+MBS.	21	O	PU	Partners
11	Isolate Vtg	4	O	PU	Partners
12	Vtg Conc. I Plasma + MBS	10	O	PU	Partners
13	Vtg Conc. II Plasma + MBS	21	O	PU	Partners
14	Data Correlation : I	10	O	PU	Partners, EC, Research. Jour. WWW
15	Data Correlation : II	22	O	PU	Partners, EC, Research. Jour. WWW
16	Interim Report	12	R	PU	Partners, EC, Research. Jour. WWW
17	Final Report	24	R	PU	Partners, EC, Research. Jour. WWW

¹PU=Public, RE =restricted to a group specified by the consortium(including EC services), CO = confidential, only for members of the consortium (including EC services)

² Indicate the target audience or the potential users/beneficiaries of such a deliverable

3. ROLE OF PARTICIPANTS

Figure 3 gives overall details of participants, their various tasks and the time table. Details are given below of the work of each individual participant.

Participant Nr: 1 (D)
HEINRICH-HEINE UNIVERSITY OF DÜSSELDORF

Address:
Institut für Zoophysiologie
Lehrstuhl für Stoffwechselfysiologie
Heinrich-Heine Universität
D-40225 Düsseldorf
Germany

Scientific Team
Prof. Dr. C.R. BRIDGES; Vito Susca (Ph. D. Student), Jörg Eicker (Diploma Student) and Christel Castor (Technician).

Contractual Links to other participants: None

Objectives

Christopher R. BRIDGES (Apl. Professor) will act as Financial and Scientific Co-ordinator and will be the workpackage leader for WP1, WP3, WP 7, WP 8 and WP 9. This partner will establish stability protocol for steroids and vitellogenin.

Postgraduate Assistant Responsible for helping with the initial field sampling and then isolation, identification and development of ELISA test for vitellogenin and steroids.

Technical Assistant: Main duties involve the running of the ELISA tests, administration data correlation and editing and maintaining our WWW information exchange on both Swordfish and Tuna work.

Support Services: Full secretarial and Finance/Orders/ Account Handling provided by the Department and the University.

Contribution of the participating organisation:

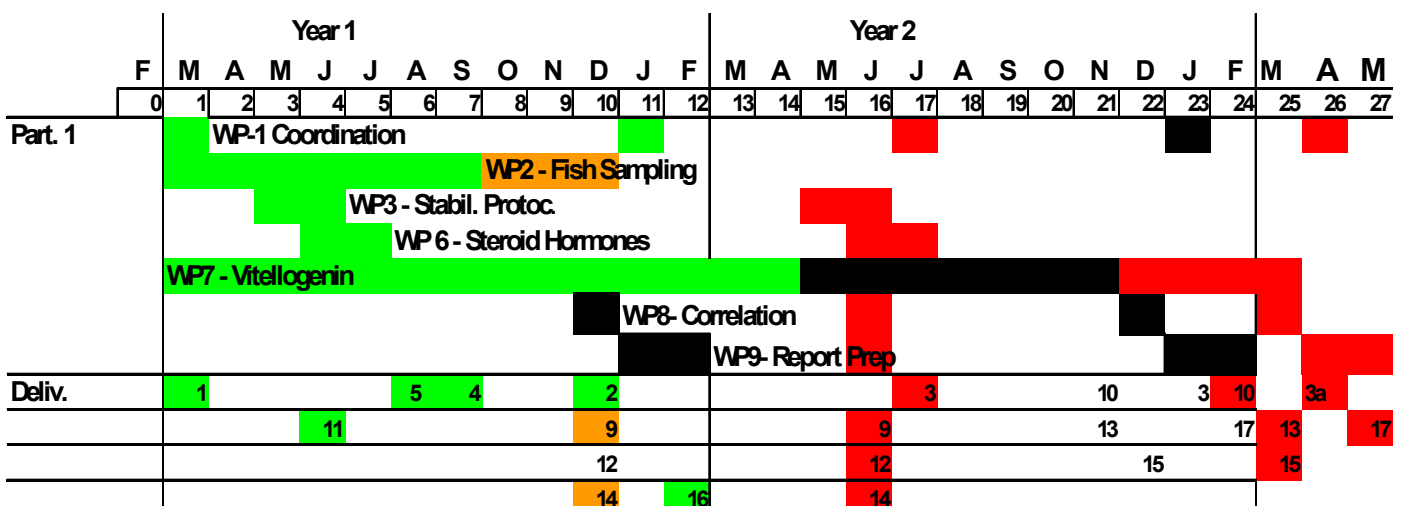
The University provides full salary for the Senior scientist, laboratory and office facilities, computing services including email and WWW access and Homepage maintenance, full library facilities and technical and financial services support.

Workpackages (With person-months)

WP1 (5); WP2 (3); WP3 (3); WP6 (4); WP7 (16), WP8 (5); WP9 (5)

See actual workpackages and figures 1 and 2 and tables 1 ,2 and 3 for details

Workplan Participant 1 with WP's and Deliverables



Research Activities during the first reporting period.

Partner 1 was involved in 7 different work packages as stated above.

WP1 : Activities will be dealt with under programme management and is made up of Appendix I and II the minutes of coordination meetings. Proposed changes are shown in red in the above flow diagram and concluded phases in green

WP2: Activities concerned with the the fish sampling included coordination of the distribution of the samples of plasma and muscle to the various participants. All frozen samples were sent to Düsseldorf first where they were logged and then distributed to other partners. Partner 1 will continue with this work and be responsible for correlation and distribution of the stored fishing samples.

WP3:

Details of the work carried out for this workpackage are given in the “Stability Protocol Report” in Appendix III. Briefly It can be concluded that the stability of testosterone in muscle samples as shown by the results in this report is high. It will be more than adequate therefore to store samples on ice before freezing.

Guidelines:

1. The amount of muscle required for a standard test is at least 100 mg.
2. Muscle taken from the ventral surface posterior to the cloaca avoiding fatty cutaneous layers.
3. Since most fish arrive dead on board only dead samples will be taken.
4. Time of sampling should be directly after catching.
5. Fish should be stored on ice if possible.
6. Muscle samples may be kept on ice and then stored at -20°C on return to the laboratory.

WP7:

COLLECTION OF PLASMA FOR VITELLOGENIN PURIFICATION

Blood samples was obtained in reproductive season during 29th May to 6th of July 2000 from female swordfishes with fish fork length (L_F) ≥ 120 cm, which may be considered as adults and in vitellogenesis. All fish were caught commercially by drift nets in the North Ionian (Gulf of Taranto, Campomarino). Juvenile females ($L_F \leq 100$ cm) and males ($L_F \geq 120$) were also sampled as controls. Soon after capture, L_F was measured and sex determined by macroscopic observation of gonads. Blood was collected from the heart with heparinized syringes and cannula (longline fish). Syringes, cannula and were been rinsed in advance with a solution containing 200 mM NaCl; 8.6 KCl, 8000 I.U. ml^{-1} Sodium heparin and 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma), pH 7.3. Blood was kept on ice after sampling at sea and then centrifuged at $5000 \times g$ for 15 min. Plasma was collected using a plastic pipette and stored at -20°C in the laboratory (usually < 4 h after capture).

IDENTIFICATION AND PURIFICATION OF Vtg

The swordfish Vtg was identified and purified as described by Susca *et al.*, (2001). In Fig. 1 & 2 is shown the purification patterns and in Fig. 3 the characterization in SDS chromatography. The purification was carried out on an ion-exchange column (Fig. 1) and the purity of the protein solution was tested with gel chromatography (Fig. 2). The molecular weight of Swordfish Vtg was calculated to be 594 kDa with this method.

A mature female specific protein eluting at 20.57 ml on the Resource Q column was identified as the same protein as that eluting at 13.71 ml on Superose 6 column. It was present in some male and immature female plasma. The separation of the female specific protein appeared as a relatively symmetrical single protein peak on Superose 6 (Fig. 2). The protein showed a major band of about 188 kDa on SDS discontinuous gradient PAGE (Fig. 3). The plasma samples were treated with 2-mercaptoethanol to obtain a better resolution.

The purified putative Vtg was deemed to be satisfactorily pure by gel filtration chromatography and electrophoresis and was used to raise the antiserum. The purification procedure yielded 9 mg ml^{-1} of swordfish-Vtg.

After successful purification antiserum against swordfish Vtg was produced in New Zealand rabbits and taken after 115 days of immunisation according to Susca *et al.*, (2001). An ELISA was established for further determination of the relative amount of Vtg in plasma and muscle samples. The Anti-sera used in this method had to be immuno-precipitated with plasma samples from male bluefin tuna.

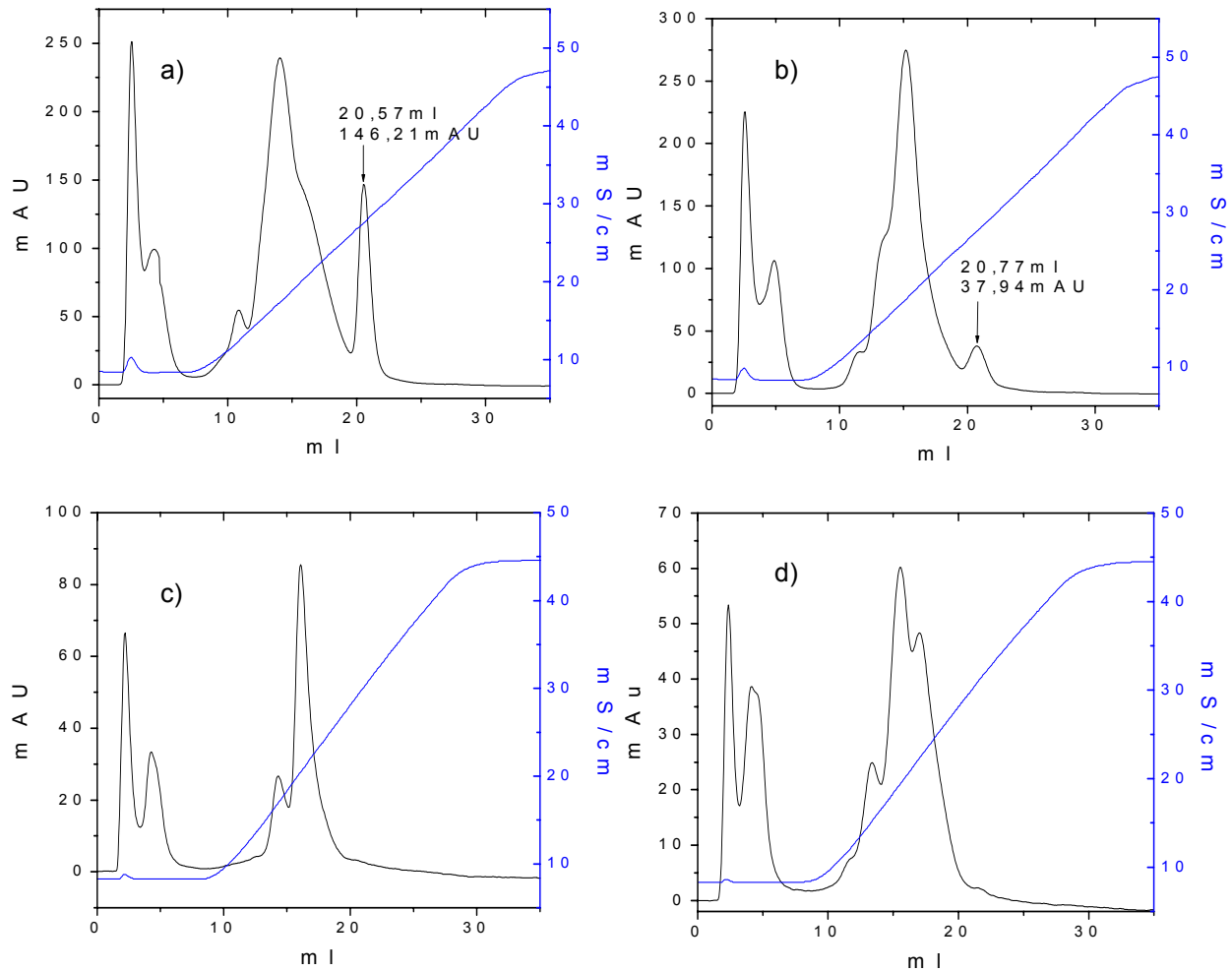


Fig. 1: Elution profiles from Swordfish plasma. The sample was precipitated with H₂O and re-suspended in Running-Buffer. Vitellogenin appears in female fish after 20,53 ml as a single sharp peak (a). The protein was also found in male fish and eluted after 20,77 ml (b). In (c) and (d) plasma samples from female, male fish respectively, were applied onto the column, whereas the protein was not present in this samples. The purification was carried out on an ion-exchange column (Resource Q, 1ml). The elution gradient for the Resource Q column was made from 0,07 mM NaCl (0 %) to 0,5 mM NaCl (100 %) with Eluent B. For further analyses and immunisation and antibody generation in New Zealand rabbits the fractions under the peak in (a) were collected, pooled and concentrated.

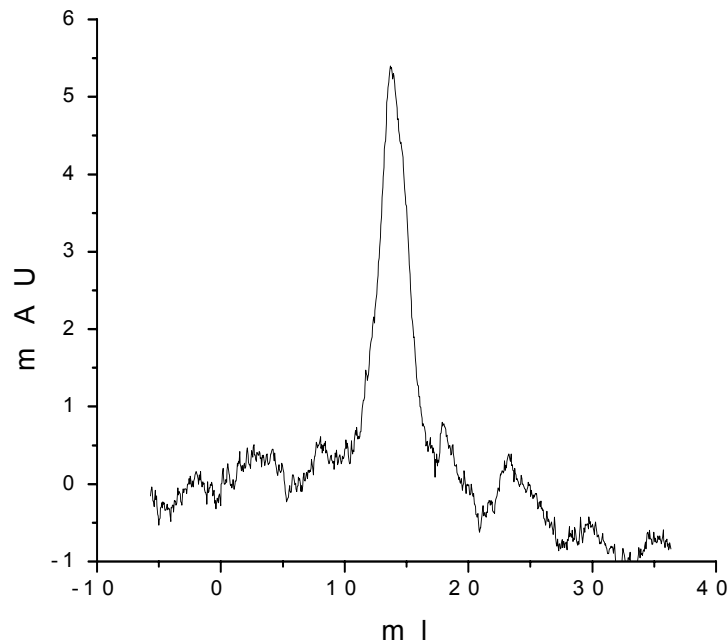


Fig. 2: Elution profile from purified Vtg. The fractions eluted with the peak (fig. 1 (a)) were collected and applied to a Superose 6 gel chromatography column. Vtg appeared as a single sharp peak after

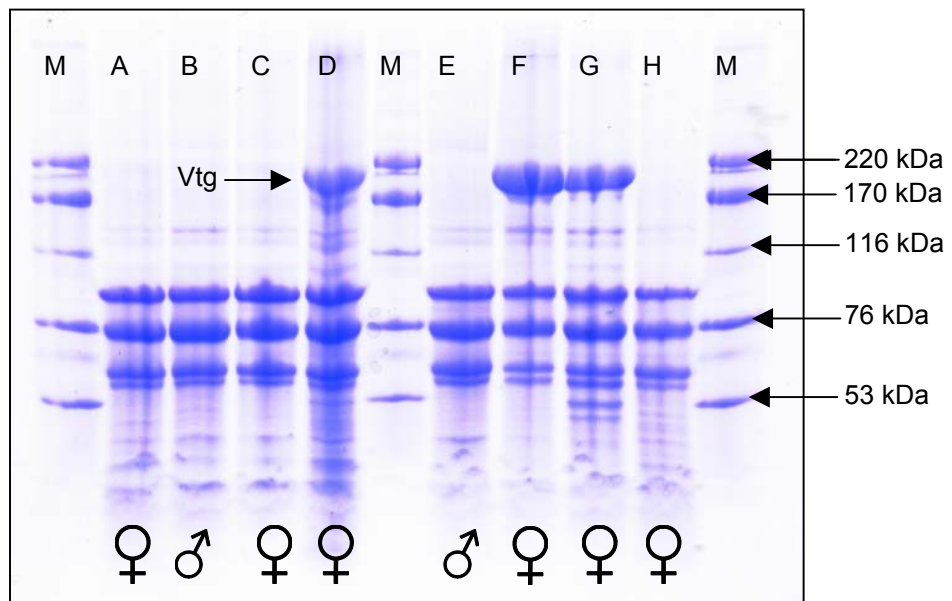
13,71 ml. The molecular mass was calculated with 594 kDa. As molecular markers Aldolase (158 kDa; $K_{AV} = 0,589$), Katalase (232 kDa; $K_{AV} = 0,566$), Ferritin (440 kDa, $K_{AV} = 0,49$) und Thyroglobin (669 kDa; $K_{AV} = 0,354$) were used.

RESULTS OBTAINED IN THE FIRST REPORTING PERIOD

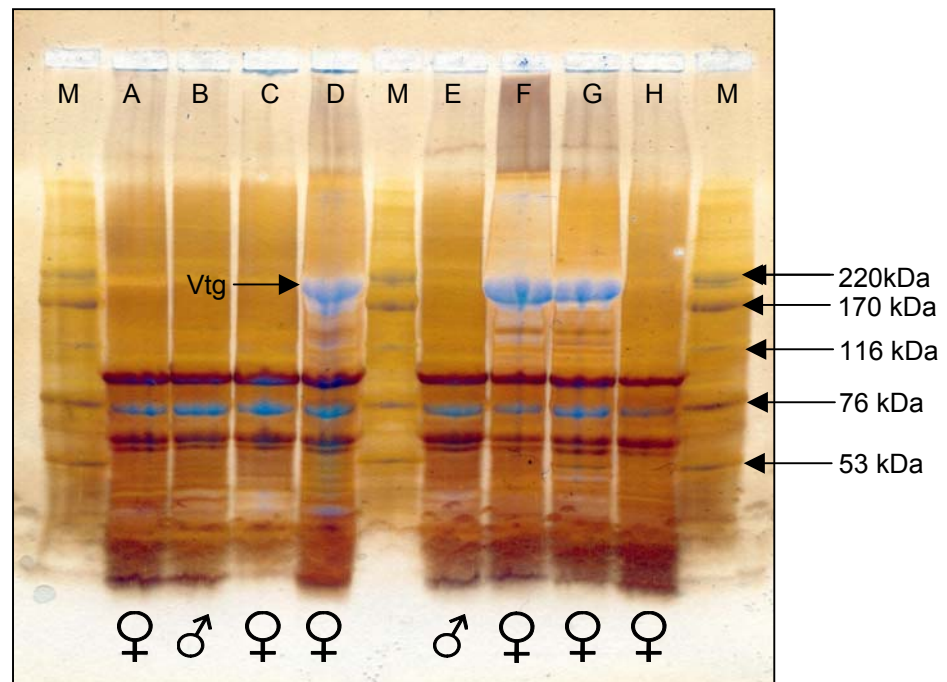
In Western Blot analysis the swordfish-Vtg manifested high specificity to vitellogenic female plasma corresponding to the purified swordfish-Vtg.

