

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 dichlormethane. 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 |
|------------|--------------------------------|

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

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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 phenylmethylsulfonylfluoride (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.

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

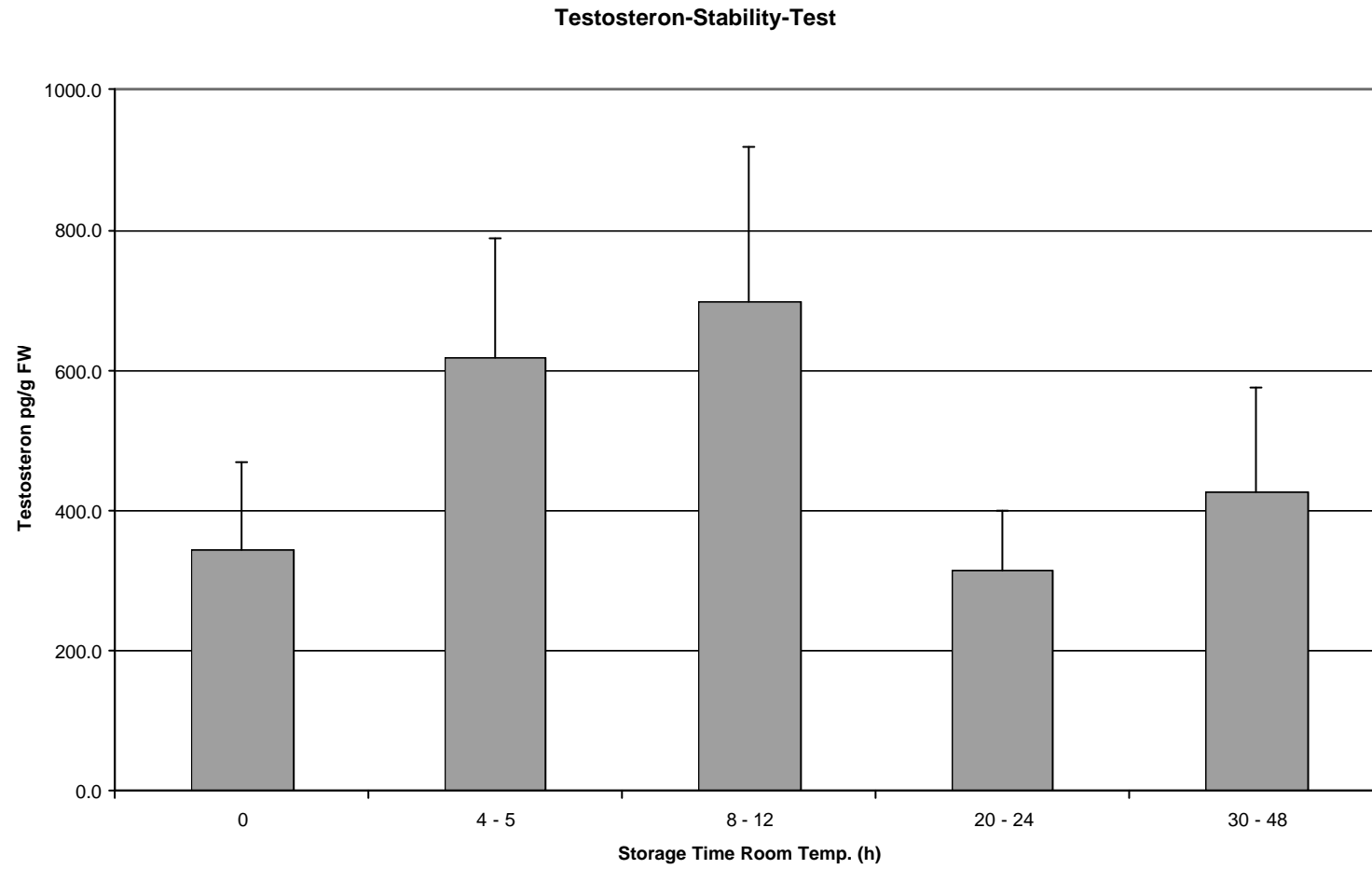
M 1: Provide clear guidelines for future sampling within fishing campaign II . Provide correction factors for the appraisal of probes taken within the first fishing campaign I. The successful completion of WP3 with corresponding positive results and the submission of the first interim report will make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



APPENDIX 4. Steroid stability at 10°C