The ELISA for swordfish-Vtg showed a competitive binding curve for vitellogenic female plasma and Vtg standards (Fig. 4). Male swordfishes and human plasma showed a cross-reactivity at low dilutions but not at high working dilutions and showed no competitive curve (Fig. 4). The logit/log transformation demonstrated the similarity between vitellogenic female plasma and the Vtg standard, are both showing nearly the same slope (Fig. 4c). The ELISA is now ready for further use to measure Vtg concentration in plasma and muscle extract.

It was realized that Vtg was not specific to only mature females but also in some males. This raises some new questions on the reproductive biology of swordfish in the Mediterranean. Similar results have been reported from the gonad morphology workpackage. These results have to be verified to see if the hermaphroditism is a natural physiological status or if pollutants induce vitellogenesis in male fish.



a)



b)

Fig. 3: Electrophoretic patterns in SDS PAGE of blood plasma.

(a) In horizontal discontinuous gradient SDS PAGE (4%-15%) with 2-mercaptoethanol Vtg appears as a major band of 188 kDa. The protein components were stained with Coomassie Brilliant Blue. As molecular marker HMW-SDS-Marker (Amersham Pharmacia Biotech, Germany) was used.

(b) The gel was additionally Silver stained (brown colour). Vtg could not be stained by this method and appears as Coomassie Blue stained bands.

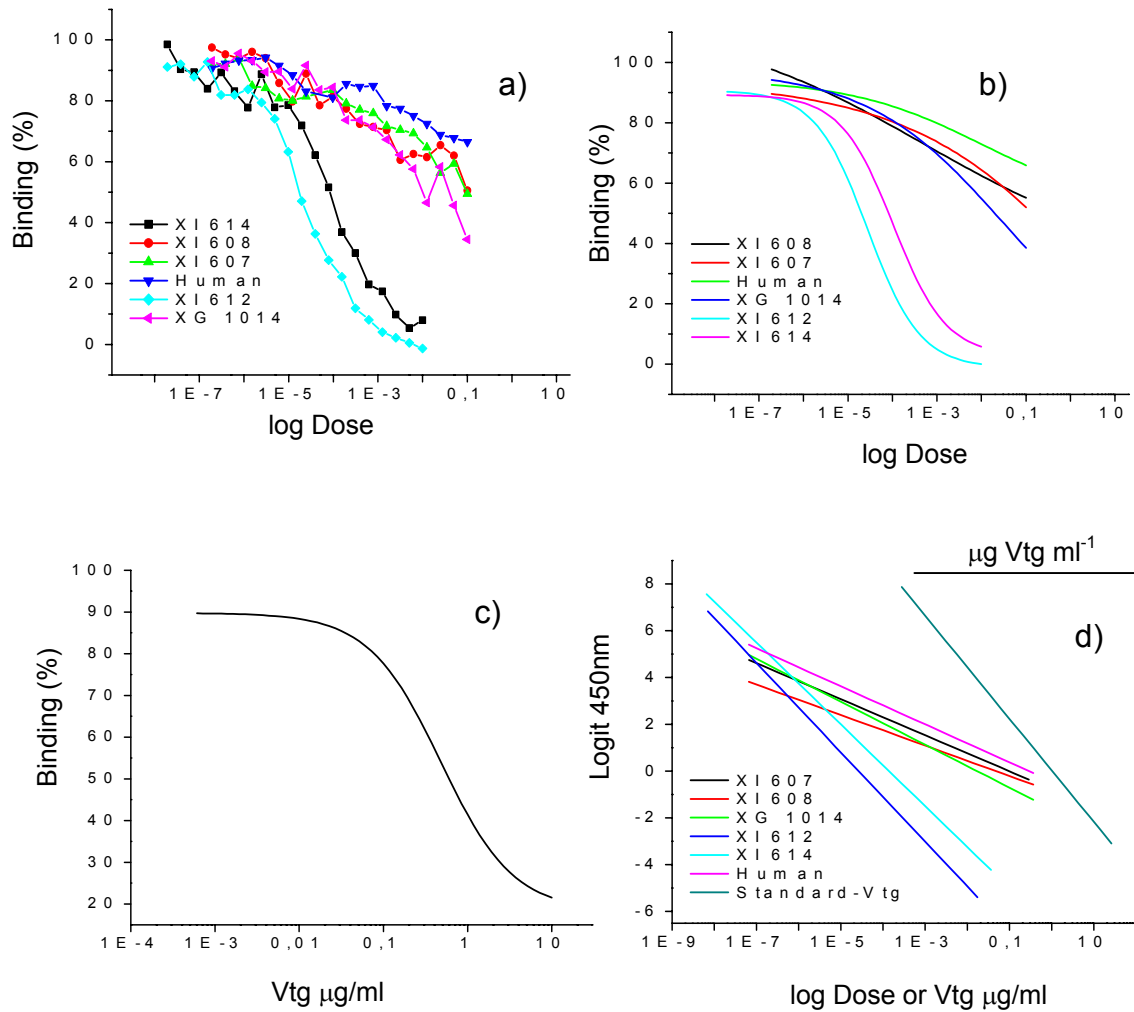


Fig. 4: Competitive binding curves of purified swordfish-Vtg, samples of plasma and human plasma. (a) Binding curves of different plasma samples from male and female fish and from human plasma in serial dilution of plasma (dose). (b) Competitive binding curve of purified Swordfish Vtg (standard curve). (c) Sigmoidal fit of curves shown in (a). (d) Corresponding logit/log transformation ($y = -\ln(B_i/B_0)/B_i$) of competitive curves shown in (a) and (b). WP 8 has been delayed until July when all the samples from fishing season I have been assessed in terms of gonad histology, steroid levels in plasma and muscle and vitellogenin in plasma and muscle.

PARTICIPATING ORGANISATION Nr: 2
UNIVERSITY OF SHEFFIELD

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Fax: +44 114 222 0002
e-mail d.kime@sheffield.ac.uk

Scientific Team

Dr. David KIME

Contractual Links to other participants: None

Objectives

Research and team involved:

David KIME (Senior Experimental Officer) : General supervision of the steroid hormone part of the project (approx. 6 person-months). Salary is funded by the University of Sheffield. This partner will be workpackage leader for WP 6.

Technical Assistant: Funded by the project (19 person-months). The assistant appointed, who will have expertise in fish reproduction, will be responsible for the experimental studies in measuring steroid hormones and the setting up of the ELISA tests at the University of Sheffield.

Support Services: Full secretarial support is provided by the Department.

Contribution of the participating organisation:

The University provides salary costs of the senior scientist, laboratory facilities, full library, computing, technical and secretarial back-up.

Workpackages (With person-months)

WP1 (1); WP6 (17); WP7 (5), WP8 (1); WP9 (1)

See actual workpackages and figures 1 and 2 and tables 1 ,2 and 3 for details of methods and deliverables

Workplan Participant 2 with WP's and Deliverables

	Year 1												Year 2															
	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Part 2	WP-1 Coordination																											
	WP6- Steroid Hormones																											
	WP7- Vitellogenin																											
													WP8- Correlation															
													WP9- Report Prep															
Deliver.	1									2		16										10	15	3	10	15	3a	
										9												13		17	13		17	
										12							12											
										14																		

Research Activities during the first reporting period.

Workpackage 6 - Measurement of steroid hormones

At the start of the project acetylcholinesterase labels were prepared for estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT) using established methodology (Cuisset, B., Pradelles, P., Kime, D. E., Kühn, E. R., Babin, P., Davail, S. and Le Menn, F. (1994). Enzyme immunoassay for 11-ketotestosterone in plasma of Siberian sturgeon. *Comp. Biochem. Physiol.* **108C**, 229-241; Nash, J. P., Davail-Cuisset, B., Bhattacharyya, S., Suter, H. C., LeMenn, F. and Kime, D. E. (2000). An enzyme linked immunosorbant assay (ELISA) for testosterone, estradiol and 17,20 β -dihydroxy-4-pregnen-3-one using acetylcholinesterase as tracer: application to measurement of diel patterns in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **22**, 355-363). Conditions were established for standard curves for assay of these hormones. Samples of plasma and muscle tissue were collected from swordfish and sent to Sheffield via the co-ordinator in Düsseldorf. A considerable number of the samples consisted of whole blood rather than plasma and were impossible to assay. Of those received 138 plasma samples were assayed for T, E2 and 11KT. Approx. 50 further samples have been received in the last few weeks from the Spanish catch and will be assayed within the next month. We did not assay for the maturational inducing steroids 17,20 β P or 20 β S since macroscopic examination indicated that few fish were at the stage of oocyte final maturation. Muscle biopsy samples were also received, but many of the early samples were not satisfactory and contained a high proportion of fat and skin. Modified protocols for collection of plasma and muscle improved the quality of the later samples for the first fishing season and a full discussion was held at the co-ordination meeting in Athens in January 2001 which should ensure better quality samples for the 2001 sampling season. Several methods were tested for preparation of muscle tissue for analysis. Glass homogenisers as designed in Düsseldorf were insufficiently robust and we have found that use of an Ultraturrax T25 homogeniser produced the most satisfactory results. As a standard method 100 mg tissue was homogenised in 0.5 ml buffer (20 mM Tris/HCl, 2% NaCl pH 8) at the full power setting. The homogenising tool was then washed (0.05 % Tween, 0.02 M potassium phosphate saline, distilled water, pH 7.4) and rinsed with distilled water between each sample. The homogenate was centrifuged at 5000 rpm, and then extracted once with 5 ml dichloromethane, and the solvent evaporated. Tissue homogenates have been extracted and will be assayed in the next month.

Results obtained in the first reporting period:

Since full histological data is not yet available, data is based only on macroscopic examination of gonads of sampled fish. We are already aware from the histology so far completed that many of the macroscopic examinations have given erroneous results for sex and maturational stage. In particular there is often a confusion between post-spawned regressing fish and those at an early stage of recrudescence which may have very similar gonadosomatic indices (GSIs). The data presented in this report will be re-interpreted when all of the histological data is available.

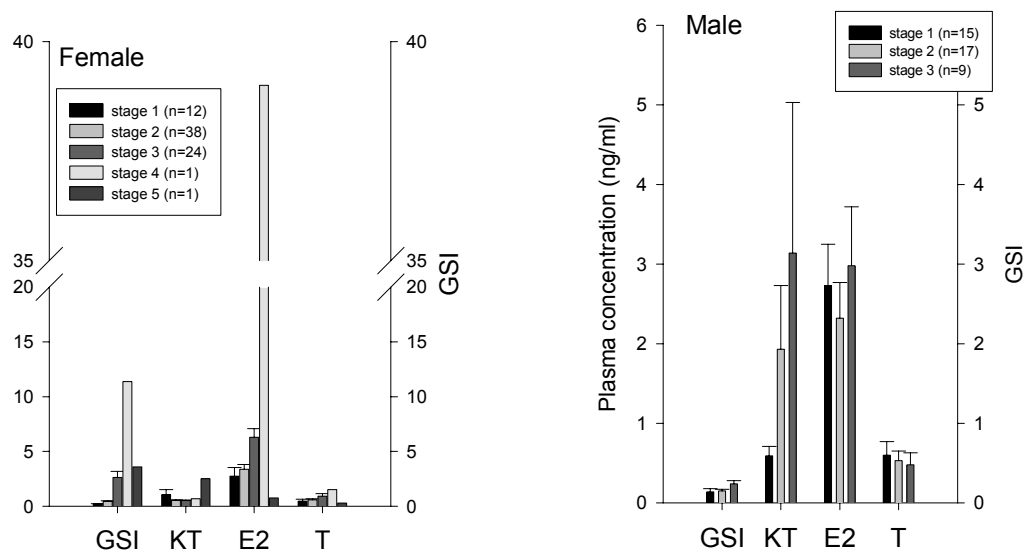
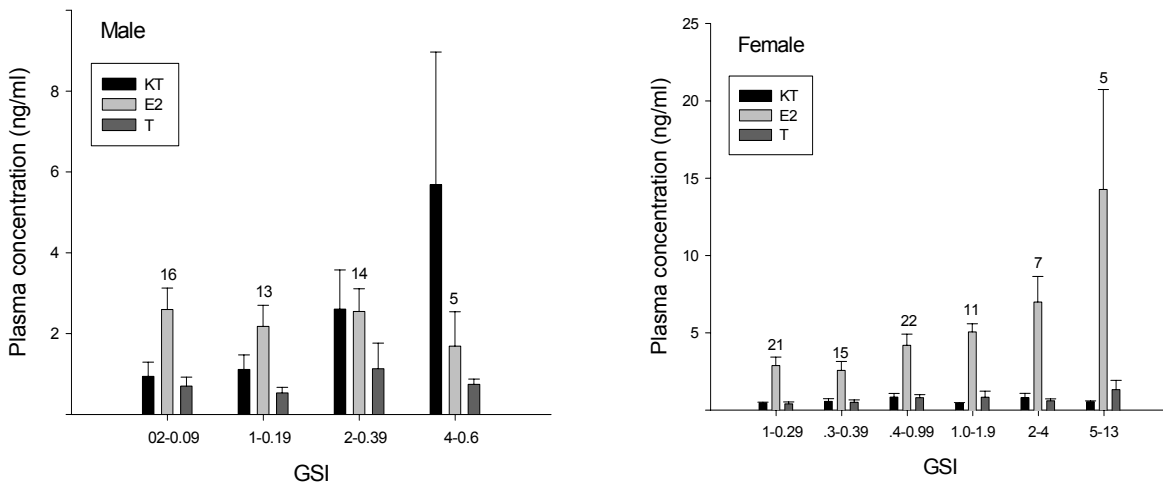
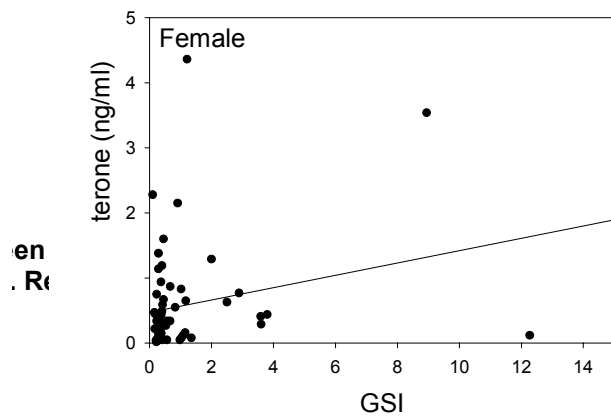
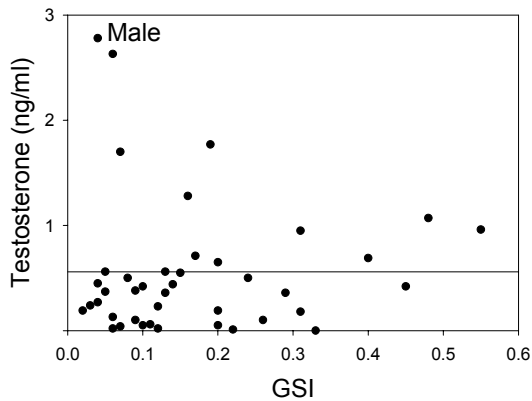
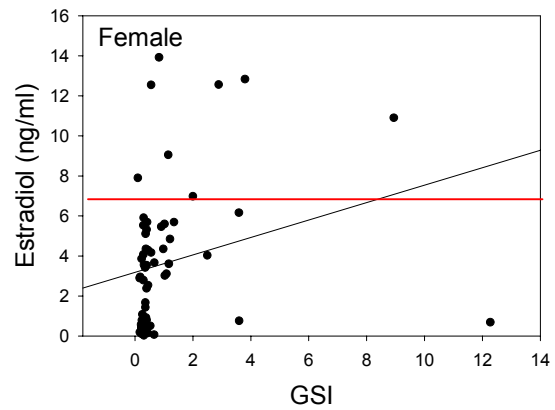
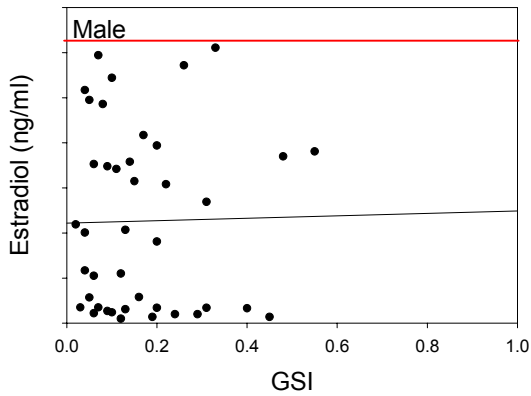
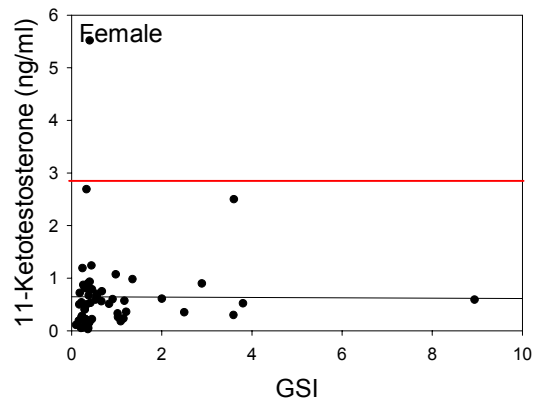
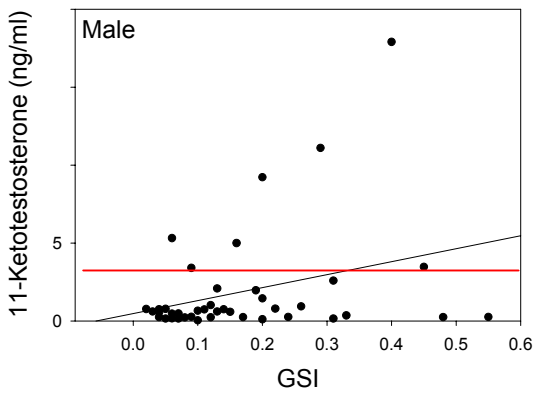


Fig. 1. GSI and plasma steroid levels in male and female swordfish. (mean ± SEM) arranged according to macroscopic stage of gonadal development



The steroid data obtained is consistent with previous reports on seasonal hormone levels in showing an increase of 11KT in males and of E2 in females during gonadal recrudescence (Figs 1, 2). It was, however, apparent from the data that there were some stage 1 fish with exceptionally high GSI, while some at Stage 3 had GSIs more typical of Stage 1 fish. We have therefore plotted steroid concentrations according to both Stage (1-5) (Fig. 1) and according to ranges of GSI (Fig. 2). Unfortunately we had only one female at each of Stage 4 and 5 and no males greater than Stage 3. The patterns observed in both Figs. 1 and 2 support our suggestion that 11KT and estradiol might be suitable indicators of sexual and reproductive status. 11KT was high (>3 ng/ml) in Stage 3 males or those with GSI>4, while E2 was high (>5 ng/ml) in Stage 3 females and those with GSI> 1. Mean 11KT was below 3 in all except one female and mean E2 below 3 in all males.



The standard errors in Figs 1 and 2 are comparable to those in similar studies, but examination of individual data shows that the range of values is very high as shown in the scatter plots for individual fish (Fig. 3). Regression lines show a significant correlation between 11KT and GSI in males ($P < 0.01$) but not females, and between GSI and E2 in females ($P < 0.001$) but not males. Testosterone was not correlated with GSI in either sex and is probably not a useful indicator for the purpose of this project. There are few previous data for E2 in males or 11KT in females as these hormones are rarely measured due to the common conception that they are zero, but there is increasing evidence from other workers that significant amounts of E2 are found in some males and 11-ketotestosterone in females (personal communications).

It is not clear whether such “abnormal” hormones are natural or the result of endocrine disruption. It may be relevant that some of the fish caught in this sampling season showed evidence of intersex gonads (See Workpackage 5 and 7). All fish with E2 >6.16 ng/ml were females, while 9/10 of the fish with 11KT > 3.0 ng/ml were males according to macroscopic examination. There was an apparent shortage of sexually mature specimens in the fish so far analysed and at the moment maturity stage cannot be reliably assigned from the macroscopic data available.

The above data will be re-analysed when all samples have been assayed and when histological data becomes available.

PARTICIPATING ORGANISATION No.3 (EL)
UNIVERSITY OF ATHENS

Address

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Panepistimiopolis, Athens, GR 15784 (GREECE)

Scientific Team

Maria Apostolopoulou (Professor)
Persefoni Megalofonou

Contractual Links to other participants: None

Objectives

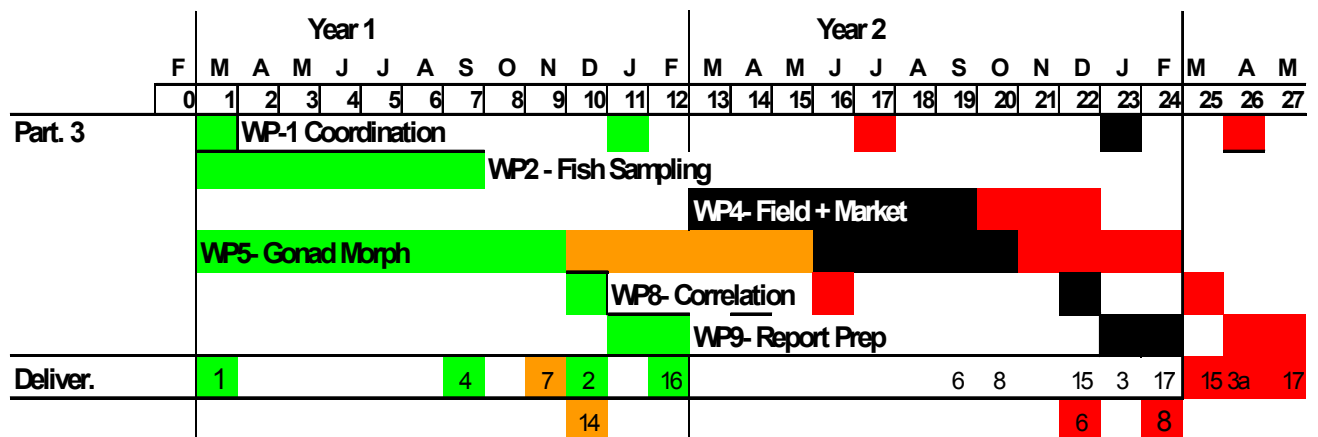
This partner will be responsible for fish sampling, biometrics and providing plasma, muscle tissue and gonadal morphometrics for 200 fish over the two year period. This partner will also assist in the collation of other swordfish fishery data.

Workpackages (With person-months)

WP1 (1); WP2 (7); WP4 (11), WP5 (10); WP8 (1);WP9 (1)

See actual workpackages and figures 1 and 2 and tables 1 ,2 and 3 for details of methods and deliverables

Workplan Participant 3 with WP's and Deliverables



Research Activities during the first reporting period.

Detailed Report of Swordfish Sampling in Greece

Swordfish blood sampling in Greece was carried out on professional fishing boats using the standard or American type swordfish longline. Their home port was Kastelli in Crete island (S. Aegean Sea) and their activities cover a vast area : S. Aegean Sea, S. Ionian Sea and Levantine basin (S. Crete, Libya, Egypt, Cyprus).

Fishing trips varied from 1 to 15 days depending on the weather, the catches and fuel tank capacity. Their distance from the coast was usually more than 10 miles, it's an open sea fishing activity. They rarely fished in coastal areas and therefore spend the night out in the sea.

Because of this, successful blood sampling in Greece could be accomplished only by choosing the boats that return at their home harbor within 24 hours or spend the night in a nearby harbor so that centrifugation and extraction of serum is possible. This was not an easy task since : Fishermen don't schedule their trips, but make decisions "on the spot" based on weather, remaining fuel and catches. Entering a harbor during night is a 1 to 2 hours delay in the morning longline retrieval and extra fuel consumption. Although numerous times they return close to land, they do not "catch" on the dock, staying anchored 100-200 meters off the coast. It's a "safety measure" against port police inspections. Some small harbors are more a night refuge and don't have even the essential facilities like water and electricity.

SAMPLING TIME SCHEDULE

Situation 1 – Spending the night in a harbor or returning to home harbor (serum available)

13:00	Depart from home harbor – Steam to setting position
16:00	Start longline setting
19:00	End of longline setting – Course to nearby night harbor
21:00	Arrive to night harbor

Spending the night

Next day

05:30	Depart from night harbor – Detect the longline transmitter-repeater
07:00	Start longline retrieval. Samples on Board (Immediately fish blood, muscle, gonad and liver samples were taken on board). Details as in Protocol. Samples kept on ice.
13:00	End of longline retrieval.
13:00 – 16:00	Head to new fishing destination, rest.
16:00	Start longline setting
19:00	End of longline setting – Course to nearby night harbor (or home harbor if trip is over)
21:00	Arrive to harbor. Centrifuge samples.
22:00	Place serum in boat's deep freeze (or in cooperatives deep freeze if in home harbor).

(Above schedule may be continued till fishing trip is over)

Situation 2 – Spending the night out in the sea (only whole blood available)

Day 1 Depart from home harbor – Steam to setting position (Trip may take as much as 2 days - e.g. : off the coast of N. Africa)

Day 2 (or 3)

17:00	Start longline setting
21:00	End of longline setting – Preparation to spend the night on board

Spending the night out in the sea, few miles from the gear – night shifts on deck

Next day

06:30	Detect the longline transmitter-repeater
07:00	Start longline retrieval. Samples on Board (Immediately fish blood, muscle, gonad and liver samples were taken on board). Details as in Protocol. Samples kept on deep freeze.
14:00	End of longline retrieval.

14:00 – 17:00	Head to new fishing destination, rest.
17:00	Start longline setting

21:00 End of longline setting – Preparation to spend the night on board

(Above schedule may be continued for several days till fishing trip is over)

Sampling summary description in Greece

Fishing trips vary from 1 to 15 days depending on the weather, the catches and fuel tank capacity. Their distance from the coast is usually more than 10 miles, it's an open sea fishing activity. They rarely fish in coastal areas and therefore spend the night out in the sea.

Because of this, successful blood sampling in Greece can be accomplished only by choosing the boats that return at their home harbor within 24 hours or spend the night in a nearby harbor so that centrifugation and extraction of serum is possible. This is not an easy task since :

- Fishermen don't schedule their trips, but make decisions "on the spot" based on weather, remaining fuel and catches.
- Entering a harbor during night is a 1 to 2 hours delay in the morning longline retrieval and
- Extra fuel consumption.
- Although numerous times they return close to land, they do not "catch" on the dock, staying anchored 100-200 meters off the coast. It's a "safety measure" against port police inspections.
- Some small harbors are more a night refuge and don't have even the essential facilities like water and electricity. (Occasionally samples have been centrifuged in a local police station or tavern!)

*) *Safe and reliable centrifugation can be accomplished only on solid ground with the centrifugation device in a horizontal position. Slight shaking or vibrations during operation may result in poor plasma separation.*

SAMPLING TIME SCHEDULE

Situation 1 – Spending the night in a harbor or returning to home harbor (serum available)

13:00 Depart from home harbor – Steam to setting position
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 19:00 End of longline setting – Course to nearby night harbor
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 21:00 Arrive to harbor. Centrifuge samples.
 22:00 Place serum in boat's deep freeze (or in cooperatives deep freeze if in home harbor).

(Above schedule may be continued till fishing trip is over)

Situation 2 – Spending the night out in the sea (only whole blood available)

Day 1 Depart from home harbor – Steam to setting position (Trip may take as much as 2 days - E.g. : off the coast of N. Africa)

Day 2 (or 3)

17:00 Start longline setting
21:00 End of longline setting – Preparation to spend the night on board

Spending the night out in the sea, few miles from the gear – night shifts on deck

Next day

06:30 Detect the longline transmitter-repeater
07:00 Start longline retrieval. Samples on Board (Blood, muscle, gonads, liver). Details as in Protocol. Samples kept on deep freeze.
14:00 End of longline retrieval.

14:00 – 17:00 Head to new fishing destination, rest.
17:00 Start longline setting
21:00 End of longline setting – Preparation to spend the night on board

(Above schedule may be continued for several days till fishing trip is over)

Sampled Data Statistics (2000)

General Data	
Sampling Months	6
Sampled Fish	84

Blood sampling Data	
	No
Total	64
Whole blood	26
Serum	38

Tissue Data	
	No
Ventral area muscle collected	69

Liver Data	
	No
Liver histological sections (EE)	3
Liver in formaline	80

Gonads Data	
	No
Total	79
Gonades in formaline	79
Gonades frozen sample	54
Gonads histological sections (EE)	22
Gonads histological sections (Domagk)	43

Sex & Size Data	LJFL (cm)				
	No	Min	Max	Average	S.D.
Total	84	70,7	182,5	123,5	26,0
Male	45	82,0	175,0	118,3	21,5
Female	35	70,7	182,5	129,7	31,4

Maturity Data	LJFL (cm)				
	No	Min	Max	Average	S.D.
GSI	79	0,032	6,338	0,389	0,813
GSI Male	44	0,032	0,638	0,170	0,139
GSI Female	35	0,038	6,338	0,680	1,176

Sex Determination	
Accordance in Macroscopic-Microscopic Sex Determination (65 samples)	100%

PARTICIPATING ORGANISATION No. 4 (E)
 INSTITUTO ESPANOL DE OCEANOGRAFIA

Address

Centro Oceanografico de Malaga
 Apartado 285,
 29640 Fuengirola, Malaga (SPAIN)

Scientific Team

Josè Miguel de la Serna

Contractual Links to other participants: None

Objectives

This partner will be responsible for fish sampling, biometrics and providing plasma, muscle tissue and gonadal morphometrics for 200 fish over the two year period. This partner will also assist in the collation of other swordfish fishery data

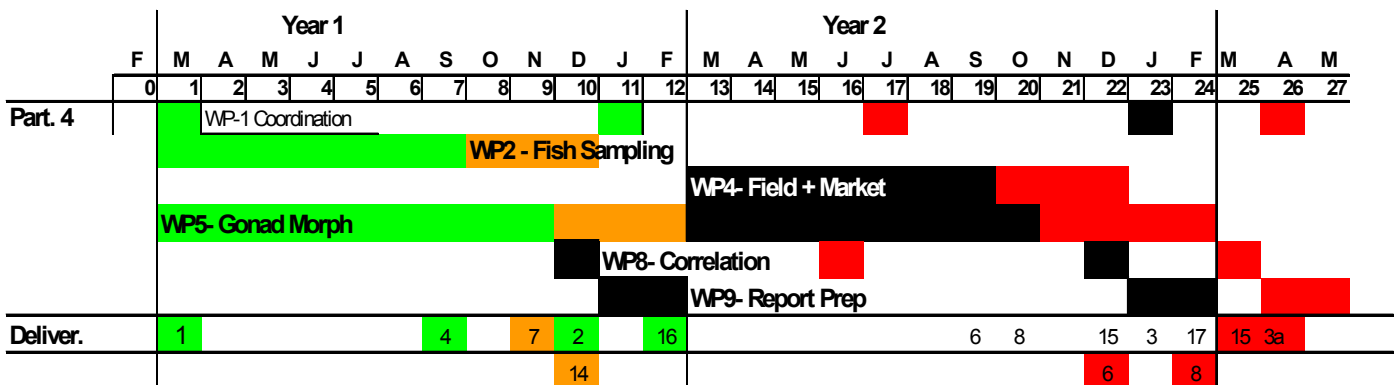
Josè Miguel de la Serna, is the scientist responsible for the Investigation Project on Mediterranean Tuna at IEO (C.O. de Malaga-Fuengirola). He is member of the Bluefin tuna and Swordfish stocks evaluation groups of SCRS and ICCAT, as well as participant in Consultation CGPM/ICCAT and several European Project (DG-XIV-CEE). He is also concerned with tagging campaigns; working as Chief of the campaigns carried out in the Mediterranean and Cantabric Seas (Bluefin tuna and Albacore).

Workpackages (With person-months)

WP1 (1); WP2 (5); WP4 (6), WP5 (3); WP8 (1);WP9 (1)

See actual workpackages and figures 1 and 2 and tables 1 ,2 and 3 for details of methods and deliverables

Workplan Participant 4 with WP's and Deliverables



Research Activities during the first reporting period.

DETAILED REPORT OF THE SWORDFISH SAMPLING IN SPAIN

The sampling in Spain was carried out on board of commercial vessels using the standard long line, the semi-pelagic long line piedra-bola and traps.

Their base ports were Carboneras and Águilas in the Southeast Mediterranean Spanish coast and they operate over a vast area of the Balearic and Alboran sea; and the traps of Tarifa (Southwest Spanish Atlantic coast).

Fishing trips varied from 1 to 15 or more days depending upon weather, catches, tonnage and autonomy of the vessels. The fishing areas extend from areas near to the coast to the open sea. Usually the long-line operates during darkness and the vessels arrive in port during the daytime. The long line remains in the water for about eight hours.

The fishes were not gutted on board, so the sampling of liver and gonad tissues only was possible to be done at landing. Usually the blood and muscle sampling were done on board after the fish was carried on board. Only the damaged fishes were gutted on board and in these scarce case the sampling was completed on board.

ON BOARD INCIDENCES:

It was only possible on board to obtain fresh blood, but:

- The trips were usually longer than one day and the vessels had not goods refrigerators on board, so even if the observer had a centrifuge on board and he could obtain a very good plasma samples these samples were not preserved adequately.
- In several cases the observers decided to take the blood samples only the previous days to landing at pilot ports in order to obtain a better plasma samples and optimise they conservation, but they haven't got a good refrigerator, and the blood or plasma samples was on board more than three or four hours before to be frozen at -20°C .
- During the sampling period the centrifuge was damaged so the observer only can obtain complete blood samples and no plasma ones.
- So, only the blood which was extracted a few hours before they enter harbour was useful like plasma or serum.

AT LANDING INCIDENCES:

Only at landing was possible to obtain complete samples consisting of plasma, muscle, liver and gonad, but:

- We have a very good centrifuge, we have refrigerator (even a liquid nitrogen tank), but we didn't get fresh fish to sample. Nobody gutted the swordfish on board. The swordfish only were gutted in the second sales market, and in some scarce cases in the first sales markets, so the fish wasn't very fresh, but we made a complete sampling.
- In several cases the observers tried to trace the swordfish sampled on board in order to complete the sampling at landing but in all the occasions they wasn't be possible.
- In the last sampling, we contacted with a company that carries the fish directly to trucks, but the swordfishes were fished one, two, three or more days before. In this case, the freshness of the fish was the same that in the previous case.
- Only in traps was possible obtaining good complete samples including plasma ones but the traps only operate during the short period from May to June.

In summary, the circumstances under which the sampling have been carried out during the last year have been very diverse but in any case complicated. The non-gutting of the fishes on board complicate obtaining complete samples. The deficiency of basic infrastructures on board make difficult the conservation of the obtained plasma samples. The sampling circumstances it does not seem that they are going to be different throughout this year, although will try the improvement of the conservation of the samples on board by means of the use of liquid nitrogen tanks and the selection of vessels with a single day trips.

PARTICIPATING ORGANISATION N° 5

UNIVERSITY OF BARI

Address

Department of Animal Production,
University of Bari,
Via Amendola, 165/a -
70126 Bari,
Italy

Scientific Team

Prof. G. DE METRIO

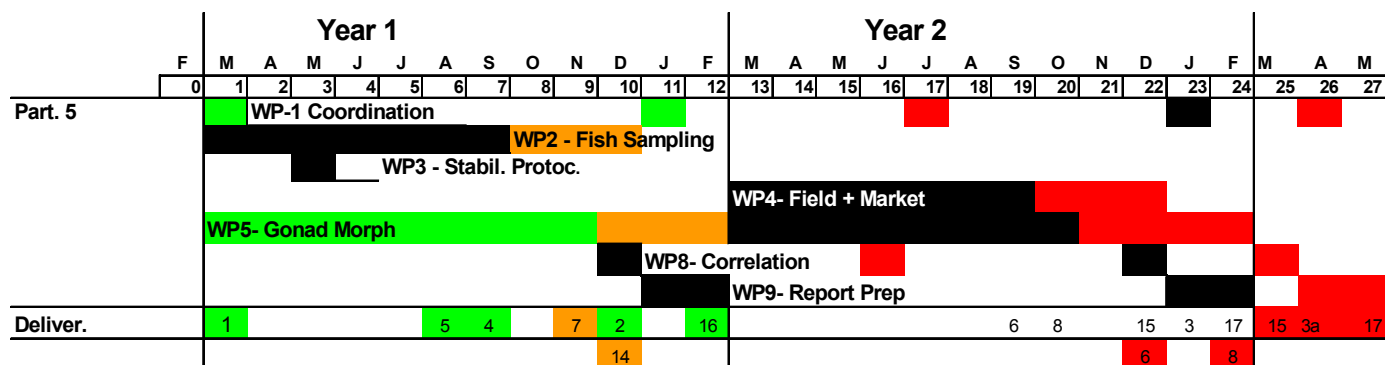
Contractual Links to other participants: None**Objectives**

This partner will be responsible for fish sampling, biometrics and providing plasma, muscle tissue and gonadal morphometrics for 200 fish over the two year period. This partner will be workpackage leader for WP 2, WP 4 and WP 5 . This partner will also assist in the collation of other Mediterranean swordfish fishery data

Workpackages (With person-months)

WP1 (1); WP2 (5); WP3 (1); WP4 (4), WP5 (4); WP8 (1);WP9 (1)

See actual workpackages and figures 1 and 2 and tables 1 ,2 and 3 for details of methods and deliverables

Workplan Participant 5 with WP's and Deliverables

Research Activities during the first reporting period.

WP 2: – Fish sampling

Start date: March 2000

N° of the partner responsible: 5

N°s of other partners involved: 1, 3, 4

Objectives

Fish sampling in the first season

- a) Procurement of Biometrics Data
- b) Procurements of Biological Samples

The sampling of swordfish biological material started in April 2000 in the following areas: North western Mediterranean along Spanish coasts, North Ionian Sea (Gulf of Taranto), Cretan and Lybian seas.

Plasma, muscle and gonad samples were taken from a total of 413 fish according to the sampling protocol.

From all the fish sampled, LJFL (length from the tip of the lower jaw to the caudal fork) and dressed weight were measured at the nearest centimetre and hectogram respectively. Gonad weight of most of the specimens sampled was measured at the nearest gram. Catch geographical co-ordinates and sea surface temperature were recorder whenever possible.

Detailed Report of Swordfish Sampling in Italy

In the North Ionian Sea, the sampling was carried out on board of boats using two different gears: long-line and drift net. A general description of the fishing operations and the characteristics of the gears used are reported below.

Swordfish long-line (SWO-LL)

The fishing period generally begins in the month of May and lasts until the end of September.

The gear used in the North Ionian Sea to catch swordfish has a main line whose length ranges between 28000 and 50000 m and is equipped with about 1000-1800 hooks with a length of 7 - 10 cm (No 3 - 0). The diameter of the main line ranges between 1.6 and 1.8 mm, the length of the branch line is 6 m, 1.4 mm \varnothing if it is monofilament or 1.2 mm \varnothing if it is double monofilament, and the distance between the branch lines is 28 m. Frozen *Scomber scombrus* is mainly used as bait.

Generally the boats leave the port at 2:00 pm and reach the fishing areas after a 3-4 hours trip. Then the gear is shot and the shooting operation is over before midnight. The retrieval operation begins early in the morning, from the last hook put at sea, and it lasts for a number of hours (from 6 to 9), depending on the length of the portion of the gear in the water, on the conditions of the sea and on the quantity of fish caught: therefore, the first hook remains underwater for about 12 hours, whereas the last one only one or two hours. Since the depth where the hooks lie depends on the construction of the gear, on the distance of the catch branch line from the floats and on the intensity of the sea currents, it has been estimated that the hooks are put in at a theoretical depth of 15 m, even if a depth-range between 0 and 75 m has been noted during observations carried out using depth-sensors.

Drift net (DN)

Since 1998, according to EU regulation, the traditional net (spadara) has been abandoned and a new net, called "ferrettara", has started to be used. It is smaller than the previous one and its characteristics are the following: the length is 2500 m and height ranges from 18 to 25 m with a mesh of 180 mm.

Generally the boats leave the port at 2:00 pm and reach the fishing areas after a 3-4 hours trip. Then the gear is shot, and the shooting operation lasts about 3-4 hours. The gear retrieval begins after midnight and it lasts for about five hours, depending on its length at sea, on the weather and sea conditions and on the quantity of fish caught.

The fishermen use the same gear to target both bluefin tuna and swordfish specimens; the first target species is mainly caught during the spring (March-May) and the second during both the spring and summer.

WP: 5 – Gonad morphometrics

Start date: March 2000

N° of the partner responsible: 5

N°s of other partners involved: 3, 4

Objective

To obtain complimentary data on GSI, gonad maturity, gonad histology and oocyte morphometry on the fish from which plasma and muscle samples have been taken.

Work done

A total of 302 gonad samples were taken from the sampled fish. Whenever possible, the gonads were removed soon after the capture of the fish and were weighted to the nearest gram for the calculation of gonado-somatic index according to the formula:

$$GSI = 100 \text{ GW/BW}$$

where: GSI = gonad-somatic index; GW = gonad weight; BW = dressed body weight.

Macroscopic maturity stage of the gonads was determined by means of the Mayer scale for partial spawning species.

Fragments of gonads were immediately fixed in 10% neutral formaline or Bouin's solution.

The fixed gonad samples were then dehydrated in ethanol and paraffin embedded.

Microtome sections (5 μm tick) were stained with histological (Hematoxylin-Eosin and Mallory's trichrome) and basic histochemical methods (Alcian, Pas, Pyronine). Oocyte diameters were measured on histological slides using Quantimet (Leica, Cambridge, UK) image analyser.

Ovaries and testes of 189 specimens have already been classified, according to the histological appearance, in 6 maturity stages (tabb. 1, 2). The gonads of 113 specimens have still to be classified.

Stage	GSI				Microscopic appearance
	N.	Mean (\pm S.E)	Min.	Max.	
1. Quiescent	2	0.798 \pm 0.048	0.750	0.846	Only primary growth oocytes.
2. Recrudescence	3	0,663 \pm 0,1506	0.500	0.964	Primary growth and cortical alveoli oocytes.
3. Ripening	11	2.017 \pm 0.338	0.897	3.795	Primary growth to yolked oocytes; atresia of yolked oocytes
4. Mature	6	9.581 \pm 1.824	1.389	14.243	Primary growth to FOM; atresia of yolked oocytes.
5. Post-ovulatory	1	3.667	3.667	3.667	POFs; atresia of yolked oocytes.
6. Regressing	19	1,356 \pm 0,233	0.400	3.947	Primary growth and cortical alveoli oocytes; late stage of atresia.

Tab. 1 - Gonad developmental stage for adult (LJFL \geq 140 cm) female swordfish. GSI, gonadosomatic index; FOM, final oocyte maturation; POF, post-ovulatory follicle.

Tab. 2 - Gonad developmental stage for adult (LJFL \geq 100 cm) male swordfish.

Stage 1 Immature/Quiescent	Prevalence of spermatogonia. Rare spermatocytes and spermatids. Low density of spermatids and spermatozoa could be observed in tubule lumina of young specimens caught during summer and autumn.
Stage 2 Early spermatogenesis	Prevalence of meiotic cysts. Rare spermatidic cysts and spermatids in tubule lumina.
Stage 3 Late spermatogenesis	Abundance of spermatidic cysts. Low density of spermatids and spermatozoa in tubule lumina.
Stage 4 Mature	High sperm density in tubule lumina. Decrease of both meiosis and germinal epithelium height.
Stage 5 Regressing	Sperm density decrease. Arrest of meiosis. Minimum epithelium height.
Stage 3' Autumnal spermatogenesis	Increase of germinal epithelium height. Renewal of meiosis. Low sperm density in tubule lumina.

Female mean GSI monthly trend showed the presence of a peak during June and July. This peak corresponds to the presence of specimens with mature gonads (fig. 1). The maximum GSI values observed during this period were 12,28 in June and 14,24 in July.

In males two GSI peaks were observed: one in July and one in October (fig. 2). The autumnal GSI peak was histologically correlated to a renewal of the spermatogenesis.

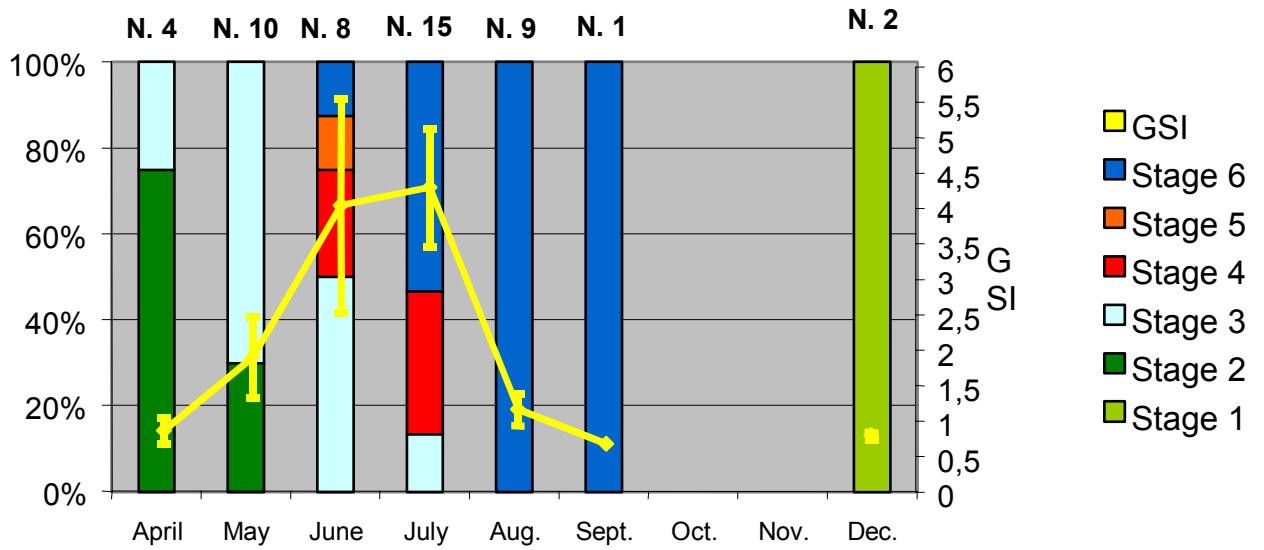


Fig. 1 – Monthly trend of GSI and histological maturity stage frequencies of adult female swordfish (LJFL ≥ 140 cm).

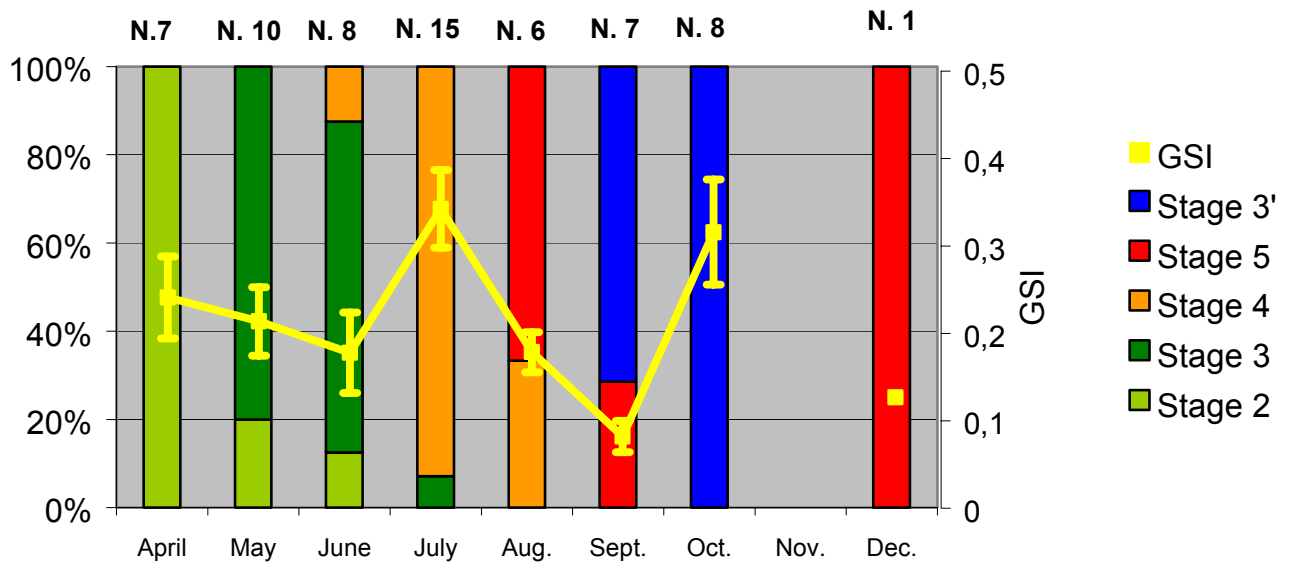


Fig. 2 – Monthly trend of GSI and histological maturity stage frequencies of adult male swordfish (LJFL ≥ 100 cm).

4. Project Management and Coordination

As can be seen from Figure 1 Plenary planning meetings took place in Düsseldorf in March 2000 and again in January 2001 at the university of Athens. A new date has been fixed for a third plenary planning meeting in the UK in July 2001 and a final meeting has been put back from January 2002 until April 2002. This is to allow for fish sampling corrections to be made during fishing season II. An up-to date workplan is shown in Figure 2 and this will be used for the next part of the project

A report is attached in Appendix I which gives details of the Agenda and the Minutes of the meeting in Düsseldorf. A similar report is provided in Appendix II with the agenda, the minutes and the appendices for the new fishing protocol are included.

5. Exploitation and Dissemination Activities

Publications

To date no publication have been made but from a previous project the basis for further publications has been provided.

V. Susca, A. Corriero M. Deflorio, C.R. Bridges, G. De Metrio (2000) New results on the reproductive biology of the bluefin tuna (*thunnus thynnus*) in the Mediterranean.
ICCAT Scientific Report : SCRS /00/91

C.R. Bridges, P. Schröder ,V. Susca A Corriero, M. Deflorio and Gregorio De Metrio (2000)
A New Muscle Biopsy Technique for Sex and Sexual Maturity Determination in Large Pelagic Fishes
ICCAT Scientific Report SCRS/2000/192

V. Susca, A. Corriero, C.R. Bridges and G. De Metrio. 2001. Study of the sexual maturity of female bluefin tuna: purification and partial characterization of vitellogenin and its use in an enzyme-linked Immunosorbent assay. Journal of Fish Biology 58, 815-831

Presentations at Meetings:

A New Muscle Biopsy Technique for Sex and Sexual Maturity Determination in Large Pelagic Fishes.
Bridges, C.R., Susca, V., A. Corriero & De Metrio, G
Eurocean Conference 2000 Sept. Hamburg

A11.1 Fishy Business" in the Mediterranean - Tuna, Tonnara and Testosterone
C. R. Bridges and V. Susca and J. Eicker (Düsseldorf); A. Corriero and G. De Metrio (Bari) SEB Meeting Canterbury 2001.

Fishy business" in the mediterranean - tuna, tonnara and testosteron?
C.R. Bridges,V. Susca, J. Eicker, A. Corriero, G. De Metrio, P. Megalofonou, M. de la Serna, D.Kime.
Seminar :Rosenstiel School of Marine and Atmospheric Science . Univ. Miami and NOAA, Miami. February 2000

European Perspectives – Examples from CFP and FP5 Programmes and Lessons learned for FP6
C.R. Bridges,V. Susca, J. Eicker, A. Corriero, G. De Metrio, P. Megalofonou, M. de la Serna, D.Kime.
DEMA meeting Liverpool April 2001

Posters:

A11.14 Vitellogeninof the Mediterranean Swordfish (*Xiphias gladius*)- Purification, partial characterisation and measurement of plasma levels.
J.Eicker, V.Susca, C.R.Bridges, D, Kime, M. de La Serna, P. Megalofonou, A. Corriero; S.and G. De Metrio . SEB Canterbury 2001

A11. 15 Determination of First Sexual Maturity in Mediterranean Bluefin Tuna (*Thunnus Thynnus*)
V. Susca, C.R. Bridges, A. Corriero; S.and G. De Metrio. SEB Canterbury

The presence of the project on the internet has been achieved by free access to our web page at

<http://www.uni-duesseldorf.de/WWW/MathNat/Zoophys/bridges/swordfish.htm>

This page contains details of the project aims, partners addresses etc and also photos or recent research trips. The interim stability report is also available as a PDF file and a list of abstracts and presentations are given. Abstracts will be included shortly. A short press statement at a recent conference is also available and individual slide presentations outlining the objectives of the work will be made available shortly.

6. Ethical Aspects and Safety Provisions

During the study period so far no ethical problems have arisen for any of the participants

Quality of Life and Management of Living Resources

Proposal Nr. QLRT-PL1999-01567

**Sexual Identification and Development in the Swordfish –
Improved Determination Tools for more Efficient Stock
Assessment and Implementation of Control Measures**

SIDS

INTERIM REPORT

APPENDICES

**1.1.1 - 5. Sustainable agriculture, fisheries and forestry and
integrated development of rural areas including mountain areas**

**1.1.1- 5.1. 2 .Sustainable fisheries and aquaculture
1.1.1- 5. 4. 3. Monitoring and enforcement of the CFP**

INTERIM REPORT APPENDIX I MINUTES OF THE FIRST COORDINATION MEETING HELD FROM THE 3rd - 5th MARCH IN DÜSSELDORF

Present: C.R. Bridges, A. Corriero, G. De Metrio, D. Kime, P. Megalofonou , José Miguel de la Serna,
V. Susca

1. Organisation of the meeting

The Co-ordinator outlined the daily schedule and the organisation of the meeting.

2. Additions or changes to the agenda

No additions or changes were made

3. Report from co-ordinator with regards administration of the project

The Co-ordinator confirmed that work had commenced on the project by all partners on the 1st of March 2000 and this information would be communicated to Brussels

4. Financial matters and transfer of initial funding

The Co-ordinator reported that as soon as the initial financing had been received by Düsseldorf that within 30 days the individual parties would be paid. All parties had submitted their Bank Account Nrs to the Co-ordinator. The Co-ordinator would confirm the arrival of the first advance payment in Düsseldorf and partners should confirm the arrival of their advance payment by email. On transferring money the admin should note both the abbreviation for the project title, the project number and the name of the participant responsible.

E1 Cost statements were discussed and also the use of Person/ hours . or Person Months.

(D.Kime to send Forms to all participants)

Email list

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bridges@uni-duesseldorf.de

5.Consortial Contract

All participants had agreed to sign the consortium agreement and these were in various stages of completion. The Co-ordinator pointed out the various aspects of the contract (Copy enclosed) and a PCC was appointed consisting of:

C.R. Bridges, Düsseldorf

G. De Metrio, Bari

D. Kime, Sheffield

P. Megalofonou , Athens

José Miguel de la Serna, Malaga

Meetings may be called within 15 days with prior written notice to the co-ordinator. The voting rights (2/3 Quorum) and the appointment of deputy's was discussed as was voting via email on policy majority decisions. The responsibilities of the co-ordinator , the participants and the task leaders was discussed.

5. Discussion of the Workpackages

a) Goals of the tasks

The goal of the present study is to develop a muscle biopsy from which sex and sexual maturation of the sword fish can be determined. To this end fish sampling has a very high priority and can be characterized by three parameters:-

- 1) Geographical location
- 2) Seasonality
- 3) Fish size

It was agreed that the IEO Malaga concentrated on the western Med. and also some Atlantic fish if possible. UniBari on the central Med. and Uni.Athens on the eastern Med. IEO would principally sample from May till October but would endeavour to obtain some specimens for March and April comparisons. Uni. Bari would be sampling from March through till September / October.

Uni.Athens would sample from April until September. All three sampling groups were asked to try and obtain one or two specimens in Nov. Dec. and Jan, for baseline work. The role of size was discussed under a later point (See fish sampling)

b) Task leaders ;

WP 1 : Task Leader Düsseldorf, Co-ordination- All partners to deliver reports in Excel and Microsoft Word. Test samples for Partner2 delivered from Düsseldorf.

WP 2 : Task Leader Bari

Standardisation of the methodologies and sampling timetable and numbers of fish.

Sampling and Measurements for Partners 3,4 and 5:

- a) Sea surface temperature measurements using thermometer probes: Position marked using GPS co-ordinate system. Correlate with sea surface satellite data. Correlation to be done by Düsseldorf via internet data access.
- b) Fish lower jaw fork length (cm) + Eviscerated weight (kg)
- c) Blood sample from the heart. - Tubes heparinized and treated with Ringers + PMSF - Freshly made up each day. Two separate samples of blood 10ml each then centrifuged to extract plasma or stored on ice, centrifuged on shore and then individual small plasma samples frozen.
- d) Muscle Biopsy: Two samples to be taken from the ventral surface between the cloaca and tail fin. The samples should be approximately 1cm x 2 cm and placed in separate plastic bags and stored on ice before freezing.
- e) GSI : Determine Gonad weight / Eviscerated fish weight (Length only in exceptional cases)
- f) Gonad sample - middle section segmental sample from wall to lumen fixed in Bouin's for 4 hrs
- g) Liver sample taken and fixed as above.
- h) Hardparts for age determination . Second spine of anal fin.

It is hoped by the second fishing season that biopsy needle will be available for use for all samples

Fish Sample Number Codes

These must be strictly adhered to.

All samples from Bari coded with **BAR** and then numbered from 1 - 499

All samples from Malaga coded with **MLG** and numbered from 500 - 999

All samples from Athens coded with **PER** and numbered from 1000-1500

Fish Sampling timetable (See Table 1 for details)

Members of the Düsseldorf group will demonstrate to both Spanish and Greek partners the method for obtaining plasma and tissue samples.

TABLE 1. MINIMUM FISH SAMPLING QUOTA'S SWORDFISH PROJECT: QLK5-CT1999-01567

	Year 2000												Year 2001															
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A
BARI (TASK LEADER)																												
Full Sampling (C)			5	15	15	15	15	10	10	10					5	15	15	15	15	10	10	10						
Muscle Only* (C)			>5	>15	>15	>15	>15	>10	>10	>10					>5	>15	>15	>15	>15	>10	>10	>10						
Full sample if possible#											3	3	3	3									3	3	3	3		
ATHEN																												
Full Sampling (C)			5	15	15	15	15	10	10	10					5	15	15	15	15	10	10	10						
Muscle Only* (C)			>5	>15	>15	>15	>15	>10	>10	>10					>5	>15	>15	>15	>15	>10	>10	>10						
Full sample if possible											3	3	3	3									3	3	3	3		
MALAGA																												
Full Sampling (C)			5	15	15	15	15	10	10	10					5	15	15	15	15	10	10	10						
Muscle Only* (C)			>5	>15	>15	>15	>15	>10	>10	>10					>5	>15	>15	>15	>15	>10	>10	>10						
Full sample if possible#											3	3	3	3									3	3	3	3		

C = Contractual

Full Sampling = Sampling of all parameters

Muscle Only* = Indicates as many samples as possible together with length

Full sample if possible# = Efforts should be made to obtain one to three samples during this period

NB

1. Full sampling only on freshly caught fish. No coagulated blood!
2. Sampling spread out over a monthly period !
3. Three size range categories < 100 cm ; 100 -140cm; >140cm . No more than two fish <100cm !
4. Forward monthly return sheets punctually by the 7th of the next month to task leader and coordinator

WP 3 : Task Leader Düsseldorf

The Düsseldorf and Bari teams will collaborate to provide standardised methods for the other groups and to determine stability of the steroids before proceeding further. This will take place in Port Cesario at the end of May beginning of June when fish are approaching maturity.

WP 4: Task Leader Bari

For fish numbers and fish sampling see table 1. The aim of this workpackage like WP2 but this time only the number of the fish will be notified to Düsseldorf and Sheffield and no information on sex or histology. At a later stage this double blind data will be correlated by an independent observer. Simulated market conditions means that partners 3,4 and 5 will take muscle samples after similar time and storage conditions as found at the market.

WP5: Task leader Bari + Athens

All three partners will carry out the work described as a, b, and c in WP5. Part d and e the histology and oocyte morphometry will be carried out by Bari and Athens. Together they will develop standard scales for determining gonad maturity and oocyte morphometry.

It was agreed that all results must be presented for correlation in November 2000 for fishing season I (D7) and October 2001 for fishing season II (D8).

WP6: Task leader Sheffield.

Muscle extraction protocols will be exchanged between Düsseldorf and Sheffield. Measurements will begin in October in Sheffield but prior to this test measurements and methodology comparisons will be made.

WP7: Task leader Düsseldorf

The Workpackage will be carried out as stated. Düsseldorf and Bari will be responsible for obtaining the initial plasma samples (30ml) from sexually mature females (Stage 4+ Gonadal maturity) for antibody preparation.

WP8: Task leader Düsseldorf.

The co-ordinator reminded all members to stick to the time table when submitting drafts of reports which should be in WORD + EXCEL spreadsheets

WP9: Task leader Düsseldorf

6. Co-ordination of the distribution and measurement of sample parameters

On receiving the sample return sheets for each month the co-ordinator will contact the partners 3, 4 and 5 and instruct them to send stored samples of plasma and muscle to the laboratories in Düsseldorf, Sheffield 5 working days before shipment.

Shipment should be in dry ice and with notification of transport via email or fax at least 24hrs prior to shipment. On shipping the tracking code number should immediately be sent to the co-ordinator and to the receiving laboratory

8. Deadlines for delivery of results to the co-ordinator

All partners were again advised of the deadlines as laid down in the original contract for the deliverables and milestones. The co-ordinator would send out reminders in good time such that the information required could be correlated for interim and final reports. The Düsseldorf group would place all results on their web-page for ease of access.

9. Preparation of interim report I after stability measurements

This report will be prepared by the Düsseldorf group with help from the Sheffield group in terms of the stability experiments for steroid hormones. The participants from Düsseldorf and Sheffield reaffirmed their confidence in a positive outcome to these field tests as laboratory tests have shown a high stability for steroids.

10. Next meeting

It was agreed to hold the next co-ordination meeting in the Mediterranean area. The colleagues from Athens have kindly agreed to hold the next co-ordination meeting in January 2001 sometime at the end of the month.

11. Any other business

The state of sampling in the BFTMED programme was discussed to determine the number of samples which could be expected adjacent to the Swordfish program. The partners from Spain and Greece reported that samples from the BFT would be taken in the fishing season which was about to commence.

The meeting closed at 16:00

APPENDIX A SAMPLING PROTOCOL (V. Susca)

APPENDIX B GONAD SAMPLING (A. Corriero)

APPENDIX C SAMPLING RETURN SHEETS

APPENDIX A

SAMPLING PROTOCOL FOR PLASMA (or Serum) AND MUSCLE

Preparation before sampling:

Fill a Styrofoam container with ice.

Prepare the heparin/PMSF solution: take a small amount of heparin powder with a spatula and place it in the 25 ml bottle full with salt solution (150 mM, if possible, if not use distilled water). Place about 5 drops of the PMSF stock with a pipette in the bottle and shake vigorously. This solution can be used for 3 to 5 days if kept in refrigerator.

Label tubes and rinse the tube and syringes with the heparin/ PMSF solution. Store the solution (in case needed later) and the rinsed tubes and syringes in a cool box (with freeze packs).

On board:

1. Note GPS and surface temperature of sampling site.
2. Measure fork length of fish and open the body of the fish with a sharp knife on ventral site so that you can reach heart, liver and gonad. Weigh the gonad and tag the fish for later identification and weight determination (→ GSI, weight determination can also be done later). Note the sex if possible.
3. Take with a prepared syringe blood from the heart (about 10 ml) and put the blood in a labelled centrifuge tube on ice. Take two muscle samples of about 3 cm from the position posterior to the anus and put it in different labelled plastic bags. Keep the tissue samples on ice.

On land:

4. Store the muscle samples in the freezer (at about -20°C).
5. Centrifuge the blood until plasma is completely separated from the erythrocytes. Centrifugation conditions depends from the centrifuge and rotor. Aspirate the plasma (upper layer) with a pipette and distribute it to different labelled tubes of 1,5 ml (eppendof tubes). Store the plasma at in freezer like muscle samples. - If you have no centrifuge see point 7.
6. Send one of the muscle samples and one tube of plasma from each fish to David Kime. The rest of plasma and the other muscle samples to Chris Bridges. (When instructed from information received via sampling returns sheet)

7. Put in the solution to rinse syringes and tubes no heparin and proceed as described by point 3. Use instead of large tubes directly 1,5 ml tubes. Leave the blood in this tubes for about 12 h on ice and then freeze them as mentioned above.
-

APPENDIX B

Procedura di campionamento di fegato e gonadi

di pesce spada

Preparazione dei fissativi

- 1) Formalina al 10%. Diluire la formalina del commercio (formaldeide 40%) 1:10 in acqua distillata. Sarebbe raccomandabile neutralizzare la formalina nel seguente modo: aggiungere abbondante carbonato di calcio (CaCO_3) in polvere, agitare, lasciare riposare per qualche giorno, filtrare.
- 2) Liquido di Bouin. E' costituito da: soluzione satura di acido picrico, formalina ed acido acetico nella seguente proporzione volumetrica 15:5:1. Miscelare l'acido picrico e la formalina nella proporzione corretta e nella quantità che si prevede sufficiente (es: 150 cc di soluzione satura di acido picrico e 50 cc di formalina del commercio) e mettere in una provetta a parte la quantità necessaria di acido acetico (es: 10 cc). Aggiungere l'acido acetico alla soluzione di acido picrico e formalina solo immediatamente prima dell'uso. Conservare in frigo il liquido di Bouin avanzante al massimo per un giorno.

Prelievo a bordo

- a) Fegato. Prelevare un campione di circa 1 cm^3 di fegato e riporlo in una boccetta contenente una quantità di formalina 10% (meglio se neutra) sufficiente a ricoprirlo abbondantemente. Il prelievo deve essere effettuato nella porzione centrale del fegato.

b) Gonade

Operazioni da effettuarsi quando almeno un esemplare utile venga pescato:

- 1) versare l'acido acetico nella boccetta contenete la soluzione di acido picrico e formalina (liquido di Bouin).
- 2) riporre la boccetta così preparata nella borsa frigo in attesa di utilizzo.
- 3) prelevare una sezione trasversale completa dello spessore di 1 cm dalla parte centrale della gonade, riporre il tutto in una boccetta e versare liquido di Bouin fino a coprire abbondantemente il tessuto.

- 4) dopo circa 4 ore svuotare la boccetta dal liquido di Bouin e versare etanolo 70 % fino a coprire abbondantemente il tessuto;
- 5) cambiare l'etanolo 70% per almeno 4 volte ogni ora (nel caso in cui l'alcool che viene scartato è ancora molto giallo, ripetere il lavaggio);
- 6) Lasciare i campioni in etanolo 70%.

N.B.

Il liquido di Bouin, una volta preparato (cioè dopo l'aggiunta dell'acido acetico), può essere conservato massimo per 24 ore in frigo.

Dalle gonadi mature, dopo aver tagliato una sezione trasversale completa, prelevare solo uno spicchio che comprenda tutto lo spessore della gonade dalla parete al lume.

Non prelevare campioni di gonade più spessi di un cm.

INTERIM REPORT APPENDIX II**Minutes of the Coordination Meeting held in the University of Athens , Dept, Biology Jan. 26th - 27th 2001.**

Present: C.R. Bridges, A. Corriero, G. De Metrio, D. Kime, P. Megalofonou ,

1. Organisation of the meeting

The coordinator outlined the organisation off the meeting and thanked Dr Megalofonou for organising the meeting. Apologies had been received from Miguel de la Serna and Vito Susca who could not attend the meeting

2. Additions or changes to the agenda**2a. Acceptance of the minutes of the previous meeting**

The minutes of the last meeting had been circulated and were accepted as a true record of the meeting

2b. Correspondence received.

The coordinator had received an email from the Spanish group pointing out some of their problems. It was decided that the coordinator should write to the Spanish group outlining where misunderstandings had occurred in terms of fishing returns etc. **(ACTION COORDINATOR)**

3. Reports: Task Leaders Work Packages:**4. Coordination (Düsseldorf)**

The coordinator reported there had been no major problems apart from the late submission of fishing returns. It had been agreed at the last meeting that The Task leader (Bari) and the Coordinator should receive a copy by the 7th of each month at the latest of the the numbers of fish caught. Due to circumstances this deadline had not been kept to and the coordinator pointed out it was difficult to correlate the number of fish caught and make contingency plans for obtaining more monthly samples if the data was not available. All sampling participants were asked to provide the return sheets as agreed.

The coordinator would provide new fishing return forms in hardcopy and as an excel template **(Appendix B)** to make sure both an email copy and a fax copy are sent. **(ACTION COORDINATOR)**

5. Fish Sampling (Bari)

From the submitted returns 168 fish had been caught in Spanish waters, 173 in Italian and 75 from Greece waters. The total target of 300 fish per year had been met but Greece was below their target of 100 fish / fishing season. They hope to increase their quota for the next fishing season and will try and obtain more samples. **(ACTION GREEK GROUP)**

Although the fish quota was reached there were a number of problems with incomplete samples i.e. plasma, gonads and tissue samples from the same fish. Complete statistics can be drawn from the definitive spread-sheet sent to all partners **(ACTION COORDINATOR)**

6. Stability Protocol (Düsseldorf)

The Düsseldorf group reported that together with colleagues from Italy field stability experiments had been carried our during May and June 2000. The stability tests were carried out for steroids and a successful interim stability report has been sent to the commission. The first results showed that steroids were quite stable at room temperature when measured up to 24hrs later. Full report enclosed in Interim Stability Report.

The stability protocol will be repeated from the 15th – 29 May in Italy together with the procurement of Vtg standard from mature female fish. This time Vtg stability will be investigated along with steroids.

7. Gonad Morphometrics (Bari)

The Bari group reported that they had histologically analysed 164 gonad samples of which 61 were males and 103 females from Italy and 25 samples (12 male and 13 female) so far from Malaga. Approximately a further 64 samples from Greece and 50 samples from Spain remain to be delivered to Bari. **(ACTION ATHENS AND MALAGA).**

The Greek group also reported that they had to purchase some of their gonad samples. The samples, which had been histologically examined, were also macroscopically staged for maturity a detailed report will be made in the interim report.

The coordinator pointed out the importance of macroscopic sex determination and stage determination on board ship. These values which are placed in the original return sheets should not be altered at a later stage but used to calculate the accuracy of the macroscopic estimation of sex and sexual maturity compared with the absolute value given by histological examination and the estimation using Vtg or sex-hormone ratio's. **(ACTION ALL SAMPLING GROUPS BARI, ATHENS and MALAGA)**

8. Steroids (Sheffield)

All samples which could be measured for steroids had been examined however the following comments were made:

Swordfish Samples

Plasma samples of poor quality— about 50% were whole blood and unusable, packaging was erratic, some vials had loose or separated tops – screw caps would be better. Snap-on caps must be firmly pressed in before despatch. All samples received were processed

Tissue samples had both a tough skin and underlying muscle which had to be separated – generally there was enough muscle, but sampling with scalpel giving a V-shaped sampled had predominantly skin. Packaging was poor and erratic – plastic bags and vials with poorly fixed snap-on caps – screw caps would be better.

Homogenisation with electric homogeniser is better than the glass pestle and mortar – practice runs done and now ready to process most samples.

All sampling groups were asked to provide better plasma samples and no frozen whole blood samples. **(ACTION SAMPLING GROUPS)**

9. Vitellogenin (Düsseldorf)

Vitellogenin had been obtained from ripe females caught in Italy during the stability protocol work of the Düsseldorf and Bari Group. Vtg had been purified via FPLC and an antibody raised which had been delivered to Düsseldorf just prior to January 1st 2001. The Vtg assay is now being validated and we expect to have a working ELISA for plasma and tissues shortly. Some of the first interesting findings were that in FPLC measurements measurable amounts of Vtg were present. This led to a re-examination of male gonad sections which showed at least 8 specimens with oocytes present in the testes.

10. Financial matters and transfer of initial funding

Although some problems were encountered with the transfer of the first instalment to all participants through the administration in Düsseldorf this had been remedied but participants should inform the coordinator as soon as possible if their funds have not arrived. **(ACTION ALL PARTICIPANTS)**

The coordinator pointed out that travel outside participating countries may have to be applied for if one strictly considers the guidelines below:

88. Travel / subsistence costs

The amount for travel and subsistence costs of personnel categories working for the project, calculated on the basis of the usual practices of the participant. The prior agreement of the Commission will be required for any destination outside the territory of a Member State, an Associated state or a third country where a principal contractor or assistant contractor is established. The inclusion of such costs in this form does not constitute a request for such approval. Upon successful completion of the contract negotiations a short description of these travels and the estimated costs, agreed between the consortium and the Commission

officer, should be included in Annex I to the contract. Any subsequent travel, not included in the list in Annex I to the contract, must be agreed in writing with the scientific project officer.

The travels should be briefly described, for example, travels inside the EU and associated states and travels outside the EU and associated states, and the total amount(s) should be entered in this line.

11. Fishing Programme 2001

- a) Standardisation of the methodologies / sampling, Stability Protocol
- b) Sampling timetable and numbers of fish; Market Sampling

Due to sampling problems from the first Fishing programme 2000 it was evident that the quota of 300 full samples to be achieved by the sampling group could not be met.

The sampling protocol has been revised (**See Appendix A**) to make sure that no frozen whole blood samples are taken.

Approximate figures for full samples taken were 55 for Spain, 150+ for Italy and 13 for Greece. However a large number of incomplete samples were obtained which will provide supporting data e.g. muscle samples, gonads e.t.c.

Since a minimum of 300 full samples per fishing season was planned it was thought prudent to sample at least 300 fish as detailed in **Annex 2** again using the same length classes and wherever possible full sampling. The quota for each month has been revised in the Greek sector due to the closure of the fishery in October. (**ACTION ALL SAMPLING GROUPS**).

Again it is essential that the Task leader and Coordinator be kept aware of the monthly catches for full sampling. Hence the return sheets should be dispatched on time:

Marcoscopic sex and stage should not be included in the sheets to Bari or Düsseldorf to simulate double-blind conditions. As many **extra** muscle samples together with macroscopic sex determinations should also be made. (**ACTION ALL SAMPLING GROUPS**).

12. Co-ordination of the distribution and measurement of samples.

Muscle and Plasma sent directly to Düsseldorf via Express Courier or Direct Transport.

Shipment should be in dry ice and with notification of transport via email or fax at least 24hrs prior to shipment. On shipping the tracking code number should immediately be sent to the co-ordinator and to the receiving laboratory.

13. Deadlines for delivery of samples and results to partners.

DEADLINE HISTOLOGICAL SAMPLES : Sent **monthly** to Bari

DEADLINE PLASMA AND MUSCLE SAMPLES: Sent every **2 months** to Düsseldorf.

FISHING RETURNS : **7th of every month** to Bari and Düsseldorf as a fax an Email Excel Spread sheet.

DEADLINE HISTOLOGY RESULTS Microscopic Sex and Stage March 1st 2001

DEADLINE HISTOLOGY RESULTS Microscopic Sex and Stage 1st Jan. 2002

DEADLINE STEROIDS RESULTS March 1st 2001

DEADLINE STEROIDS RESULTS Jan. 1st 2002

DEADLINE VTG RESULTS 1st July 2001

DEADLINE VTG RESULTS 1st Jan. 2002

14. Preparation of interim report

All participants had received a copy of the guidelines for drawing up the interim report:

ftp://ftp.cordis.lu/pub/life/docs/a_guidelines_sc.doc

An interim report is planned for the 1st March 2001. This will require input from Task Leaders (see minutes of previous meeting) and also the cost statements (E1) of each participant (three signed originals) without VAT.

<http://www.cordis.lu/fp5/management/provisions/r-modcost-4.htm#Part E-1>

For personnel costs you must certify that “full supporting documentation to justify the costs hereby declared, including time sheets as referred to in Article 23 (1) (a), third subparagraph of Annex II to the contract, is available for audit by the Commission and its authorised representatives or the Court of Auditors and reflects the costs actually incurred.”

9. Next meeting

Dr Kime kindly offered to host the next meeting in the UK. It was thought pertinent that we meet during the second fishing season at the end of July to establish that all was running smoothly and make any corrections for missing or unfilled quota's.

Meeting closed at 16:00

APPENDIX A (Revised 12.2. 2001)

SAMPLING PROTOCOL FOR PLASMA AND MUSCLE

Preparation before sampling:

Fill a Styrofoam container with ice.

Prepare the heparin/PMSF solution: take a small amount of heparin powder with a spatula and place it in the 25 ml bottle full with 1.5 % sodium chloride in distilled water. Place about 5 drops of PMSF stock with a pipette in the bottle and shake vigorously. This solution can be used for 3 to 5 days if kept in refrigerator. (Syringes, canula and tubes should be rinsed in advanced with this solution.)

Label screw top tubes and rinse the tube and syringes with the heparin/PMSF solution. Store the solution (in case needed later) and the rinsed tubes and syringes in a cool box (with freeze packs). Use various sizes of plastic bags to store muscle and plasma samples and then one large bag for all the samples..

NUMBERS MALAGA MG2000-2500; BARI IT2501-3000; GREECE AT3000-3500:

On board:

8. Note GPS and surface temperature of sampling site.
9. Measure fork length of fish and open the body of the fish with a sharp knife on ventral site so that you can reach heart, liver and gonad. Weigh the gonad and tag the fish for later identification and weight determination (→ GSI, weight determination can also be done later). Note the macroscopic sex and if possible also the macroscopic stage of development.
10. Take with a prepared syringe blood from the heart (about 10 ml) and put the blood in a labelled centrifuge tube (rinsed with heparin/PMSF solution) **on ice**. Take 2 muscle samples of about 3 ccm* from the position posterior to the anus and put it in different labelled tubes with screw caps (Sheffield and Düsseldorf). **Keep the tissue samples on ice.**

On land:

11. Store the muscle samples in the freezer **noting the time between capture and storage (hrs) on the bag** (at about -20°C).
5. Centrifuge the blood until plasma is completely separated from the erythrocytes. The centrifugation condition depends from the centrifuge and rotor. Aspirate the plasma (upper layer) with a pipette and distribute it to different labelled appropriate screw top tubes (1 for Düsseldorf, 1 for Sheffield and the rest in a single other tube). Store

the plasma **immediately** at about -20°C in freezer like muscle samples.

ONLY IN AN EMERGENCY

If you have no centrifuge to get plasma:

- a. Put in the **PMSF** solution to rinse syringes and tubes **no heparin**
Use small tubes directly. Leave the blood in these tubes for about **12 h on ice to clot.**
- b. After about 12 h (the next day!) **when the blood is coagulated** you may extract **serum by centrifugation or pushing down the clot and obtain the supernatant.** Freeze the tubes with the serum as mentioned above by -20°C for plasma and tissue.

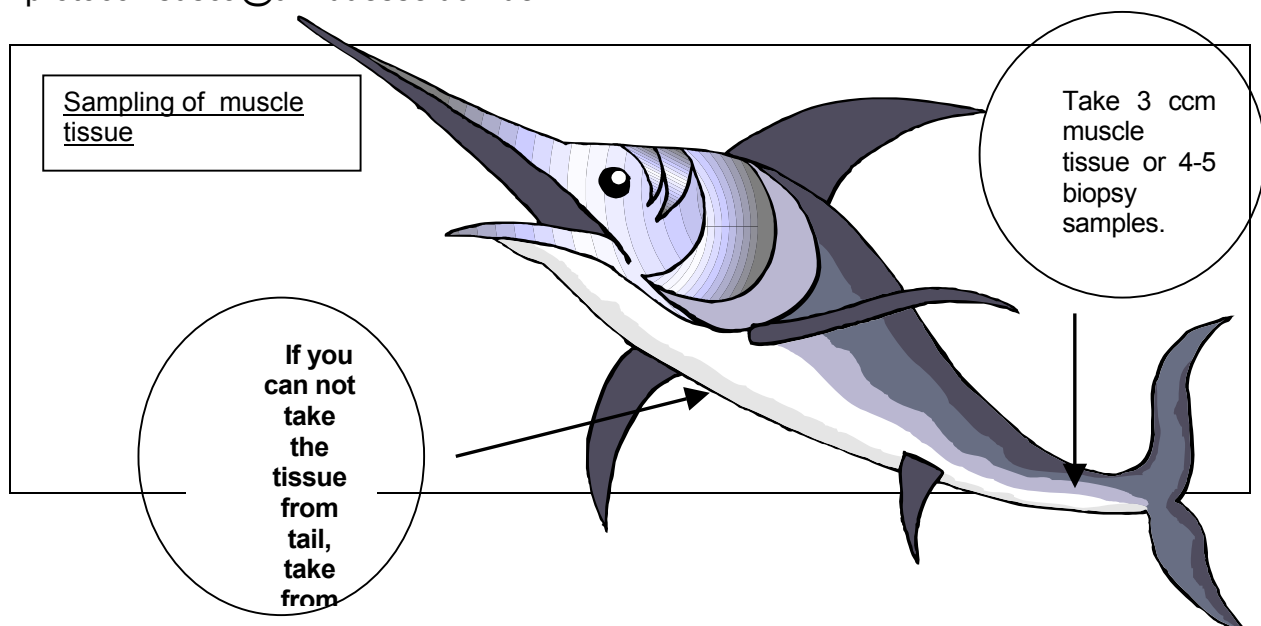
Please do not freeze whole blood or blood which has not clotted; it will be not usable for the analyses!

The fish should be fresh and the time of death not more than 3 hrs before sampling otherwise you will not be able to obtain blood plasma.

Note the time of sampling from fish and that of freezing the samples.

* If you are going to use the biopsy needle take 4 – 5 samples for Sheffield and 4 –5 for Düsseldorf per fish from the same area (from the position posterior to the anus). If it is not possible to take any samples from this position, then take 2 muscle samples of about 5 cm strip length from the ventral site paying attention to take specimen with **muscle tissue** and **not only fat or skin!**

Please contact Vito Susca if you have difficulties or questions regarding the sampling protocol: susca@uni-duesseldorf.de



A FULL SET OF SAMPLES IS PLASMA, GONAD, MUSCLE+ BIOMETRIC**DATA –SEX****Procedura di campionamento di fegato e gonadi
di pesce spada*****Preparazione dei fissativi***

- 3) Formalina al 10%. Diluire la formalina del commercio (formaldeide 40%) 1:10 in acqua distillata. Sarebbe raccomandabile neutralizzare la formalina nel seguente modo: aggiungere abbondante carbonato di calcio (CaCO_3) in polvere, agitare, lasciare riposare per qualche giorno, filtrare.
- 4) Liquido di Bouin. E' costituito da: soluzione satura di acido picrico, formalina ed acido acetico nella seguente proporzione volumetrica 15:5:1. Miscelare l'acido picrico e la formalina nella proporzione corretta e nella quantità che si prevede sufficiente (es: 150 cc di soluzione satura di acido picrico e 50 cc di formalina del commercio) e mettere in una provetta a parte la quantità necessaria di acido acetico (es: 10 cc). Aggiungere l'acido acetico alla soluzione di acido picrico e formalina solo immediatamente prima dell'uso. Conservare in frigo il liquido di Bouin avanzante al massimo per un giorno.

Prelievo a bordo

- b) Fegato. Prelevare un campione di circa 1 cm^3 di fegato e riporlo in una boccetta contenente una quantità di formalina 10% (meglio se neutra) sufficiente a ricoprirlo abbondantemente. Il prelievo deve essere effettuato nella porzione centrale del fegato.

b) Gonade**Operazioni da effettuarsi quando almeno un esemplare utile venga pescato:**

- 7) versare l'acido acetico nella boccetta contenete la soluzione di acido picrico e formalina (liquido di Bouin).
- 8) riporre la boccetta così preparata nella borsa frigo in attesa di utilizzo.
- 9) prelevare una sezione trasversale completa dello spessore di 1 cm dalla parte centrale della gonade, riporre il tutto in una boccetta e versare liquido di Bouin fino a coprire abbondantemente il tessuto.
- 10) dopo circa 4 ore svuotare la boccetta dal liquido di Bouin e versare etanolo 70 % fino a coprire abbondantemente il tessuto;
- 11) cambiare l'etanolo 70% per almeno 4 volte ogni ora (nel caso in cui l'alcool che viene scartato è ancora molto giallo, ripetere il lavaggio);

12) Lasciare i campioni in etanolo 70%.

N.B.

Il liquido di Bouin, una volta preparato (cioè dopo l'aggiunta dell'acido acetico), può essere conservato massimo per 24 ore in frigo.

Dalle gonadi mature, dopo aver tagliato una sezione trasversale completa, prelevare solo uno spicchio che comprenda tutto lo spessore della gonade dalla parete al lume.

Non prelevare campioni di gonade più spessi di un cm.

TABLE 1. MINIMUM FISH SAMPLING QUOTA'S SWORDFISH PROJECT: QLK5-CT1999-01567revised 19.02.2001

	Year 2001															
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A
BARI (TASK LEADER)																
Full Sampling (C)			5	15	15	15	15	10	10	10						
Muscle Only* (C)			>5	>15	>15	>15	>15	>10	>10	>10						
Full sample if possible#	3	3									3	3	3	3		
ATHEN																
Full Sampling (C)			5	15	15	15	15	15	15							
Muscle Only *(C)			>5	>15	>15	>15	>15	>10	>10							
Full sample if possible	3	3									3	3	3	3		
MALAGA																
Full Sampling (C)			5	15	15	15	15	10	10	10						
Muscle Only* (C)			>5	>15	>15	>15	>15	>10	>10	>10						
Full sample if possible#	3	3									3	3	3	3		

C = Contractual

Full Sampling = Sampling of all parameters

Muscle Only* = Indicates as many samples as possible together with length

Full sample if possible#= Efforts should be made to obtain one to three samples during this period

NB

1. Full sampling only on freshly caught fish. No coagulated blood!
2. Sampling spread out over a monthly period !
3. Three size range categories < 100 cm ; 100 -140cm; >140cm . No more than two fish <100cm !
4. Forward monthly return sheets punctually by the 7th of the next month to task leader and coordinator

INTERIM REPORT ANNEX III

Quality of Life and Management of Living Resources

Contract Nr. QLRT-PL1999-01567

Sexual Identification and Development in the Swordfish – Improved Determination Tools for more Efficient Stock Assessment and Implementation of Control Measures

SIDS

1.1.1 - 5. Sustainable agriculture, fisheries and forestry and integrated development of rural areas including mountain areas

**1.1.1- 5.1. 2 .Sustainable fisheries and aquaculture
1.1.1- 5. 4. 3. Monitoring and enforcement of the CFP**

FIRST INTERIM REPORT – STABILITY PROTOCOL Sept. 1st 2000

Objectives

To determine in the first phase the best method of taking and storing plasma and muscle samples from swordfish in the field. Determine the stability of both steroid hormones and vitellogenin in plasma and muscle during May and June when steroid levels and vitellogenin levels are high. Provide a basis for the full funding of the project through an interim report at the end of month 6.

In compliance with the specific objectives listed in Annex 1 of contract nr Contract Nr. QLRT-PL1999-01567:-

“Specific Objectives

- a) Identification and isolation of sex hormones and vitellogenin in plasma and muscle biopsy samples (MBS) taken from the swordfish. Confirm stability of steroids under field sampling conditions and their suitability as sex and gonadal development markers”.

The following short interim report concerning Workpackage 3 (See Appendix 1) is submitted.

As can be seen in Appendix 1 the main objective of the stability protocol was to determine in the first fishing phase the best method of taking and storing plasma and muscle samples from swordfish in the field. To determine the stability of both steroid hormones and vitellogenin in plasma and muscle during May and June when steroid levels and vitellogenin levels are high. Provide a basis for the full funding of the project through an interim report at the end of 6 months.

Description of the Work:

The fishing season commenced in March 2000 and within the first three months extensive sampling has been carried as detailed in the other workpackages. Successful sampling of both plasma and muscle samples took place with morphometric and biometric sampling. These will be the subject of a full report in March 2001.

During a period from the 30.05.2000 until 30.06.2000 a stability protocol was carried out in Porto Cesario and Campo Marina together with plasma sampling from mature female for the isolation swordfish vitellogenin.

Material and Methods

Fish samples were obtained by working directly with a commercial fisherman in this area. Drift nets were set at about 18.00 and retrieved approximately 3- 6 hrs later. Their position (GPS) and surface water temperature were noted. As soon as animals were retrieved out of the net blood muscle and gonad samples were taken. In almost all cases the fish were dead when they came on board, however the maximum duration of time after death was approximately 6hrs. On a few occasions fish were alive but in future protocols only dead fish will be used. All fish sampled yielded blood samples directly from the heart which were placed in heparinised tubes with PMSF. In some cases dual samples were taken in which only PMSF was added and heparin omitted. This was to obtain serum samples. Muscle samples were taken by cutting a strip of muscle from the ventral edge of the incision made to remove the gonads and intestines of the fish. The best area was posterior to the cloaca on the ventral surface although samples from the heart were also taken. These muscle samples were divided into 5- 6 individual portions and placed in a plastic container and labelled. With regard to freezing samples in this study liquid nitrogen was used to freeze one sample for time zero. The samples were then either stored at 10°C or room temperature (26°C). At regular time intervals samples were then placed in liquid nitrogen. In future liquid nitrogen is impractical to use on a fishing boat. Dry ice or normal ice is the usual means of storing samples and all plasma samples were placed on ice in this study.

Laboratory and Extraction and Measurements

In the laboratory further muscle samples were frozen at time intervals in liquid nitrogen then at -20°C and plasma separated from red cells by centrifugation and also frozen. All samples were transferred in dry ice back to the laboratory in Düsseldorf.

To extract steroid hormones from tissues 100-200 mg of tissue were homogenized with 400 μl of buffer then centrifuged at 10,000 g for 15 minutes. The supernatant was then extracted for steroids using dichloromethane. The extracted steroids were resuspended in buffer and measured with an ELISA technique (Cuisset et al., 1994). Vitellogenin was isolated using FPLC techniques (Susca et al, 2000) from mature female plasma samples.

Results

It was decided that for the stability protocol only one primary sex steroid, testosterone, would be used initially as this is a marker for sexual maturation in both male and female fish. After determining testosterone concentrations the extracted samples were re-frozen and a detailed analysis of the other hormones E2 and 11-KT together with vitellogenin determinations will be presented in Interim Report 2 in March 2001. It is envisaged that the ELISA test for vitellogenin will be ready by December 2000.

Appendix 2 outlines the biometric and raw data of the stability protocol. In total 17 fish were studied made up of 7 female and 3 male fish incubated at room temperature and 7 female fish stored on ice at 10°C . The weights of the fish varied from 22 to 83 kg in the first series with GSI ranging from 0.17 – 11.4 and 32 – 73 kg in the second series with GSI values between 0.37 and 11.4.

Appendix 3 illustrated that at room temperature (26°C) no significant difference in the mean values for muscle Testosterone were observed over a period of up to 48 hrs after landing. The values ranging around 400 pg/g. This compared with values of 1400 pg/ml measured in the plasma of these fish. In a second series of experiments where samples were stored on ice or in the refrigerator before freezing again no statistical difference was shown between the mean Testosterone concentrations over a period of 48 hrs. In these fish the mean plasma concentrations of testosterone were 970 pg/ml compared to approximately 400-500 pg/g in muscle.

Vitellogenin was successfully isolated, then purified and concentrated for specific antibody generation for a standard ELISA. The samples are currently at a commercial facility for antibody-generation. The first results are awaited in December.

Discussion

From the evidence provided by this first workpackage there is no evidence for the breakdown of testosterone after storage at room temperature (26°C) for two days or when stored at 10°C . This is similar to the results in the laboratory with steroid standards which are stable over long periods of time. However in muscle tissue enzymes will be present which catalyze the breakdown of steroids. A detailed study of all steroids will be presented in the next report as there was insufficient time to carry out a complete study, but we do not foresee any great differences. After 48hrs at room temperature, which we infer as the extreme case, the muscle samples were certainly not in an edible state. Storage on ice or immediate sale on landing appear to be the normal storage methods commercially on board ship which should therefore not affect steroid levels. From the differences in levels measured between muscle and plasma it is clear that the present ELISA techniques are sensitive enough to detect steroid hormones in tissue. Further confirmation of the stability of vitellogenin will be given in the next report after the ELISA protocol has been developed. A similar study will be carried out at the same time next year to confirm the present findings.

Conclusions

It can be concluded that the stability of testosterone in muscle samples as shown by the results in this report is high. It will be more than adequate therefore to store samples on ice before freezing. Swordfish vitellogenin has been successfully isolated from female plasma samples and the ELISA should be ready by December this year. The generation of the required species specific anti-body requires 3-4 months alone.

Guidelines:

1. The amount of muscle required for a standard test is at least 100 mg.
2. Muscle taken from the ventral surface posterior to the cloaca avoiding fatty cutaneous layers.
3. Since most fish arrive dead on board only dead samples will be taken.
4. Time of sampling should be directly after catching.
5. Fish should be stored on ice if possible.
6. Muscle samples may be kept on ice and then stored at -20°C on return to the laboratory.

References:

Cuisset et al (1994) Enzyme immunoassay for 11-KT using acetylcholinesterase as label: application to the measurement of 11-KT in plasma of Siberian sturgeon. *Comp. Biochem. Physiol.* 108C,229-241.

Susca et al. (2000) Submitted *J. Fish Biology*.

APPENDIX 1

WP3	Workpackage description
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Workpackage number: 3
Start date or starting event: Month 3
N° of the partner responsible 1
N°s of other partners involved: 5
Person-months per partner: 1 (3), 5(1): Total = 4

Objectives

To determine in the first phase the best method of taking and storing plasma and muscle samples from swordfish in the field. Determine the stability of both steroid hormones and vitellogenin in plasma and muscle during May and June when steroid levels and vitellogenin levels are high. Provide a basis for the full funding of the project through an interim report at the end of month 6.

Description of work

Stability and Verification Protocol and Standardisation

Muscle sampling:

-Whole fish experiments (30 Fish - 15 Female + 15 Male):

1. Choice of muscle to use/ amount required?
2. Live or dead fish ?
3. Time of sampling ?
4. Storage of fish before sampling ?
5. Storage muscle sample after sampling ?

-Plasma samples

1. With or without phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (1%) added to syringe
7. Direct freezing in liquid nitrogen
8. Freezing in dry ice.
9. Unfrozen kept cool – time course experiments.
5. Room temperature stability.

Deliverables

D5: Provide stability protocol to determine the best sampling method for plasma and tissues and also the most stable method of storing probes after sampling. Determine the breakdown rate of steroids and vitellogenin in plasma and muscles under various storage conditions. Provide first interim report to confirm the suitability of the methods used after 6 months to confirm further funding.

Milestones and expected results

M 1: Provide clear guidelines for future sampling within fishing campaign II . Provide correction factors for the appraisal of probes taken within the first fishing campaign I. The successful completion of WP3 with corresponding positive results and the submission of the first interim report will make the full funding of the project possible.

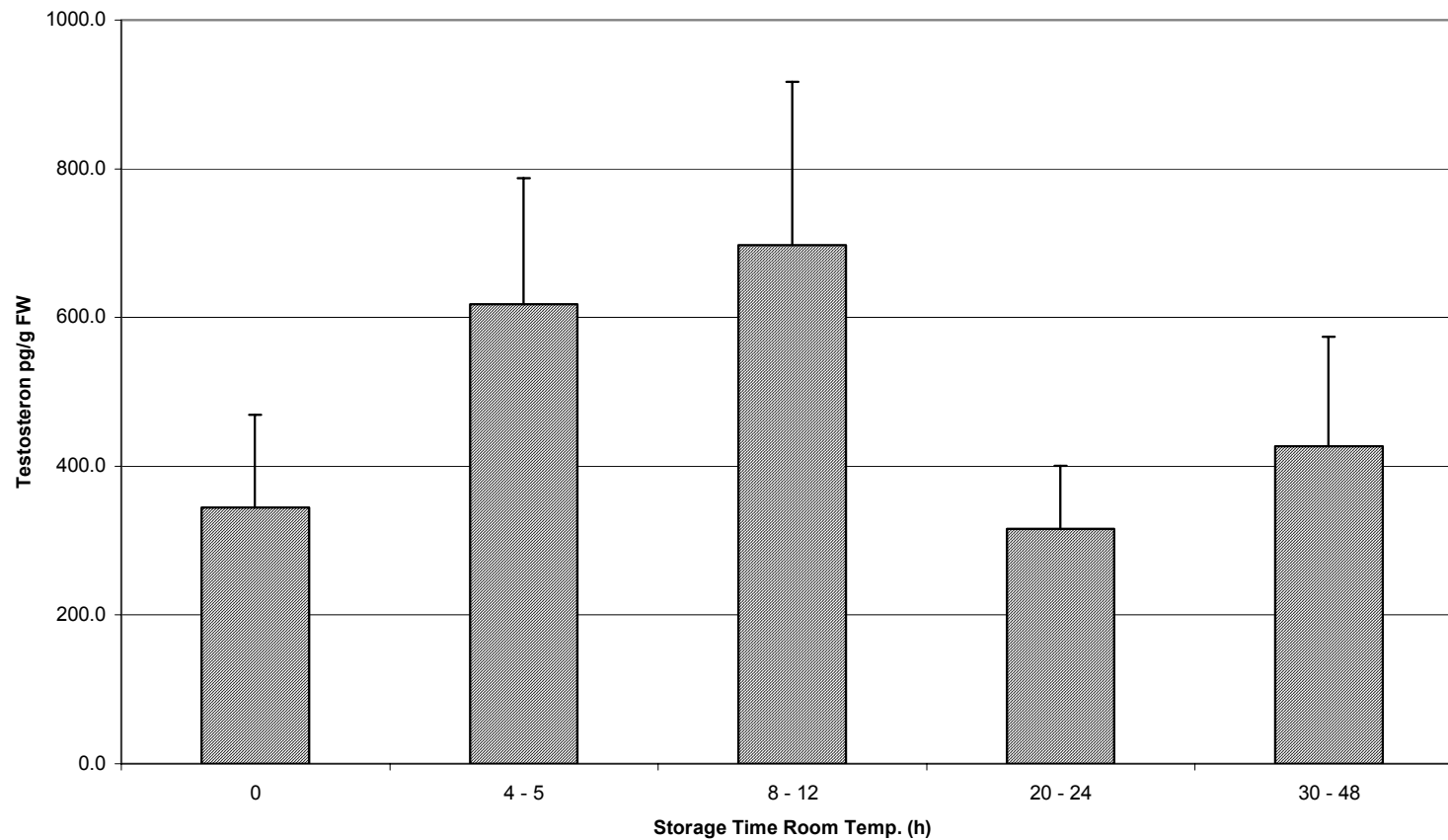
APPENDIX 2- Biometrics Data on Study Fish together with Raw Data from the stability protocol

Samples for stability protocol						Testosterone Concentration after incubation (h) at room temperature				
						0	4 - 5	8 - 12	20 - 24	30 - 48
Datum	Sex	Weight (kg)	LF (cm)	ID	GSI					
28 May	F	50	141	600	1.08	91		109	473	89
28 May	M	40	130	601	0.15	82	277		443	
30 May	M	12	109	602	0.17	90	274	1342	571	785
30 May	F	83	192	603	3.79	445	272	755	784	846
30 May	F	51.5	143	604	3.59	203	1928	120	242	86
30 May	F	58	160	605	1.21	798	929	661	0	327
2 June	M	22	130	607	0.45	169	459	134	0	
2 June	F	39	150	611	0.89	1333	551	660	102	
2 June	F	48	158	614	1.87	141	598	2221	229	
01-Jul	F	73	180	622 (Muscle)	11.4	95	276	271		
F = Female				M		344.7	618.2	697.0	316.0	426.6
M = Male				SD		393.1	507.1	660.1	254.6	329.9
All specimen from the Ionean Sea.				SEM		124.3	169.0	220.0	84.9	147.5

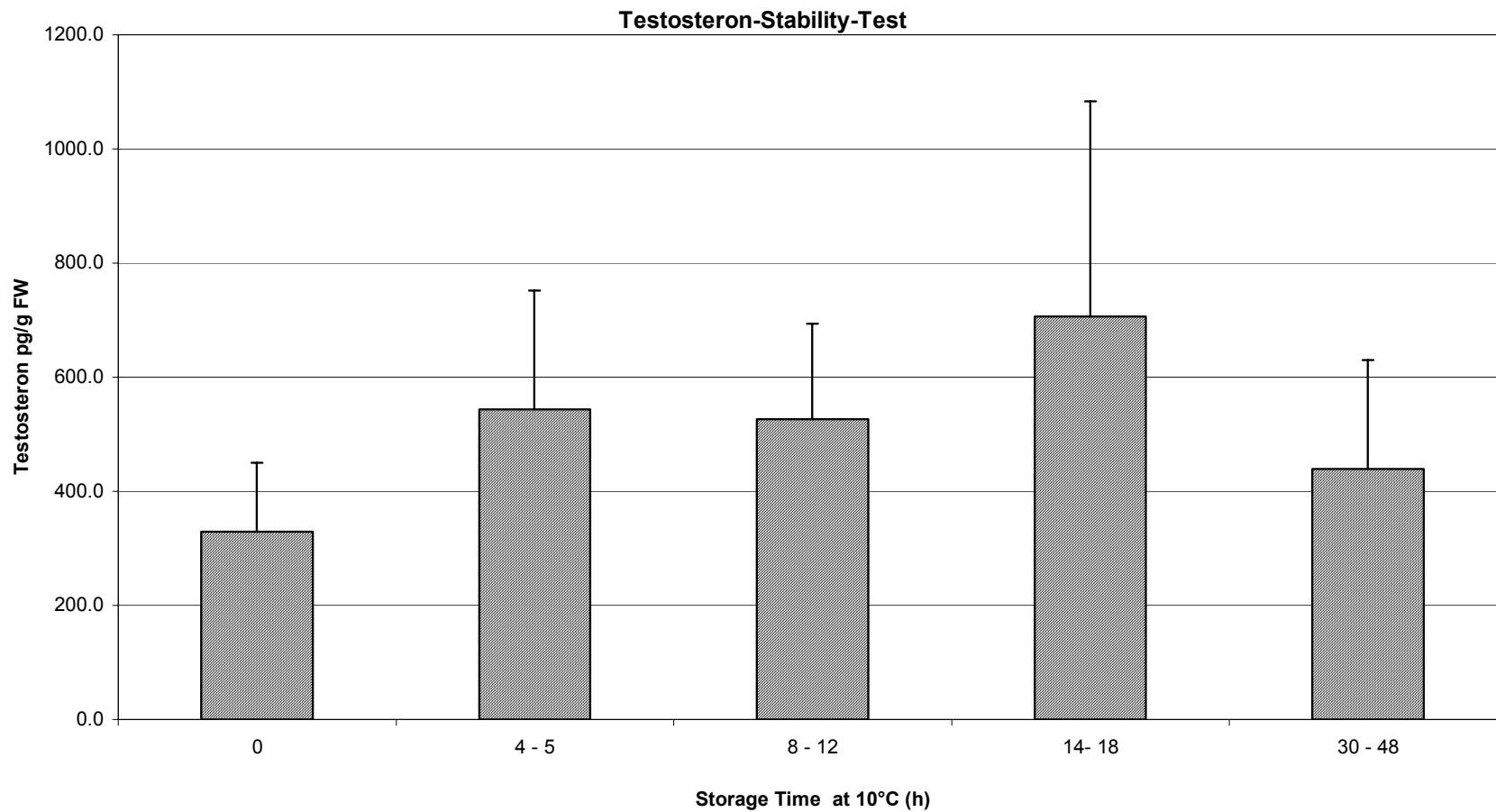
Samples for stability protocol						Testosterone Concentration after incubation (h) at 10°C				
						0	4 - 5	8 - 12	14- 18	30 - 48
29 June	F	45	160	612 (Heart)	3.67	1093	1590	206	377	189
28 June	F	45	160	612 (Muscle)	3.67	134		185	305	
28 June	F	36	136	615 (Heart)	0.67	95	164	628	139	156
28 June	F	36	136	615 (Tissue)	0.67	198	95	171	202	97
28 June	F	36	136	615 (Muscle)	0.67	321	0	1010		
21 June	F	32	125	619	0.37	221	820	1327	2928	1313
1 July	F	73	180	622 (Heart)	11.4	242	593	159	289	
F = Female				M		329.1	543.7	526.6	706.7	438.8
M = Male				SD		319.1	549.9	441.7	996.3	505.8
All specimen from the Ionean Sea.				SEM		120.6	207.9	166.9	376.6	191.2

APPENDIX 3 Steroid stability at room temperature

Testosteron-Stability-Test



APPENDIX 4. Steroid stability at 10°C



ANNEX IV INTERIM REPORT**SCRS/00/91****NEW RESULTS ON THE REPRODUCTIVE BIOLOGY OF THE BLUEFIN TUNA
(*THUNNUS THYNNUS*) IN THE MEDITERRANEAN**

by

V. Susca¹, A. Corriero², M. Deflorio², C.R. Bridges¹, G. De Metrio²**SUMMARY**

To deepen the knowledge of the reproductive biology of the Mediterranean bluefin tuna (BFT), plasma concentrations of the steroid hormones Testosterone (T), 11-Ketotestosterone (11-KT) and 17 β -Estradiol (E₂) and the yolk precursor Vitellogenin (Vtg) have been determined and correlated with gonadal maturity stage. In female BFT we found rising levels of E₂, T and Vtg approaching the spawning period. In male BFT, T and 11-KT increase together with gonadal maturation. Characteristic sex hormone profiles for different sex and reproductive maturity stage have been observed. 11-KT has been detected in low levels in females and does not seem to play any role in ovary maturation; on the other hand E₂ and Vtg have no role in male sexual maturation. These findings can be summarised in the formula $[(E_2/11-KT)]/[T]$, which can be used, in addition with the detection of Vtg, to distinguish between females and males and to estimate sexual maturity stage.

KEYWORDS

Thunnus thynnus; Tuna fisheries; Sexual maturity; Sex ratio, Sex determination; Reproductive cycle; Proteins, Juveniles; Animal reproductive organs; Fish physiology

1. INTRODUCTION

In spite of the economic importance of the bluefin tuna (BFT), the knowledge of its reproductive biology is limited to research based on the distribution of eggs and larvae (Piccinetti *et al.*, 1977, 1997; Cavallaro *et al.*, 1997; Nishida *et al.*, 1997), on seasonal variations of the gonadosomatic index (I_G) (de la Serna & Alot, 1992), and on macroscopic classification of gonad maturity stage (Rodríguez-Roda, 1964, 1967). A histological study of Western Atlantic BFT ovaries has been carried out by Baglin (1982).

Since knowledge of reproductive biology is an extremely important tool for determining a correct policy for the management of fish stocks, in our laboratories studies on the correlation between endocrinological patterns and gonad maturity are in progress. Here the results obtained till now are presented, underlining the possible utility of the techniques used for the determination of sex, maturity stage and size at first sexual maturity

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2. MATERIAL AND METHODS

2.1. Specimen collection

Blood, tissue and gonad samples were obtained, during the months of March, May, beginning of June and August 1998, from fish caught commercially by longlines and drift nets in South Adriatic, North Ionian (Gulf of Taranto) and Ligurian seas and by traditional traps (Tonnare) operating in Carloforte and Portoscuso (Sardinia, Italy). Soon after the capture, fish fork length (L_F) was measured and sex was determined by macroscopic observation of gonads. Blood was collected, centrifuged at 5000 rpm for 15 min, and plasma collected and stored at -20°C . Fragments of ovaries were fixed in Bouin's solution.

2.2. Sex steroid and Vtg measurement

11-Ketotestosterone (11-KT), Testosterone (T) and 17β -Estradiol (E_2) have been extracted with Dichloromethane and the concentration measured by ELISA as described by Cuisset *et al.* (1994). In our laboratories we have established ELISA's with a sensitivity of 1 pg/ml for 11-KT and T and 3 pg/ml for E_2 in the assay.

BFT vitellogenin (BFT-Vtg) has been purified and an ELISA established as reported for other fish species (Bon *et al.*, 1997 and Mosconi *et al.*, 1998). Dot blots analysis have been made using plasma diluted $> 1:2000$. The membrane has been blocked with 3% powdered skimmed milk then the anti BFT-Vtg serum applied, washed and the secondary antibody labelled with alkaline phosphatase added. Visualisation was performed enzymatically.

2.3. Histology of gonads and immunohistochemical detection of Vtg-like material

Gonad samples were embedded in paraffin wax. Sections ($5\ \mu\text{m}$ thick) were stained with haematoxylin – eosin. Immunohistochemical detection of anti-VTG positive material was performed on ovary sections using the abBFT-Vtg. The immunoreaction was revealed by the avidin-biotin-peroxidase complex method.

2.4. Statistical analysis

Plasma levels of steroids, vitellogenin and percentage of vitellogenic oocytes in Figure 3 have been expressed as mean \pm SEM and significant changes have been assessed by Students t-Test. All statistical analysis has been made with the software package Sigma Stat (Jandel Scientific, Erkrath, Germany). Statistical relevance was accepted at $p < 0.05$. Statistical analysis of the identity of two linear regressions in Figure 1A has been performed by F-test using the formula:

$$F = s^2_{y-x \text{ verb}} / s^2_{y-x p}$$

$s^2_{y-x \text{ verb}}$ = the difference of the sum of square of residues around the regression lines;

$s^2_{y-x p}$ = pool of the variance around both regression lines.

3. RESULTS

3.1. Sex and sexual maturity stage estimation

Testosterone (T) plasma levels rise in both sexes approaching reproductive period. The T concentration begins to decrease before spawning as reported for many other fishes (Kime, 1993). In female BFT the rise of T plasma level is followed by the increase of E_2 ; then both decrease just before spawning. After spawning the E_2 level is higher than T. In the reproductive season 11-Ketotestosterone (11-KT) is present in very low levels (0.68 ± 0.10 ng/ml; $N = 38$) in the plasma of adult females. In males 11-KT rises approaching spawning to high levels in plasma (up to 40 ng/ml) and follows the T peak as for E_2 in female. In spent males 11-KT is present in higher concentrations than T. E_2 in male is present in relatively low concentrations (0.56 ± 0.11 ng/ml; $N = 60$) and does not seem to play any role in sexual maturation.

We converted the different sex steroid concentrations into a mathematical relationship applying the Steroid Sex ratio Formula (SSF): $[(E_2/11-KT)]/[T]$. We plotted SSF vs. T concentration on a logarithmic scale, Testosterone representing a marker for sexual

maturation for both males and females (Fig. 1A). We found 2 statistically different regression lines ($p < 0.001$, assessed by F-test for the comparison of linear regression). The regression lines show that SSF values for different sexes, result from specific steroid profiles in the different maturation phases. The regression line with the higher SSF values has been calculated for females, the one with lower values for males. Fig. 1B shows, for 4 males and 4 females, the correlation between steroid profile and gonad maturity stage evaluated histologically.

The limit of the ability of the SSF to determine sex and sexual maturity stage is represented by immature fish or post-spawning fish because of their low levels of plasma T. In some case quantitative Vtg estimation (by ELISA) or simply qualitative VTG analysis (by dot blot) can clarify the sex and maturity stage (Fig. 2). In fact, Vtg can be detected in ripening, mature and post-spawning females, while it is nearly undetectable in males.

3.2. Size at first sexual maturity

The results obtained correlating BFT-Vtg and E_2 plasma levels and histological analysis by female are summarised in Figure 2.

In the recrudescence period (April) there is no significant difference in sexual maturation by different size classes. In the ripening Period (May) fish with L_F ranging from 110 and 120 cm showed a significant increase of E_2 and Vtg plasma concentrations (12.98 ± 1.53 ng/ml and 25.70 ± 6.86 mg/ml respectively). The mean oocyte diameter increased significantly, in respect to the previous length class (from 55 to 90 μm), and the histological analysis demonstrated the presence of oocytes in late vitellogenic stage. Fish with $L_F > 120$ cm showed an increase of Vtg plasma level and mean oocyte diameter (32.54 ± 8.36 mg/ml and 106 ± 10.12 μm respectively) in respect to the previous length class and a significant increase of percentage of vitellogenic oocytes (from 5 ± 1.2 % of the previous class to 11.2 ± 3.67 %).

The immunohistochemical staining of ovaries with anti BFT-Vtg serum revealed the presence of Vtg-like material in oocyte having a minimum diameter of 220 μm . Immunopositive oocytes were observed only in ovaries of specimens caught in May with $L_F \geq 110$ cm.

4. DISCUSSION

Determination of reproductive status is a key requirement for any fisheries management programme. Vtg has been widespread used in aquaculture species as an indicator of sexual maturity (Bon *et al.*, 1997 and Mosconi *et al.*, 1998).

In this study we showed the successful use of both steroid and VTG plasma levels for sex determination and estimation of sexual maturity stage of adult bluefin tunas. Sex determination of fish with nearly undetectable steroid and VTG levels, such as juveniles or quiescent fishes, remain to be resolved.

The next objective of our researches is the use of tissues for the sex determination and maturation stage estimation. Since tissue steroid levels are very low, it is important to establish good steroid extraction methods and sensitive detection systems. The ELISA's established are very sensitive and reliable. If we confirm on tissue the results obtained in this study we will be able in future to assess reproductive status and sex ratio using not more than 200 mg of tissue. This seems an important objective if considering that plasma and gonads from fishes caught commercially are very difficult to obtain.

The preliminary study on the first sexual maturity indicates that in our sample no female BFT with $L_F < 110$ cm could be considered to be adult. This seems to be in agreement with Rodríguez-Roda (1967) who, by means of macroscopic evaluation of gonad maturity stage, found 100% Eastern Atlantic BFT females mature over 120 cm (L_F). This findings need to be confirmed in a larger fish sample including sampling at the different phases of the reproductive cycle. It is notable that pre-adult females (L_F ranging from 100 to 110 cm) showed a slight gonadal development, characterised by the appearance of oocytes in lipid stage. This finding occurred contemporaneously to both the appearance of E_2 and Vtg in the

plasma. The simulation of gonadal development has already been reported by Baglin (1982) in sexual immature Western Atlantic BFT females.

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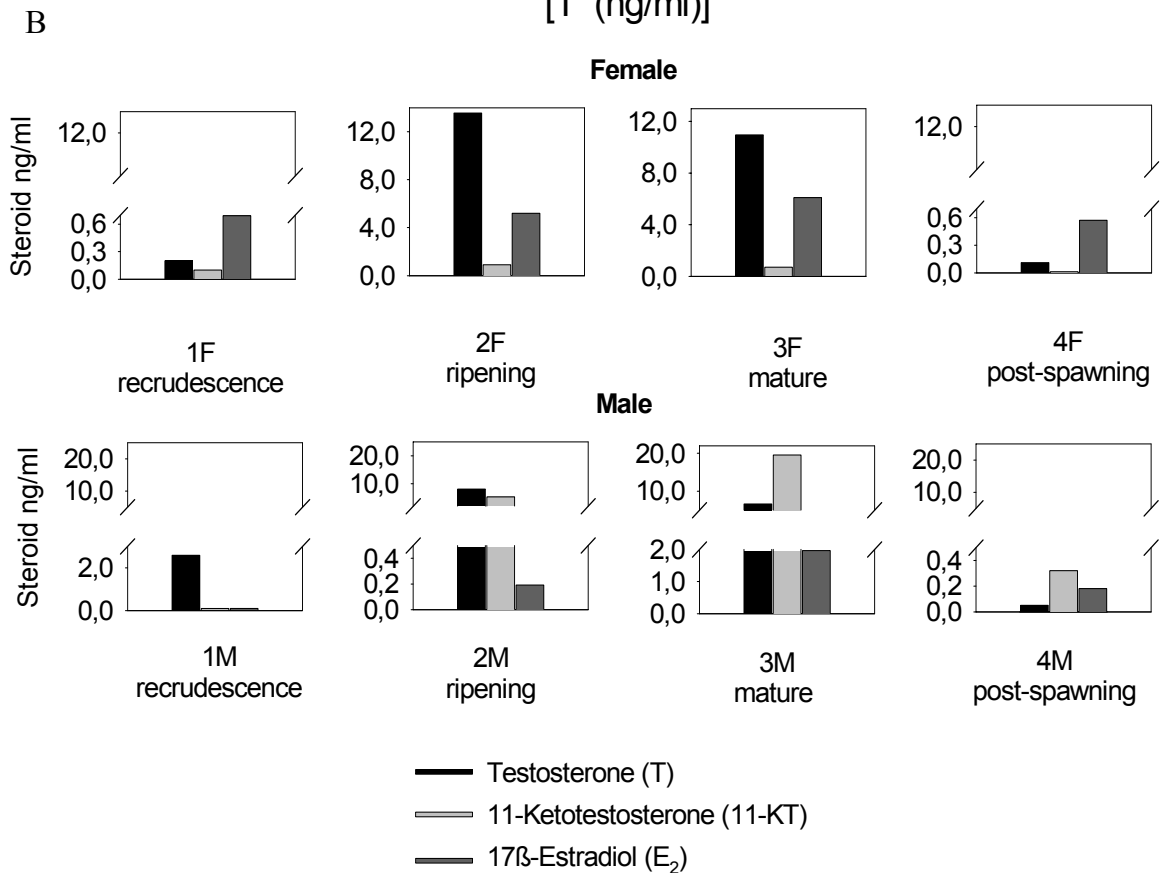
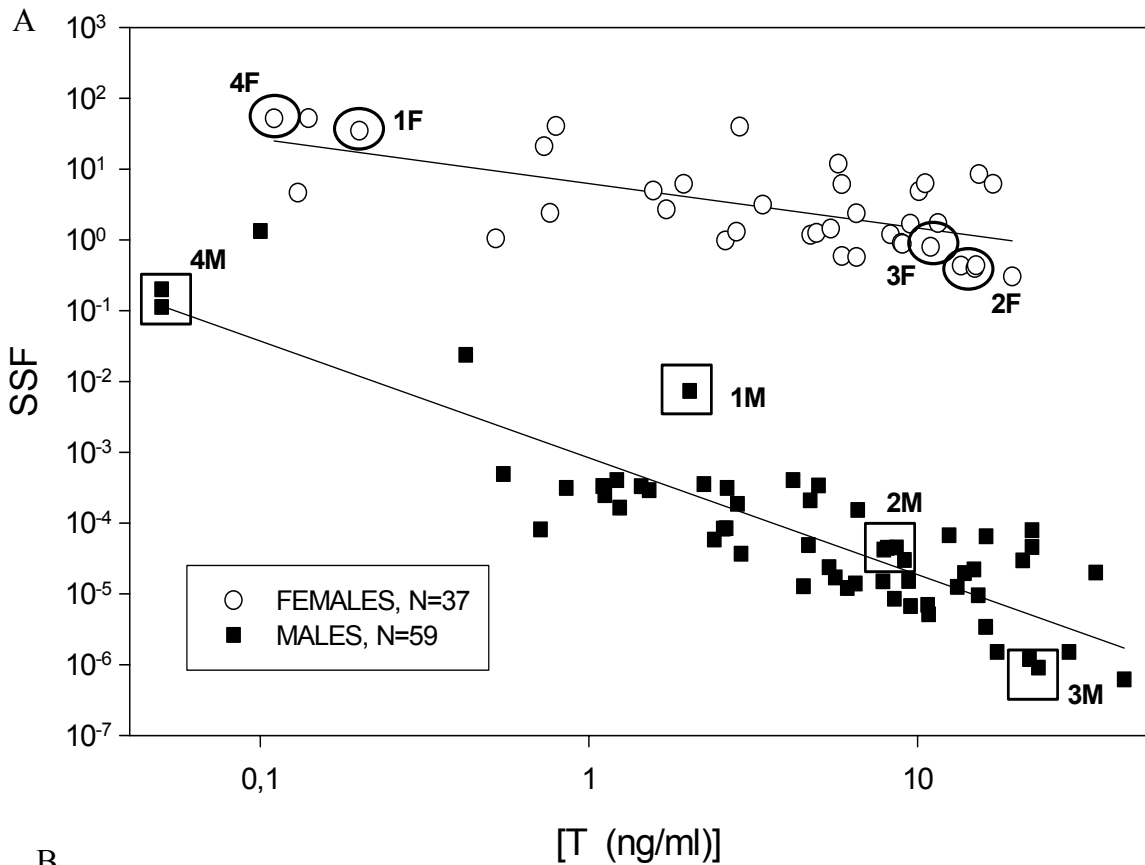


Fig. 1: Sex determination and maturity stage estimation by adult BFT. A) Depending from the SSF (Steroid Sexratio Formula, $[(E_2/11-KT)]/[T]$) value and the concentration of Testosterone (T) females and mals can be clearly distiguished in a logarithmic plot. B) Different maturity stages from single individuals show specific steroid profile with a specific SSF value evidenced in A.

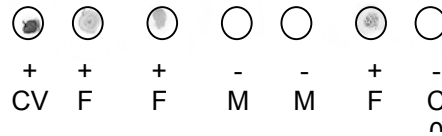


Fig. 2: Use of dot blot analysis for plasma BFT-Vtg to determine the sex. Positive reaction (+) for Vtg is detected by females in reproductive status (F). By males (M) the test is negative (-). This simple test can be used for sex determination when the measurement of steroids gives no precise results. Highly specific is the western blot

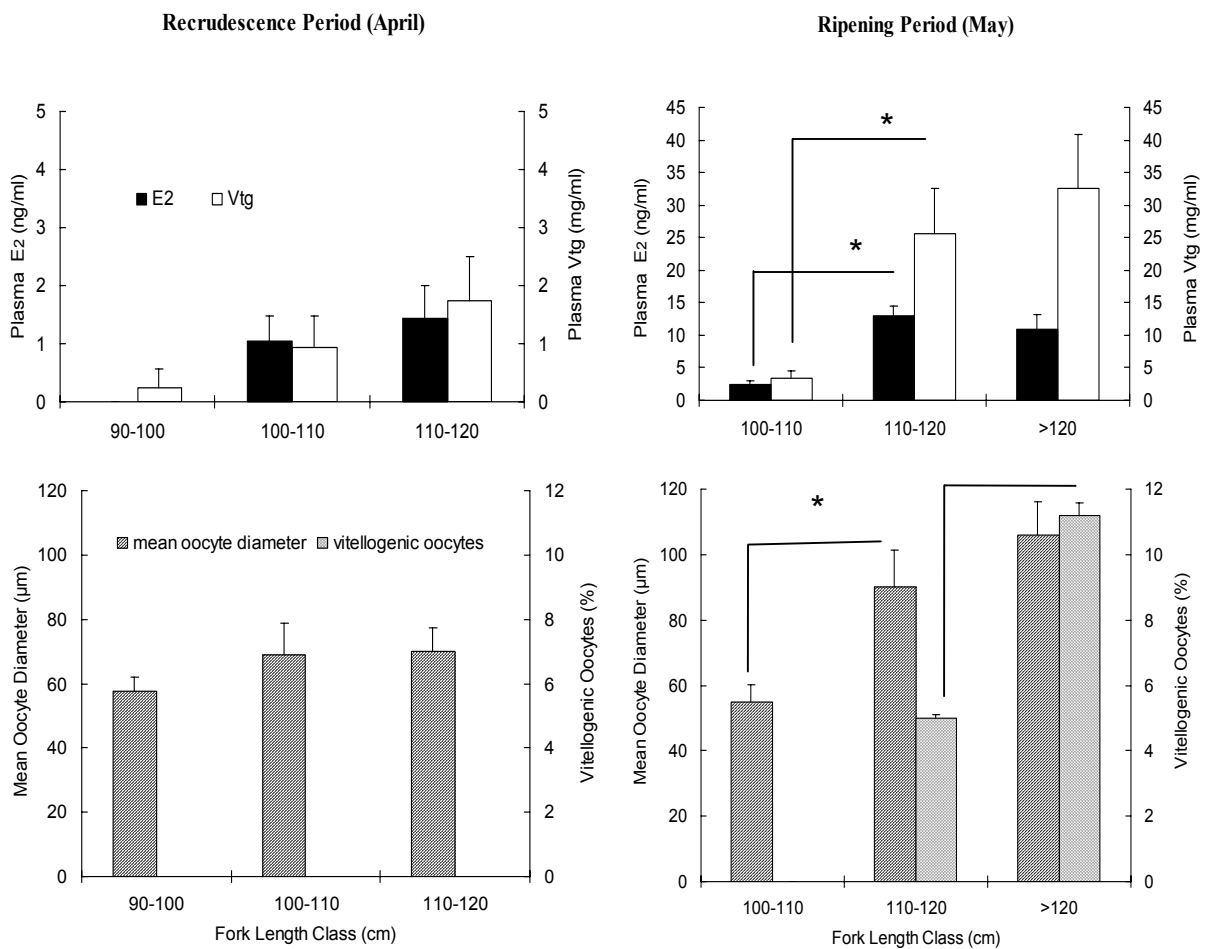


Fig. 3: Determination of the size at first sexual maturity of female BFT using changes in plasma levels of 17β-Estradiol (E₂), Vitellogenin (Vtg), mean oocyte diameter and percentage of vitellogenic oocytes in two different periods of the female BFT reproductive cycle: recrudescence (April) and ripening (May). Significant differences in the parameters considered are observed in the ripening period (May) between the length classes 100-110 and 110-120.